Activiation of the Action Potential Na⁺ Ionophore by Neurotoxins

AN ALLOSTERIC MODEL*

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The alkaloid neurotoxins aconitine, veratridine, grayanotoxin, and batrachotoxin activate the action potential Na⁺ ionophore by interaction with a common binding site. Concentration-response curves are fit by simple Langmuir isotherms. The fraction of Na⁺ ionophores activated at saturating concentrations of neurotoxin are: aconitine, 0.02; veratridine, 0.08; grayanotoxin, 0.51; and batrachotoxin, 0.95. For the partial activators, the primary effect of scorpion toxin is to increase the passive Na⁺ permeability of electrically excitable neurons. The jump in Na⁺ permeability reflects activation of the action potential Na⁺ ionophore. The binding of scorpion venom to the action potential Na⁺ ionophore is examined in vitro. 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 (11, 12). Veratridine, batrachotoxin, and aconitine increase the passive Na⁺ permeability of electrically excitable neuroblastoma cells (13, 14). Two kinds of evidence indicate that this increase in Na⁺ permeability reflects activation of the action potential Na⁺ ionophore. (a) the increase is completely inhibited by low concentrations of tetrodotoxin (13, 14), and (b) variant neuroblastoma clones specifically lacking the depolarizing phase of the action potential (13) do not respond to veratridine.

Equilibrium concentration-response relationships indicate that veratridine, aconitine, and batrachotoxin interact competitively with a single class of binding sites in activating the action potential Na⁺ ionophore of electrically excitable neuroblastoma cells (14, 15). Venom of the scorpion Leiurus quinquestriatus, and a polypeptide toxin purified from that venom, act cooperatively with veratridine, batrachotoxin, and aconitine to activate the action potential Na⁺ ionophore (15, 16). Batrachotoxin enhances the binding of [3H]monoiodo scorpion toxin to the action potential Na⁺ ionophore (17). These observations suggest that the alkaloid toxins and scorpion toxin bind to two functionally separate regulatory components that interact allosterically in controlling the ion transport activity of the action potential Na⁺ ionophore. The binding of scorpion toxin to the action potential Na⁺ ionophore is examined in detail in the preceding report (17). In this report, I present more extensive data analyzing the interactions among the alkaloid neurotoxins and scorpion toxin in activating the action potential Na⁺ ionophore and describe a two-state allosthetic model which quantitatively accounts for the observations.

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1 For brevity, the term "alkaloid neurotoxins" is used to refer to aconitine, veratridine, batrachotoxin, and grayanotoxin as a group.

In fact, grayanotoxin contains no nitrogen and thus, strictly speaking, is not an alkaloid.
EXPERIMENTAL PROCEDURES

Materials—The sources of materials and the growth of neuroblastoma cells is described in the preceding manuscript (17). Grayanotoxin I was kindly provided by Dr. T. Narahashi (Department of Pharmacology and Pharmacology, Duke University) and Dr. B. Wurtz (Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health).

Measurements of Na\(^+\) Uptake—Unless otherwise indicated, the following procedure was used. Cell cultures were incubated with the concentrations of toxins indicated in the figure legends for 30 min at 36\(^\circ\)C in 0.25 ml of medium consisting of 135.4 mM KCl, 50 mM Hepes\(^\text{a}\) (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 and the cells were rinsed twice in 15 s with 1 ml of medium consisting of 5.4 mM KCl, 150 mM choline chloride, 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. The cells were then incubated for 30 s 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 \(\mu\)Ci/ml of \(^{22}\)NaCl. Finally, the cells were washed three times with 3 ml of medium consisting of 183 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 washing conditions, extracellular \(^{22}\)Na\(^+\) is effectively removed, whereas intracellular \(^{22}\)Na\(^+\) is retained (16). The cells were suspended and radioactivity was determined as described previously (15).

Cells were labeled with 4.5-\(^{3}\)H]leucine by growth for 24 h in growth medium containing 0.5 \(\mu\)Ci/ml of \(^{3}\)H]leucine. The concentration of \(^{3}\)H]leucine in individual cultures after the experiment was used to normalize the results from different cultures. Protein was determined by a modification of the method of Lowry et al. (20).

Data were analyzed and fit to possible mathematical models using the PALS System (Division of Computer Research and Technology, National Institutes of Health). In experiments where the data are expressed as fractional activation of the \(^{22}\)Na\(^+\) ionophore, the maximum rate of uptake of \(^{22}\)Na\(^+\) estimated from a computed fit of a batrachotoxin titration curve in the presence of 100 \(\mu\)M scorpion toxin was assigned a value of 1.0.

RESULTS

Relationship between Measured \(^{22}\)Na\(^+\) Influx and \(P_{Na}\)—The goal of these experiments is to make detailed measurements of the concentration dependence of activation of the action potential \(^{22}\)Na\(^+\) ionophore by neurotoxins. Since the fraction of ionophores activated is proportional to \(^{22}\)Na\(^+\) permeability (\(P_{Na}\)), it is important to establish that the measured \(^{22}\)Na\(^+\) influx varies linearly with \(P_{Na}\). Goldman (21) and Hodgkin and Katz (22) have derived a relationship between ionic flux and ion permeability which is obeyed by many excitable cells.

\[
J_{Na} = P_{Na}[Na]_{out} \frac{F}{RT} \left( \frac{V}{\mu V_{R}} - 1 \right)
\]

This relationship predicts that \(^{22}\)Na\(^+\) influx \((J_{Na})\) is linearly proportional to \(^{22}\)Na\(^+\) permeability only if the membrane potential \((V)\) is constant. However, \(V\) depends on ionic permeabilities and concentrations according to Equation 2 (20).

\[
V = \frac{RT}{F} \ln \frac{P_{k}[K]_{in} + P_{Na}[Na]_{in} + P_{Cl}[Cl]_{in}}{P_{k}[K]_{in} + P_{Na}[Na]_{in} + P_{Cl}[Cl]_{in}}
\]

Thus, in general, when cells are treated with toxins which increase \(P_{Na}\), the relationship between \(J_{Na}\) and \(P_{Na}\) is nonlinear. The conditions used in these experiments are designed to eliminate these difficulties.

The toxins studied in these experiments require up to 60 min to equilibrate with their sites of action (14). During this time \(^{22}\)Na\(^+\) permeability is dramatically increased. In order to prevent entry of \(^{22}\)Na\(^+\) into cells, the incubations are carried out in Na\(^+\)-free medium. In addition, it was noted during previous experiments (16) that some loss of intracellular K\(^+\) occurred during these incubations because the \(^{22}\)Na\(^+\) ionophore is not absolutely specific for Na\(^+\). Since loss of intracellular K\(^+\) depolarizes the cells (Equation 2), it alters the relationship between measured flux and \(P_{Na}\). To prevent loss of intracellular K\(^+\), cells were incubated with toxins in medium containing 135 \(\mu\)M K\(^+\). The experimental protocol therefore involves incubation of the cells with neurotoxins for 30 to 60 min under conditions \((K^+)_\text{out} = 135 \mu\text{M}\). \(^{22}\)Na\(^+\) influx was then measured with \(^{22}\)Na\(^+\) uptake was then measured in a choline-substituted medium containing ouabain to inhibit \((Na^+\text{-}K^+)-\text{ATPase and having } [K^+]_\text{out} = 5 \text{ mM and } [Na^+]_\text{out} = 5 \text{ mM. Under these conditions the membrane potential is approximately } \pm 41 \text{ mV (23). A low concentration of Na\(^+\) is chosen so that the membrane potential is unaffected by the increased Na\(^+\) permeability caused by neurotoxin treatment and therefore \(J_{Na}\) remains directly proportional to \(P_{Na}\) as the concentration of neurotoxin is varied. The uptake of \(^{22}\)Na\(^+\) remains linear with time for at least 2 min. Membrane potential measurements with microelectrodes indicate that batrachotoxin (the most effective toxin studied) causes at most a small depolarization of neuroblastoma cells in medium containing 10 \(\mu\)M Na\(^+\). A more quantitative test of this point is suggested by Equation 1. Thus, at low Na\(^+\) concentrations where \(V\) is constant, \(J_{Na}\) should increase linearly with extracellular Na\(^+\) concentration. At higher concentrations, the relationship should become nonlinear. An experiment testing this point is illustrated in Fig. 1A. After incubation with batrachotoxin, \(J_{Na}\) varies linearly with \([Na^+]_\text{out}\) up to 10 \(\mu\text{M}\) in this experiment. In some experiments, linearity was maintained up to 15 \(\mu\text{M}\) Na\(^+\). Therefore, in this concentration range, \(J_{Na}\) is proportional to \(P_{Na}\). Subsequent experiments were carried out in medium containing 5 \(\mu\text{M}\) Na\(^+\).

The membrane potential of N18 cells can be varied between \(-41\) and 0 mV by increasing extracellular K\(^+\) (23). To further test Equation 1, \(J_{Na}\) was measured at different membrane potentials and the results were plotted according to Equation 1 (Fig. 1B). The linear relationship observed between \(J_{Na}\) and \(VF/(RT_\text{exp}(VF/RT) - 1)\) confirms the validity of Equation 1.

![Fig. 1. The dependence of \(^{22}\)Na uptake on Na\(^+\) concentration and membrane potential. A. N18 cells were incubated for 30 min as described under “Experimental Procedures” either with (•) or without (○) 1 \(\mu\text{M}\) batrachotoxin. \(^{22}\)Na\(^+\) uptake was then measured for 80 s as described under “Experimental Procedures” in medium with the indicated Na\(^+\) concentration plus choline so that Na\(^+\) + choline\(^+\) = 130 \(\mu\text{M}\). B. N18 cells were incubated with or without batrachotoxin as in A. \(^{22}\)Na\(^+\) uptake was then measured in medium with 5 \(\mu\text{M}\) Na\(^+\) plus choline chloride and KCl so that choline + K\(^+\) = 130 \(\mu\text{M}\). The membrane potentials under these conditions are: \([K^+]_\text{out} = 5.4 \text{ mV}, \Delta V = -41 \text{ mV}; [K^+]_\text{out} = 10 \text{ mV}, \Delta V = -37 \text{ mV}; [K^+]_\text{out} = 25 \text{ mV}, \Delta V = -28 \text{ mV}; [K^+]_\text{out} = 60 \text{ mV}, \Delta V = -13 \text{ mV}; [K^+]_\text{out} = 135 \text{ mV}, \Delta V = 0 \text{ mV} (23)."

\(^*\) The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
and indicates a linear relationship between $J_{\text{Na}}$ and $P_{\text{Na}}$. I conclude then that measurements of $^{22}\text{Na}^+$ influx under these conditions provide an accurate measure of $P_{\text{Na}}$ and thus of activation of the action potential Na$^+$ ionophore.

**Specificity of Neurotoxin Action**—The depolarization of nerve axons by aconitine, batrachotoxin, grayanotoxin, and veratridine is inhibited by low concentrations of tetrodotoxin (1, 3, 5, 6). The increase in Na$^+$ permeability of cultured neuroblastoma cells caused by these toxins is inhibited non-competitively by tetrodotoxin with $K_v$ values of 5 to 10 nmol (13–16). In voltage clamp experiments in frog node of Ranvier, batrachotoxin at high concentration alters the properties of all the voltage-sensitive Na$^+$ ionophores (24). In denervated rat muscle (25) and in cultured rat muscle (26) both action potentials and the increase in Na$^+$ permeability caused by veratridine and batrachotoxin become relatively resistant to tetrodotoxin inhibition. These observations are all most consistent with the view that aconitine, batrachotoxin, grayanotoxin, and veratridine act specifically on the Na$^+$ ionophore involved in action potential generation.

Additional evidence in favor of that view has been derived from studies of neuroblastoma and hybrid cell clones having heritable defects in action potential generation. Previous experiments have shown that veratridine increases the Na$^+$ permeability of electrically excitable neuroblastoma and hybrid clones but has no effect on inexcitable clones (13). Table I summarizes the results of experiments testing the effect of veratridine, batrachotoxin, aconitine, and scorpion toxin on clonal lines differing in electrical excitability. The electrically excitable clone N18 responds to all four toxin treatments with large increases in Na$^+$ permeability, whereas clone N103 which lacks the action potential Na$^+$ response but retains the action potential K$^+$ response (27) has a 20-fold smaller response to all four treatments. Hybrid cell clones formed between neuroblastoma clone N18TG2 (electrical excitable) and L cell clone B82 (inexcitable) have inheritable differences in electrical excitability (29). The electrically excitable clones NL308 and NL309 respond to all four toxin treatments. In contrast the inexcitable clones NL304 and NL305 have 10-fold smaller responses to all four treatments.

In addition to these experiments, Stellwag and Cohn (29) have analyzed 19 clones derived from rat brain tumors and found seven electrically excitable clones which responded to veratridine, only one electrically excitable clone that did not respond to veratridine, 10 inexcitable clones which did not respond to veratridine, and one inexcitable clone that did respond well to veratridine.

These results taken together with the tetrodotoxin inhibition data provide strong support for the view that all four alkaloid toxins act specifically on the action potential Na$^+$ ionophore. Other authors have suggested that these drugs might act specifically on tetrodotoxin-sensitive "resting Na" channels" (30, 31) that do not participate in action potential generation. This interpretation seems unlikely in view of the results described above.

**Site of Neurotoxin Action**—Aconitine, veratridine, and batrachotoxin increase the Na$^+$ permeability of cultured neuroblastoma cells to different extents at saturation (15). Treatment of cells with a combination of a good activator and a poor activator results in inhibition of the response to the good activator by the poor activator (14, 15). The concentration dependence is consistent with competitive inhibition, suggesting that these three neurotoxins act at a common binding site. More detailed results on this point using the more rigorous experimental conditions described in this report are illustrated in Fig. 2. In this experiment, cells were incubated with increasing concentrations of batrachotoxin in the presence of 0, 10, 20, or 50 nM aconitine. Since the increase in Na$^+$ permeability caused by aconitine is less than 2% of that caused by batrachotoxin (see below) aconitine can be treated as an inhibitor of batrachotoxin action. The data, when plotted on double reciprocal coordinates, give a competitive inhibition pattern. In addition, the abscissa intercepts, when plotted versus aconitine concentration as described by Cleland (32), give a straight line. Thus aconitine is a linear competitive inhibitor of activation by batrachotoxin, indicating that the binding of the two toxins is mutually exclusive.

Grayanotoxin was not studied in my earlier experiments. As shown originally in squid giant axon (30), the increase in Na$^+$ permeability of neuroblastoma cells caused by grayanotoxin is inhibited by tetrodotoxin. The Na$^+$ permeability increase caused by grayanotoxin is also inhibited by aconitine (Fig. 3). In these experiments, cells were incubated with a fixed concentration of grayanotoxin and increasing concentrations of aconitine, and Na$^+$ permeability was determined. In the absence of scorpion toxin (Fig. 3A) aconitine activates fewer than 2% of the Na$^+$ ionophores and inhibits the Na$^+$

### Table I

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Electrophysiologic properties</th>
<th>Neurotoxin-stimulated $^{22}\text{Na}^+$ uptake</th>
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<tr>
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<td>Na$^+$ re-</td>
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<td>K$^+$ response</td>
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<tr>
<td>NL309</td>
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<td>+</td>
</tr>
</tbody>
</table>

*Sctx, scorpion toxin.

**Fig. 2.** Competitive interaction between batrachotoxin and aconitine. N18 cells were incubated for 30 min as described under "Experimental Procedures" with the indicated concentrations of batrachotoxin and 0 (A), 10 (B), 20 (C), or 50 (D) nM aconitine. The initial rate of $^{22}\text{Na}^+$ uptake was then determined as described under "Experimental Procedures." A, the data are presented as a double-reciprocal plot. B, abscissas intercepts are plotted versus aconitine concentration (31).
permeability increase caused by grayanotoxin almost completely. In the presence of scorpion toxin (Fig. 3B), aconitine activates a substantial fraction of the Na⁺ ionophores and reduces the Na⁺ permeability increase caused by grayanotoxin only to the value observed with aconitine. The concentration dependence of these effects is consistent with a competitive interaction between the two toxins. These results are identical to those described earlier with veratridine, batrachotoxin, and aconitine and support the view expressed previously (15) that all four of these toxins bind to a common binding site.

Cooperative Effect of Scorpion Toxin—Previous results have shown that the venom of the scorpion Leiurus quinquesstriatus (15) and a toxin purified from that venom (16) act cooperatively with aconitine, veratridine, and batrachotoxin to activate the action potential Na⁺ ionophore. In the present experiments, I have made a detailed analysis of the concentration dependence of this cooperative interaction using the more rigorous experimental conditions described in this report. In each experiment the concentration of one alkaloid toxin was varied over a wide range in the presence of different fixed scorpion toxin concentrations. The greatest increase in Na⁺ permeability was observed in the presence of saturating concentrations of batrachotoxin plus scorpion toxin. The value of \( V_{\text{max}} \) (extrapolated to [batrachotoxin] = \( \infty \) was determined under these conditions in each experiment and was assigned a value of 1.0. All other data were normalized to this value.

The data from these experiments are presented in Figs. 4 to 7. The curves drawn are least squares fits to a model described below. In general, the data also can be fit well by a simple Langmuir isotherm as described previously (14, 16). The curves are essentially superimposable on those illustrated. The Hill coefficients are approximately 1.0 for all the data. The parameters for fits of each curve individually to a simple Langmuir isotherm are given in Table II. \( K_{0.5} \) is the concentration of toxin required to give 50% maximum effect and \( P_a \) is the fractional activation at infinite toxin concentration. The apparent dissociation constants \( (K_{0.5}) \) for the poor activators, veratridine and aconitine, are relatively little affected by scorpion toxin. In contrast, the values of \( K_{0.5} \) for the good activators, batrachotoxin and grayanotoxin, are decreased more than 10-fold by scorpion toxin. The maximum uptake velocities (\( P_a \)) for veratridine and aconitine are substantially increased by scorpion toxin. In contrast, the values of \( P_a \) for batrachotoxin and grayanotoxin are relatively little affected by scorpion toxin.

These results are similar to those obtained previously in experiments using whole scorpion venom (15) under other experimental conditions. In the present experiments, the difference between \( P_a \) in the presence of the poor activators, veratridine and aconitine, and that in the presence of batrachotoxin is more pronounced. This is due to underestimation of \( P_a \) in the presence of scorpion toxin plus batrachotoxin in the earlier experiments (15) because relatively high concentrations of Na⁺ were used (50 mM, compare with Fig. 1) and incubations with toxins were carried out in medium with \( [\text{K}^+]_{\text{out}} = 5 \text{ mM} \) causing some loss of intracellular K⁺ (16).

Two-state Conformational Change Model—The four alkaloid toxins act competitively at a common binding site in activating the action potential Na⁺ ionophore but each causes...
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The data of Figs. 4 to 7 were fit to a modified Michaelis-Menten equation of the form \(P(A) = P_0 A/(K_{0.5} + A)\) where \(P_0\) is the fraction of Na\(^+\) ionophores activated at saturation, \(K_{0.5}\) is the concentration of toxin required to obtain 50% maximum activation, and \(A\) is toxin concentration.

### Table II

Parameters derived from fits to Langmuir isotherm

<table>
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<tr>
<th>[Sca] (^b)</th>
<th>Aconitine</th>
<th>Veratridine</th>
<th>Grayanotoxin</th>
<th>Batrachotoxin</th>
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<td>(P_0)</td>
<td>(K_{0.5})</td>
<td>(P_0)</td>
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<td>0.31</td>
<td>5.2 (\times) 10(^{-3})</td>
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</table>

\(^b\) Sca, scorpion toxin.

\[ P(A) = \frac{1}{1 + A/K_r} \]

This relationship defines the dependence of the fraction of active Na\(^+\) ionophores on the activator concentration, \(A\), and three constants, the conformational equilibrium constant, \(M_{RT}\), and the dissociation constants, \(K_r\) and \(K_H\). It is identical to the function of state of Monod et al. (33) for the special case of \(n\), the number of interacting protomers, equal to 1.

From Equation 6, in the absence of ligand,

\[ P_d(0) = 1/(1 + M_{RT}) \]

Thus, in general, some fraction of the Na\(^+\) ionophores must be in the \(R\) state in the absence of toxins. At infinite activator concentration,

\[ P_d(\infty) = \frac{1}{1 + M_{RT}} \]

Thus, the fraction of Na\(^+\) ionophores activated by saturating concentrations of toxin \(P_0\) can take on any value between 0 and 1 depending on the values of the parameters \(M_{RT}\), \(K_r\), and \(K_H\).

Equation 6 also defines the relationship between \(K_{0.5}\) and the parameters \(M_{RT}\), \(K_r\), and \(K_H\), if \(M_{RT}\) is restricted to values \(\geq 100\). By definition, \(P_0(K_{0.5})/P_0(\infty) = 0.5\). Therefore,

\[ 2 + 2M_{RT} \left( \frac{K_H}{K_r} \right) = 1 + M_{RT} \left( \frac{1 + K_{0.5}/K_r}{1 + K_{0.5}/K_H} \right) \]

For \(M_{RT} > 100, K_{0.5}\) is always much greater than \(K_r\) and thus \(1 + K_{0.5}/K_r < K_{0.5}/K_r\). Making this approximation and rearranging terms yields

\[ \frac{1}{K_{0.5}} = \frac{1}{M_{RT}K_r + \frac{1}{K_r}} \]

Thus, as a function of \(M_{RT}\), \(K_{0.5}\) varies from a maximum of \(K_r\) at \(M_{RT} = \infty\) to a minimum of \(M_{RT}K_r\) as \(M_{RT}\) becomes smaller.

In fitting this model to the experimental data, \(K_r\) and \(K_H\) are taken as constants for each of the alkaloid toxins. When \(K_r < K_H\), alkaloid toxins activate some fraction of the Na\(^+\) ionophores according to Equation 6. The effect of scorpion toxin is to reduce \(M_{RT}\). Thus, scorpion toxin alters the energy required to cause the \(T \rightarrow R\) transition.

It is not possible to define all three parameters uniquely unless \(M_{RT}\) can be defined from Equation 7. Tetrodotoxin has no effect on steady state Na\(^+\) permeability of N18 cells unless the Na\(^+\) ionophores are activated by treatment with alkaloid toxins. Therefore, the fraction of ionophores active in the...
absence of an alkaloid toxin is too small to measure. Since our experiments can detect activation of 0.5% of the Na\(^+\) ionophores, \(P_a(0) \approx 0.005\) (Equation 7) and \(M_{RT} > 200\). In the terms of the model, the effect of scorpion toxin is to reduce \(M_{RT}\). However, purified scorpion toxin does not activate a detectable fraction of the action potential Na\(^+\) ionophores (16). Thus \(M_{RT}\) in the presence of saturating concentrations of scorpion toxin must also be greater than 200. In fitting the data, \(M_{RT}\) was set at 500 in the presence of 100 nM scorpion toxin. Values of \(K_T\) and \(K_R\) were then derived by fitting the data to 100 nM scorpion toxin to Equation 6 (Table III). The ratio of dissociation constants for the \(T\) and \(R\) states (\(K_T/K_R\)) range from \(1.4 \times 10^8\) for aconitine to \(1.4 \times 10^5\) for batrachotoxin.

To fit the data at other scorpion toxin concentrations, \(K_T\) and \(K_R\) were held constant and \(M_{RT}\) was allowed to vary. The best fit parameters are presented.

### Table III

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<th>(K_R)</th>
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<td>Batrachotoxin</td>
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### Table IV

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</tr>
<tr>
<td>100</td>
<td>(5.0 \times 10^2)</td>
<td>(5.0 \times 10^2)</td>
<td>(5.0 \times 10^2)</td>
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<tr>
<td>300</td>
<td>(2.7 \times 10^2)</td>
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\(a\) Scots, scorpion toxin

**Fig. 8.** Dependence of the allosteric constant on scorpion toxin concentration. The values of \(M_{RT}\) from Table IV are plotted as a function of scorpion toxin concentration (Fig. 8). The agreement, while not perfect, is satisfactory since experiments with each different toxin were necessarily carried out on separate groups of cell cultures. The results which agree least well are those for grayanotoxin in the absence of scorpion toxin (enclosed in parentheses in Table IV). These values are the least reliable because limited supplies of grayanotoxin did not allow experiments at saturating concentrations in the absence of scorpion toxin. The model thus fits all the data with a single assumption, namely, that scorpion toxin causes a reduction in the value of \(M_{RT}\).

This model predicts quite clearly that scorpion toxin should have its major effect on \(P_a\), for poor activators and on \(K_{0.5}\) for good activators. Thus, from equation (10), \(M_{RT}K_T > K_R\) for poor activators and \(K_{0.5}\) approximately \(K_T\), even in the presence of scorpion toxin (compare values of \(K_{0.5}\) and \(K_T\) in Tables I and III). \(P_a\) for poor activators is increased dramatically as \(M_{RT}\) is decreased (Equation 5) as long as \(P_a\) is substantially less than 1. In contrast, for good activators, \(M_{RT}K_T < K_T\) and \(K_{0.5}\) varies approximately as \(M_{RT}K_T\) (Table III) and thus is decreased dramatically by scorpion toxin. \(P_a\) for good activators is near 1 and thus cannot be increased greatly by scorpion toxin.

The experimental data cannot be fit by holding \(M_{RT}\) constant and allowing \(K_T\) to vary as a function of scorpion toxin concentration. The experimental data can however be fit if \(M_{RT}\) is assumed to remain constant and \(K_T\) is allowed to vary as a function of scorpion toxin concentration. The curves derived from these two different assumptions are virtually superimposable. One would expect, however, that, if scorpion toxin affected \(K_T\), the extent of the scorpion toxin effect would vary significantly for the four different alkaloid toxins since they differ substantially in structure and binding constant. In fact, however, the increase in \(K_T\) (or in \(M_{RT}\)) is substantially less than 1. In contrast, for good activators, \(M_{RT}K_T < K_T\) and \(K_{0.5}\) varies approximately as \(M_{RT}K_T\) (Table III) and thus is decreased dramatically by scorpion toxin. \(P_a\) for good activators is near 1 and thus cannot be increased greatly by scorpion toxin.

This treatment of the heterotropic cooperative interaction between scorpion toxin and the alkaloid toxins is analogous to the treatment of heterotropic interactions between substrates, activators, and inhibitors of allosteric enzymes by Monod et al. (33).

In the model of Monod et al. the change in \(M_{RT}\) caused by heterotropic allosteric ligands is considered to be due to the preferential binding of the ligands to the \(R\) or \(T\) states. The mechanism by which scorpion toxin affects \(M_{RT}\) for activation of the Na\(^+\) ionophore by alkaloid toxins must be more complex because scorpion toxin binding is only slightly affected by batrachotoxin and thus scorpion toxin must bind well to both active and inactive states of the ionophore. This conclusion remains correct whether scorpion toxin affects \(K_R\) or \(M_{RT}\).

**DISCUSSION**

The results presented in this report confirm and extend conclusions reached in previous reports in four respects:
1. Neuroblastoma cell lines specifically lacking the Na\(^+\) response of the action potential are unaffected by veratridine, batrachotoxin, and aconitine, strengthening the conclusion (13) that these toxins act specifically on the Na\(^+\) ionophores involved in action potential generation.
2. All four of the alkaloid neurotoxins (aconitine, batrachotoxin, grayanotoxin, and veratridine), which modify the kinetic properties of the Na⁺ ionophore in electrophysiologic experiments, interact competitively in causing persistent activation of the Na⁺ ionophore. Aconitine is a linear competitive inhibitor of activation of the Na⁺ ionophore by batrachotoxin indicating that binding of these two toxins is mutually exclusive. These results provide strong support for the conclusion that all four toxins act at a specific chemically sensitive site associated with the action potential Na⁺ ionophore (15). In general, competitive inhibition can also be caused by allosteric interactions of ligands binding at separate sites. In this case, the competitive interaction arises because the allosteric inhibitor favors a conformational state that binds the second ligand poorly. In my experiments, all four alkaloid toxins are activators of the Na⁺ ionophore. Therefore, they all must bind preferentially to the active state of the Na⁺ ionophore. Since each pair of alkaloid toxins interacts competitively, four separate active states of the Na⁺ ionophore, each having specific high affinity for one toxin and low affinity for the other three, are necessary to explain the results on the basis of indirect allosteric competitive inhibition. This seems very unlikely. I conclude, then, that all four alkaloid toxins act at a common binding site.

3. In previous reports (15) I have hypothesized that the alkaloid toxins, scorpion toxin, and tetrodotoxin interact with three functionally separable components of the action potential Na⁺ ionophore. This hypothesis is strengthened by observations in this and the accompanying report (17): (a) tetrodotoxin does not affect binding of scorpion toxin; (b) the alkaloid toxins have only small effects on binding of scorpion toxin; (c) scorpion toxin, while it affects activation of the action potential Na⁺ ionophore by alkaloid toxins, does not alter the dissociation constants ($K_r$, $K_b$) for alkaloid toxins.

4. Using experimental conditions under which $P_{Na}$ is proportional to the measured Na⁺ influx, I have confirmed my earlier results (15) showing that the effect of scorpion toxin is to increase the maximum fraction of Na⁺ ionophores activated by partial activators (veratridine, aconitine, and grayanotoxin) and to reduce the concentration required for 50% activation by both partial and full activators. These results support the earlier conclusion that there are heterotropic cooperative interactions between scorpion toxin and each alkaloid neurotoxin whereas there are no homotropic cooperative interactions observed in experiments with a single neurotoxin (15).

The main purpose of this report is to describe a simple allosteric model which accommodates this heterotropic cooperative interaction. The model described under "Results" makes only two assumptions, namely, that the alkaloid toxins bind better to the active state of the Na⁺ ionophore and that scorpion toxin alters the energy required for activation of the Na⁺ ionophore by alkaloid toxins. These assumptions are identical to those made by Monod et al. in describing heterotropic cooperativity between allosteric modifiers and enzyme substrates. The theoretical curves generated by this model match the experimental data precisely (Figs. 4 to 7). Not all the parameters of the model are defined uniquely, however. The value of $M_{RT}$, the allosteric constant, must be chosen before computed fits of $K_r$ and $K_b$ can be carried out. The only constraint in choosing $M_{RT}$ is that it must be >200. To illustrate the fit of the data, $M_{RT}$ was taken as 500 at 100 nM scorpion toxin. Equally good fits of the data can be achieved by choosing $M_{RT}$ at 100 nM scorpion toxin to be larger than 500. Under these conditions, the best fit values of $K_r$ are approximately those in Table III, whereas the best fit values of $K_b$ are reduced in rough proportionality to the increase in the value of $M_{RT}$ selected. This behavior results from the fact that, under most conditions studied in these experiments, the term $1 + A/K_b$ in equation (6) is approximately equal to $A/K_b$ causing $M_{RT}$ and $K_b$ to behave as the product $M_{RT}/K_b$ when the equation is fit by iterative procedures. Values derived for $K_b$ and the ratio $K_b/K_r$ therefore depend on the value of $M_{RT}$ selected. The values of these parameters presented are near the maximum allowed by the data.

The fraction of the Na⁺ ionophores that are active at any membrane potential can be calculated from voltage clamp data using the theory of Hodgkin and Huxley (38). Assuming that the active state of the Na⁺ ionophore caused transiently by depolarization is the same as the active state caused by toxin treatment, $M_{RT}$ in the absence of toxin should equal the ratio of inactive to active ionophores calculated from voltage clamp data. At $-41$ mV, this ratio, calculated as ($m^\infty$)⁻¹ (38), is approximately 400 for squid giant axon (38), 5500 for frog muscle (39), and 6700 for frog node of Ranvier (40). Thus the values of $M_{RT}$ in the absence of scorpion toxin (Table IV) are similar to the values of ($m^\infty$)⁻¹ for Na⁺ ionophores in frog muscle and nerve.

The assumption that alkaloid toxins bind better to the active state of the Na⁺ ionophore is supported by previous electrophysiologic data. Stimulation of nerve axons by repetitive depolarizing pulses during treatment with aconitine (4), batrachotoxin (41), or veratridine (3) causes a dramatic increase in the rate of toxin action. In contrast, a single, long term depolarization has little effect on the action of alkaloid toxins in axons (4, 41) or in neuroblastoma cells. These results suggest that repetitive stimulation enhances the rate of binding of alkaloid toxins by causing the Na⁺ ionophore to become active for a few milliseconds during each stimulation, whereas long term depolarization has little effect because the Na⁺ ionophores are activated only once. These observations support the view that alkaloid toxins bind better to the active state of the Na⁺ ionophore and provide independent support for one of the two main assumptions of the allosteric model.

Although the allosteric model presented was derived from studies of activators of the Na⁺ ionophore, it makes clear predictions concerning the mechanism of action of inhibitors of activation of the Na⁺ ionophore. Simple noncompetitive inhibitors (31) should reduce $P_{Na}$ without affecting $K_{0.5}$ for both good and poor activators. Thus, my previous results show that tetrodotoxin is a simple noncompetitive inhibitor (14). Simple competitive inhibitors (31) which bind at the alkaloid toxin binding site should increase $K_{0.5}$ without affecting $P_{Na}$ for both good and poor activators. Divalent cations are therefore simple competitive inhibitors and must compete with the alkaloid toxins for a common binding site (14). In contrast, allosteric competitive inhibitors which inhibit by increasing $M_{RT}$ or $K_b/K_r$ should appear to be competitive inhibitors with respect to good activators but noncompetitive or mixed inhibitors with respect to poor activators. Yohimbine and local anesthetics may be examples of such inhibitors.

The results in this and the accompanying report suggest that the alkaloid toxins and scorpion toxin bind to and cause conformational changes in two separate components of the Na⁺ ionophore. The conformational change in the alkaloid

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3. Unpublished observations.

toxin binding component causes persistent activation of the Na\(^+\) ionophore. The conformational change in the scorpion toxin binding component alters the energy required for activation of the Na\(^+\) ionophore by alkaloids and is opposed by depolarization. The conformational changes in these two components are partially coupled so that the energy required for the conformational change in each component is dependent upon the state of the other. The partial coupling is reflected in the enhancement of scorpion toxin binding by batrachotoxin (17) and in the enhancement of alkaloid toxin activation by scorpion toxin. Experiments defining the stoichiometry and physical relationship between these binding components are required to verify these suggestions.

Hodgkin and Huxley (38) were able to describe many of the kinetic and voltage-dependent properties of the action potential Na\(^+\) ionophore of squid giant axon in terms of two independent processes, one controlling activation of the ionophore and one controlling inactivation during a maintained depolarization. In voltage clamp experiments, the venom of the scorpion Leiurus quinquestriatus inhibits specifically the Na\(^+\) ionophore of squid giant axon in terms of two kinetic and voltage-dependent properties of the action potential. The partial coupling between these two binding sites observed in my experiments may be the basis for the coupling between the processes of activation and inactivation observed in recent voltage clamp (43) and gating current (44) experiments. Binding experiments in tissues with known voltage clamp parameters are required to verify this relationship.

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