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Ion Flux Studies of Voltage-Sensitive Sodium Channels in Synaptic Nerve-Ending Particles

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SUMMARY

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Neurotoxins which activate voltage-sensitive sodium channels cause a 10-fold increase in the initial rate of $^{22}Na^+$ uptake in synaptic nerve-ending particles (synaptosomes). Batrachotoxin is a full agonist in activating synaptosomal sodium channels, whereas veratridine and aconitine are partial agonists acting at the same receptor site. The $^{22}Na^+$ uptake induced by alkaloid toxins is completely blocked by tetrodotoxin and saxitoxin. The K_i for saxitoxin (6 nM) is identical with the K_d for ^3H-labeled saxitoxin binding to sodium channels in synaptosomes. The polypeptides scorpion toxin, *Anemonia sulcata* toxin II, and Anthopleurin A enhance activation of sodium channels by alkaloid toxins, reducing the $K_{0.5}$ for all alkaloid toxins and increasing the fraction of sodium channels activated by the partial agonists. Scorpion toxin and *A. sulcata* toxin II are full agonists in this respect, whereas Anthopleurin A is a partial agonist. The three polypeptide toxins bind to a common receptor site, and the concentration dependence of binding and activation of $^{22}Na^+$ influx are closely correlated. Alkaloid toxins markedly enhance ^{125}I-labeled scorpion toxin binding to its receptor site, demonstrating bidirectional allosteric coupling between the receptor sites for polypeptide and alkaloid toxins. The concentration-effect curves for alkaloid toxin enhancement of ^{125}I-labeled scorpion toxin binding and $^{22}Na^+$ uptake are closely correlated. Depolarization enhances the effect of alkaloid toxins on ^{125}I-labeled scorpion toxin binding but does not alter the effect of saturating concentrations of scorpion toxin on batrachotoxin activation. In depolarized synaptosomes, this allosteric coupling between the alkaloid toxin and polypeptide toxin receptor sites is quantitatively fit by a model which assumes that both the alkaloid and polypeptide toxins act by binding selectively to active states of sodium channels.

INTRODUCTION

Neurotoxins are valuable probes of voltage-sensitive sodium channel structure and function. The sodium channels of peripheral nerve and neuroblastoma cells possess three distinct neurotoxin receptor sites (for review, see ref. 1). The first receptor site binds the inhibitors tetrodotoxin and saxitoxin. These compounds block the flux of sodium through the channel. The second receptor site binds the alkaloid toxins batrachotoxin, vetratridine, and aconitine. These three toxins alter the voltage dependence of activation and inactivation and cause persistent sodium channel activation. The third receptor site binds polypeptide toxins isolated from scorpion and sea anemone venoms. Scorpion and sea anemone toxins inhibit channel inactivation and interact cooperatively with the alkaloid toxins in causing persistent activation. Scorpion toxin binding to its receptor is voltage-dependent, suggesting that the polypeptide toxin receptor may be the voltage-sensing component of the sodium channel (2). Binding of the alkaloid toxins to their receptor enhances the binding of radioactively labeled scorpion toxin (3, 4).

Brain membrane preparations have a 50-fold greater neurotoxin binding capacity than do neuroblastoma cells and therefore provide a useful starting point for studies of the molecular properties of sodium channels. Nerve-ending particles (synaptosomes) from rat brain retain a membrane potential (5) and have functional sodium channels as shown by measurements of membrane potential (5), ion flux (6–8), and neurotoxin binding (4, 9, 10). Synaptosomes therefore provide a cell-free vesicular system in which sodium channel function can be studied and compared with that in intact nerve or cellular preparations. In these experiments, we have used the quantitative ion flux procedures developed in our studies of neuroblastoma cells (11, 12) to analyze the interaction of

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neurotoxins with sodium channels in synaptosomes and to correlate neurotoxin binding with physiological effects on ion flux.

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: veratridine from Aldrich Chemical Company, Milwaukee, Wis.: aconitine from K & K Laboratories, Plainview, N. Y.; scorpion (*Leiurus quinquestriatus*) venom from Sigma Chemical Company, St. Louis, Mo.; tetrodotoxin from Calbiochem, San Diego, Calif.; and carrier-free ^{22}NaCl from New England Nuclear Corporation, Boston, Mass. Batrachotoxin was a gift from Dr. John Daly (Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health). The sea anemone (*Anthopleura xanthogrammica*) toxin Anthopleurin A was supplied by Dr. T. R. Norton, University of Hawaii. Sea anemone toxin II was obtained from Dr. L. Beress, University of Kiel, Kiel, West Germany. Saxitoxin was supplied by Dr. E. Schantz, University of Wisconsin, and labeled with ^{3}H as previously reported (13). Scorpion toxin was purified and labeled with ^{125}I as previously described (3, 11). The toxin preparation used contains a single polypeptide whose M_r is 6700 and whose *pI* is 9.8 (3, 11); it is one of several toxic polypeptides in *L. quinquestriatus* venom.

Preparation of synaptosomes. Synaptosomes were prepared from rat brain by a modification of the method of Gray and Whittaker (14). The brains of male Sprague-Dawley rats were removed and homogenized in ice-cold 0.32 M sucrose and 5 mM K_2HPO_4, pH 7.4 (10 ml/g wet weight), with 10 strokes of a motor-driven Teflon-glass homogenizer. The homogenate was then centrifuged at 1,000 × *g* for 10 min. The supernatant was saved and the pellet was resuspended in 10 ml of the homogenizing solution. The pellet was homogenized and centrifuged as before. The two supernatants were combined and centrifuged at 17,000 × *g* for 60 minutes. The resulting supernatant was discarded and the pellet was resuspended in 10 ml of 0.32 M sucrose and 5 mM K_2HPO_4, pH 7.4, using 10 strokes of a glass-Teflon homogenizer. The resuspended pellet was then layered onto a step gradient consisting of 10-ml layers of 1.2, 1.0, 0.8, 0.6, and 0.4 M sucrose, all solutions also being made with 5 mM K_2HPO_4, pH 7.4. The gradient was centrifuged at 100,000 × *g* for 105 minutes. The synaptosomes distribute within the 1.0 M sucrose fraction, and this fraction was collected. The sucrose concentration of the 1.0 M fraction was reduced to 0.32 M by the dropwise addition of 5 mM K_2HPO_4, pH 7.4, with constant stirring to prevent hypotonic lysis. The synaptosome suspension was then centrifuged at 40,000 × *g* for 45 minutes and the resulting pellet was resuspended in 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes[2]-Tris (pH 7.4), and 130 mM choline chloride (1 ml/1.75 g of original wet weight of brain). Ten strokes of a loose-fitting glass-glass homogenizer were used to resuspend this final synaptosome pellet. The synaptosomes were stored in liquid N_2 with no appreciable loss of toxin binding or $^{22}Na^+$ uptake activity (less than 15%). It was necessary to freeze the synaptosomes slowly in 0.5-ml aliquots on Dry Ice before storage in liquid N_2. Prior to use, the synaptosomes were thawed at 36° for 5 min and then stored on ice.

Measurement of $^{22}Na^+$ uptake. Since most of the neurotoxins used in this study act slowly, it was necessary to incubate the synaptosomes with the toxin prior to measurement of $^{22}Na^+$ uptake. The synaptosomes were incubated with the toxins for 10 min at 36° in a 50-μl solution containing 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes-Tris (pH 7.4), 130 mM choline chloride, and BSA, 1 mg/ml. The action of all toxins reached a plateau within this time. After 10 min, 150 μl of the $^{22}Na^+$ uptake solution was added vigorously to ensure mixing. This solution contained the same toxin concentrations used in the preincubation plus 5.4 mM KCl, 0.8 mM $MgSO_4$, 55 mM glucose, 50 mM Hepes-Tris (pH 7.4), 128 mM choline chloride, 2.66 mM NaCl, 5 mM ouabain, BSA (1 mg/ml), and 1.3 μCi of carrier-free ^{22}NaCl/ml. When uptake was measured in the presence of 125 mM KCl, the choline concentration of both the preincubation and uptake solutions was reduced to maintain constant osmolarity. The synaptosomes were incubated with the $^{22}Na^+$-containing solution at 36° for 5 sec unless otherwise indicated. To stop $^{22}Na^+$ uptake, 3 ml of an ice-cold wash solution were added and the mixture was rapidly filtered under vacuum through an Amicon 0.45-μm cellulose filter (catalogue No. 041255). The filter was washed twice with 3 ml of the wash solution and placed in a scintillation vial. The wash solution was composed of 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5 mM Hepes-Tris (pH 7.4), and BSA, 1 mg/ml. Filter radioactivity was used to quantitate $^{22}Na^+$ uptake. When the length of time required to filter and wash the synaptosomes was varied from 10 to 30 sec, no change in filter radioactivity was observed, suggesting that little or no $^{22}Na^+$ efflux occurred during the filtration process. Nonspecific $^{22}Na^+$ uptake was determined using 1 μM tetrodotoxin in both the preincubation and uptake solutions. Except where indicated, the data are presented as corrected specific uptake after subtraction of nonspecific uptake.

Measurement of radioactively labeled toxin binding. Specific binding of ^{3}H-labeled saxitoxin and ^{125}I-labeled scorpion toxin was measured as described previously (4, 13). The procedure used was similar to that used to measure $^{22}Na^+$ uptake, except that incubation with the $^{22}Na^+$-containing uptake solution was omitted. The synaptosomes, diluted 4-fold from the concentration used for $^{22}Na^+$ uptake measurement, were incubated with the toxins at 36° for 10 min in 200 μl of the preincubation solution used for the uptake assay. After 10 min, the synaptosomes were filtered as previously described except that Whatman GFC glass fiber filters were used. Filter radioactivity was used to quantitate radiolabeled toxin binding. Nonspecific binding was determined in the presence of a saturating concentration of unlabeled toxin. Nonspecific binding was never greater than 15% of the

[2] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin.

total binding in polarized synaptosomes and less than 50% in depolarized synaptosomes. The binding data are reported as specific binding after subtraction of nonspecific binding.

Other methods. Synaptosome protein concentrations were measured by a modification of the method of Lowry *et al.* (15) or by the method of Peterson (16). Lysed synaptosomes were prepared by hypotonic lysis in 5 mM K_2HPO_4, pH 7.4. The lysed membranes were then centrifuged and the sediment was resuspended in the appropriate solution. Each data point given represents an average of two or three determinations.

RESULTS

Characterization of synaptosomal $^{22}Na^+$ uptake. In the absence of neurotoxins, $^{22}Na^+$ uptake into synaptosomes was slow. Batrachotoxin caused a 10-fold increase in the initial rate of $^{22}Na^+$ uptake. Tetrodotoxin completely blocked the increase in $^{22}Na^+$ uptake caused by batrachotoxin. The time courses for $^{22}Na^+$ uptake with no added toxins (●), with 2.5 μM batrachotoxin alone (▲), or with 2.5 μM batrachotoxin plus 1 μM tetrodoxin (△) are illustrated in Fig. 1A. Since tetrodotoxin specifically blocked voltage-sensitive Na^+ channels, uptake in its presence represented nonspecific $^{22}Na^+$ uptake. All of the following flux data are reported as specific uptake corrected by subtracting nonspecific uptake in the presence of 1 μM tetrodotoxin. The uptake of $^{22}Na^+$ was linear for 5 sec and then reached a plateau. Since it is desirable to measure initial rates of $^{22}Na^+$ influx, all flux measurements reported here were made at 5 sec. Our technique did not allow routine measurements with good precision at times shorter than 5 sec.

Sodium flux was related to membrane permeability by the Goldman-Hodgkin-Katz equation [i.e., equation 2.4 of Hodgkin and Katz (17)]. Flux is directly proportional to permeability only if the membrane potential remains constant during sodium channel activation. Under these conditions, sodium flux increases linearly with external sodium concentration (12). Figure 1B illustrates the relationship between $^{22}Na^+$ uptake and $[Na^+]_{out}$ in the presence of 2.5 μM batrachotoxin. Uptake was linear with increasing $[Na^+]_{out}$ up to 3.0 mM, indicating a constant membrane potential at these sodium concentrations. Beyond 3.0 mM, the curve began to plateau, indicating depolarization. The decrease in driving force upon depolarization was responsible for the decrease in $^{22}Na^+$ uptake. All flux experiments described were performed in the presence of 2 mM NaCl.

To demonstrate that the $^{22}Na^+$ uptake observed was mediated by voltage-sensitive sodium channels, the inhibition of uptake by saxitoxin was studied. Synaptosomes were preincubated with increasing concentrations of saxitoxin for 10 min. The uptake of $^{22}Na^+$ in the presence of 200 μM veratridine was then determined. Saxitoxin inhibited veratridine-stimulated sodium uptake completely, with a $K_{0.5}$ of 6 nM (Fig. 2). Since the K_i for inhibition of action potential sodium currents by saxitoxin ranges from 1 to 10 nM (1), we conclude that veratridine-stimulated sodium uptake is mediated by voltage-sensitive sodium channels in synaptosomes.

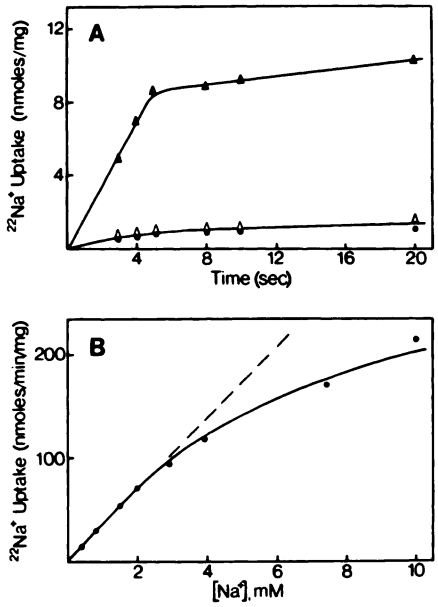


FIG. 1. *Characterization of synaptosomal $^{22}Na^+$ uptake*

A. Time course of $^{22}Na^+$ uptake. Synaptosomes were preincubated with 2.5 μM batrachotoxin (▲——▲), 2.5 μM batrachotoxin plus 1 μM tetradotoxin (△——△), or no toxins (●——●). After a 10-min preincubation, the synaptosomes were incubated with $^{22}Na^+$-containing uptake solution for the indicated time. The $^{22}Na^+$ influx was then measured by dilution, filtration, and washing as described under Materials and Methods.

B. Relationship between $^{22}Na^+$ uptake and $[Na^+]_{out}$. Synaptosomes were incubated for 10 min with 2.5 μM batrachotoxin; $^{22}Na^+$ uptake was then measured for 5 sec in the presence of the indicated Na^+ concentrations as described under Materials and Methods. Nonspecific uptake, determined in the presence of 1 μM tetrodotoxin, has been subtracted from the total uptake.

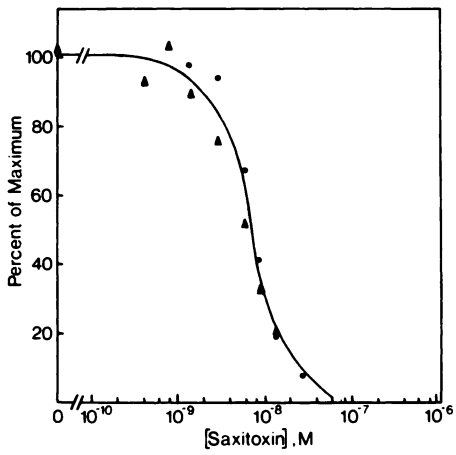


FIG. 2. *Inhibition of veratridine-induced $^{22}Na^+$ uptake and ^3H-labeled saxitoxin binding by unlabeled saxitoxin*

$^{22}Na^+$ uptake was measured in the presence of 200 μM veratridine and increasing concentrations of saxitoxin (●——●). Binding of 2 nM ^3H-labeled saxitoxin in the presence of increasing concentrations of unlabeled saxitoxin was determined (▲——▲). In both cases the synaptosomes were incubated with the toxins for 10 min prior to the measurement of $^{22}Na^+$ uptake or ^3H-labeled saxitoxin binding. Nonspecific $^{22}Na^+$ uptake and ^3H-labeled saxitoxin binding, determined in the presence of 1 μM tetrodotoxin, have been subtracted.

In parallel experiments, synaptosomes were incubated with 2 nM ^{3}H-labeled saxitoxin and increasing concentrations of unlabeled saxitoxin. At 10 min the amount of bound ^{3}H-labeled saxitoxin was determined. The concentration-effect curves for saxitoxin inhibition of $^{22}Na^+$ uptake and displacement of ^{3}H-labeled saxitoxin by unlabeled saxitoxin were superimposable (Fig. 2). Both curves were closely fit by a hyperbola, with $K_{0.5}$ = 6 nM. These results demonstrate a close correlation between binding and inhibition of sodium channels by saxitoxin in synaptosomes and confirm previous results showing that saxitoxin binds to a single class of receptor sites in synaptosomes (10, 13).

Alkaloid toxin stimulation of $^{22}Na^+$ uptake. The alkaloid neurotoxins, batrachotoxin, veratridine, and aconitine, are activators of sodium channels (1). Concentration-effect curves for the stimulation of synaptosomal $^{22}Na^+$ uptake by these three toxins are shown in Fig. 3. Batrachotoxin stimulated uptake with a $K_{0.5}$ of 0.5 μM, whereas veratridine and aconitine had $K_{0.5}$ values of 13 μM and 14 μM, respectively. These $K_{0.5}$ values are similar to those reported for alkaloid toxin-induced $^{22}Na^+$ uptake by neuroblastoma cells. Under the conditions used here, batrachotoxin is defined as a full agonist and veratridine and aconitine are defined as partial agonists.

To demonstrate that these three toxins act at the same site on the sodium channel, a competition experiment was carried out. Synaptosomes were incubated with 1.5 μM batrachotoxin and increasing concentrations of aconitine or veratridine, and $^{22}Na^+$ uptake was measured. As would be expected if the toxins compete for the same binding site, increasing concentrations of either veratridine or aconitine decrease the batrachotoxin-stimulated $^{22}Na^+$ uptake to a level corresponding to that induced by a saturating concentration of either veratridine or aconitine alone (– – –, Fig. 4). We conclude that these three alkaloid toxins act at a common receptor site to cause persistent activation of sodium channels in synaptosomes.

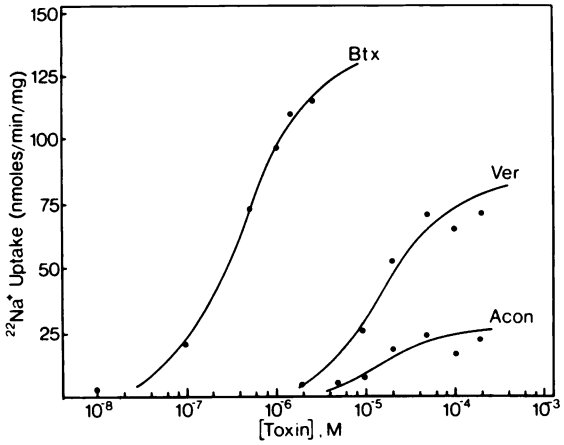


FIG. 3. *Concentration-effect curves for alkaloid toxin-induced $^{22}Na^+$ uptake*

Synaptosomes were incubated with the toxin for 10 min, and $^{22}Na^+$ uptake was measured for 5 sec as described under Materials and Methods. The effect of increasing concentrations of batrachotoxin (*Btx*), veratridine (*Ver*), and aconitine (*Acon*) on uptake are shown. Nonspecific uptake has been subtracted.

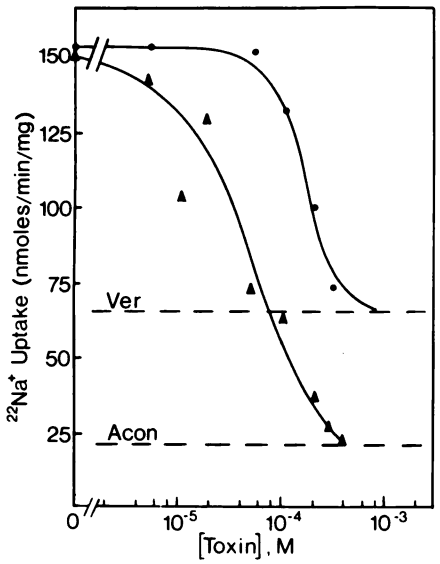


FIG. 4. *Competition between alkaloid toxins*

$^{22}Na^+$ uptake was measured following a 10-min incubation of synaptosomes with 1.5 μM batrachotoxin and increasing concentrations of either veratridine (●——●) or aconitine (▲——▲). – – –, $^{22}Na^+$ uptake induced by a saturating concentration of either veratridine (*Ver*) or aconitine (*Acon*) with no batrachotoxin present.

Polypeptide toxin enhancement of alkaloid toxin-induced $^{22}Na^+$ uptake. Scorpion toxin inhibits sodium channel inactivation but does not, by itself, cause persistent activation of sodium channels (1). However, scorpion toxin markedly enhances alkaloid toxin-induced $^{22}Na^+$ uptake by neuroblastoma cells (1). Figure 5 shows the effect of 200 nM scorpion toxin on alkaloid toxin-induced $^{22}Na^+$ uptake by synaptosomes. The effect of scorpion toxin on batrachotoxin-stimulated uptake is shown in Fig. 5A. Scorpion toxin caused a shift in the concentration-effect curve, lowering the $K_{0.5}$ from 0.5 μM to approximately 0.05 μM. Consistent with the designation of batrachotoxin as a full agonist, no change in the maximal rate of uptake was observed. Figure 5B and 5C shows the effect of scorpion toxin on veratridine- and aconitine-stimulated $^{22}Na^+$ uptake. In both cases the concentration-effect curves were shifted. Scorpion toxin lowered the veratridine $K_{0.5}$ from 13 μM to 0.9 μM and the aconitine $K_{0.5}$ from 14 μM to 1.8 μM. Scorpion toxin increased the maximal uptake rate observed with both veratridine and aconitine. The maximal uptake observed with veratridine plus scorpion toxin approached the rate seen with batrachotoxin. Thus, scorpion toxin converted the partial agonist veratridine to a full agonist. Values for $K_{0.5}$ and for the fraction of ion channels activated at saturation (P_∞) are presented in Table 1.

Polypeptide neurotoxins isolated from sea anemone nematocysts possess activities similar to those of scorpion toxin (1). Concentration-effect curves for anemone and scorpion toxin enhancement of $^{22}Na^+$ uptake stimulated by 20 μM veratridine are shown in Fig. 6. Scorpion toxin is more potent, having a $K_{0.5}$ of approximately 2 nM whereas the two anemone toxins, anemone toxin II and Anthopleurin A give values of approximately 200 nM. Both scorpion toxin and anemone toxin II, although

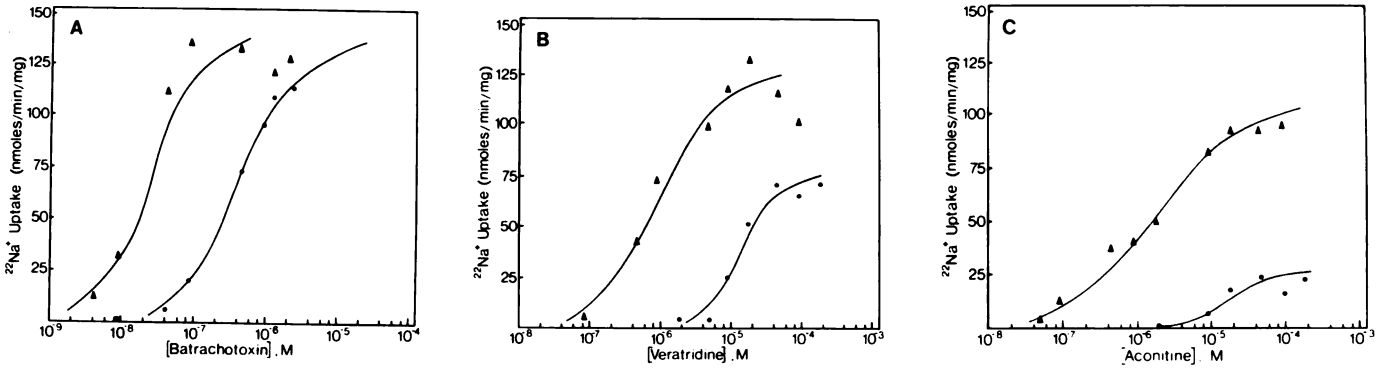


FIG. 5. *Effect of scorpion toxin on alkaloid toxin-induced $^{22}Na^+$ uptake*
Concentration-effect curves for batrachotoxin (A)-, veratridine (B)-, and aconitine (C)-induced uptake in the presence (▲——▲) or absence (●——●) of 200 nM scorpion toxin. Toxins were incubated with the synaptosomes for 10 min prior to a 5-sec flux measurement.

differing in potency, are full agonists with respect to uptake enhancement. Anthopleurin A is a partial agonist, with approximately 50% of the intrinsic activity of anemone toxin II.

Polypeptide toxin displacement of ^{125}I-labeled scorpion toxin. Scorpion toxin binds specifically to receptor sites associated with sodium channels in synaptosomes. Binding studies were used to demonstrate that the three polypeptide neurotoxins bind to the same site on sodium channels in synaptosomes. The ability of scorpion toxin, sea anemone toxin II, and Anthopleurin A to displace radioactively labeled scorpion toxin from its binding site was measured. As shown in Fig. 7, unlabeled scorpion toxin displaced ^{125}I-labeled scorpion toxin with an apparent K_d of 2 nM. The two anemone toxins were equally effective in displacing the ^{125}I-labeled scorpion toxin but had a much lower affinity for the scorpion toxin binding site than did scorpion toxin itself. The anemone toxins had an apparent K_d of approximately 300 nM. Since the anemone toxins could displace ^{125}I-labeled scorpion toxin completely, all three polypeptide toxins appeared to share the same binding site. Although anemone toxin II and Anthopleurin A had equal affinity for the scorpion toxin binding site, Anthopleurin A had approximately half the intrinsic activity of anemone toxin II with respect to enhancing veratridine-stimulated $^{22}Na^+$ uptake. The similarity between the apparent K_d values for binding of the three polypeptide toxins and the $K_{0.5}$ values for enhancement of uptake indicates a close relationship between binding and enhancement of Na^+ channel activation.

Alkaloid toxin enhancement of ^{125}I-labeled scorpion toxin binding to synaptosomes. Previous experiments showed that alkaloid toxins enhance scorpion toxin binding to both intact and depolarized synaptosomes (4, 13). The enhancement is greater for depolarized or lysed synaptosomes (13).[3] Parallel experiments were performed to compare the effects of alkaloid toxins on sodium flux and ^{125}I-labeled scorpion toxin binding. All three alkaloid toxins, batrachotoxin, veratridine, and aconitine, enhanced the binding of ^{125}I-labeled scorpion toxin to lysed synaptosomes. The concentration-effect curves for this enhancement are shown in Fig. 8. Batrachotoxin can be defined as a full agonist in this respect, whereas veratridine and aconitine are partial agonists. The $K_{0.5}$ values for enhancement of binding are 0.5 μM for batrachotoxin and 20 and 40 μM for veratridine and aconitine, respectively. The set of concentration-effect curves for the enhancement of ^{125}I-labeled scorpion toxin binding are almost identical with those for the alkaloid toxin stimulation of $^{22}Na^+$ uptake (Fig. 3). These data suggest that (a) binding of ligands at the alkaloid toxin binding site can affect the polypeptide toxin binding site and (b) the conformational change induced by alkaloid toxin binding that activates the sodium channel is also responsible for the enhancement of ^{125}I-labeled scorpion toxin binding.

The effect of the alkaloid toxins on ^{125}I-labeled scorpion toxin binding in intact synaptosomes at the resting membrane potential was relatively small. Batrachotoxin induced only a 1.8-fold increase in ^{125}I-labeled scorpion toxin binding when intact synaptosomes were used. Veratridine enhancement of binding could not be studied with confidence. Nevertheless, the small enhancement of ^{125}I-labeled scorpion toxin binding by batrachotoxin in intact synaptosomes at the resting membrane potential also correlated with the concentration-effect curve for sodium influx (data not illustrated).

In order to demonstrate that lysis of the synaptosomes

TABLE 1
Parameter values from the allosteric model
Data for $K_{0.5}$ and P_∞ from experiments like those of Figs. 3 and 5 are presented along with values of K_T/K_R calculated from equation 8 of ref. 12. The calculations were made assuming a value of $M_{RT} = 7000$ (10).

Alkaloid toxin	Scorpion toxin	$K_{0.5}$	P_∞	K_T/K_R
	nM	*M*		
Batrachotoxin	0	5.0×10^{-7}	0.95	1.3×10^5
	200	5.0×10^{-8}	>0.99	
Veratridine	0	1.3×10^{-5}	0.54	8.2×10^3
	200	9×10^{-7}	0.89	
Aconitine	0	1.4×10^{-5}	0.18	1.6×10^3
	200	1.8×10^{-6}	0.71	

[3] Nonspecific binding to lysed synaptosomes (200 fmoles/mg/nM) is greater than that to intact, depolarized synaptosomes (110 fmoles/mg/nM). Specific binding to lysed or intact depolarized synaptosomes is similar in both the presence and absence of batrachotoxin (11). Batrachotoxin causes a 5- to 10-fold increase in specific binding in both preparations.

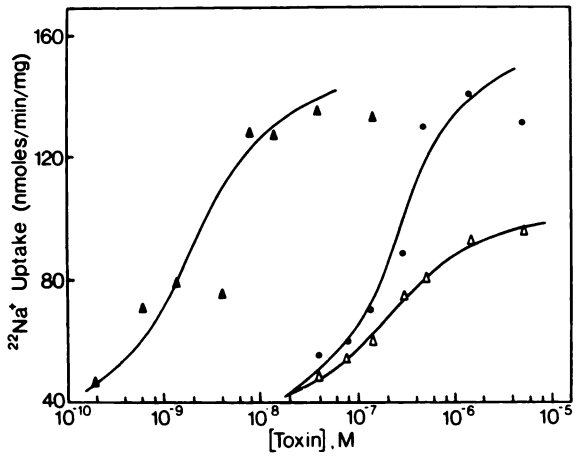


FIG. 6. *Concentration-effect curves for polypeptide toxin enhancement of veratridine-induced $^{22}Na^+$ uptake*

Synaptosomes were incubated for 10 min with 20 μM veratridine and varying concentrations of scorpion toxin (▲——▲), anemone toxin II (●——●), or Anthopleurin A (△——△); $^{22}Na^+$ influx was then measured for 5 sec as described under Materials and Methods.

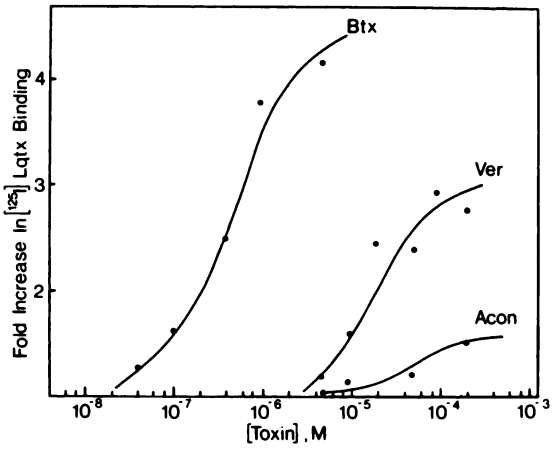


FIG. 8. *Alkaloid toxin enhancement of ^{125}I-labeled scorpion toxin binding to lysed synaptosomes*

The binding of 0.25 nM ^{125}I-labeled scorpion toxin in the presence of varying concentrations of batrachotoxin (*Btx*), veratridine (*Ver*), and aconitine (*Acon*) is shown. The lysed synaptosomes were incubated with the toxins for 10 min, diluted, filtered, and washed as described under Materials and Methods.

does not affect the relationship between alkaloid toxin-induced Na^+ uptake and enhancement of ^{125}I-labeled scorpion toxin binding, the effect of batrachotoxin on $^{22}Na^+$ uptake and ^{125}I-labeled scorpion toxin binding by intact, depolarized synaptosomes was studied in companion experiments. The synaptosomes were depolarized by using 125 mM KCl in both the preincubation and flux solutions. Figure 9 shows the concentration-effect curves for batrachotoxin-induced $^{22}Na^+$ uptake and enhancement of ^{125}I-labeled scorpion toxin binding to intact, depolarized synaptosomes. These two concentration-effect curves are superimposable, with $K_{0.5}$ values of 0.5 μM. The close similarity of concentration-effect curves for alkaloid toxin-induced $^{22}Na^+$ uptake by intact, polarized synaptosomes to those for alkaloid enhancement of ^{125}I-labeled scorpion toxin binding by lysed synaptosomes, therefore, is not an artifact of synaptosome lysis.

Our results provide direct support for the hypothesis (4, 12) that the enhancement of ^{125}I-labeled scorpion toxin binding and the activation of the sodium channel by alkaloid toxins result from the same alkaloid toxin-induced conformational change in the sodium channel. Neither depolarization nor lysis of the synaptosomes has a marked effect on the $K_{0.5}$ for batrachotoxin action (compare ion flux data of Figs. 5A and 9 or binding data of Fig. 9 and ref. 4).

The binding of scorpion toxin to its receptor site in synaptosomes is highly voltage-dependent (4). Depolarization of the synaptosomes increases K_d for scorpion toxin binding without changing the maximal number of binding sites (4). To test whether depolarization alters the coupling of scorpion toxin binding to enhancement

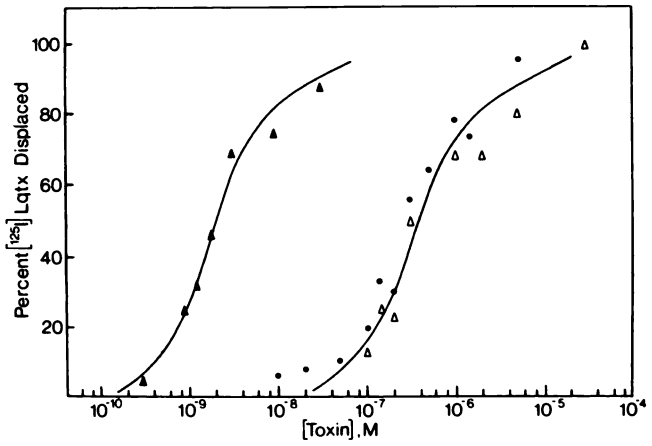


FIG. 7. *Displacement of ^{125}I-labeled scorpion toxin by polypeptide toxins*

The binding of 0.25 nM ^{125}I-labeled scorpion toxin was measured in the presence of varying concentrations of scorpion toxin (▲——▲), anemone toxin II (●——●), or Anthopleurin A (△——△). The synaptosomes were incubated with the toxins for 10 min, diluted, filtered, and washed as described under Materials and Methods.

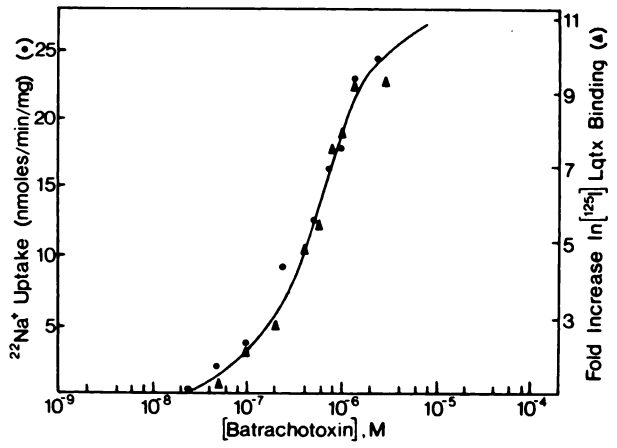


FIG. 9. *$^{22}Na^+$ uptake and ^{125}I-labeled scorpion toxin binding by intact, depolarized synaptosomes*

The synaptosomes were depolarized using 125 mM KCl in all incubation solutions. The effect of increasing batrachotoxin concentrations on $^{22}Na^+$ uptake (●——●) and scorpion toxin (*Lqtx*) binding (▲——▲) is shown. The synaptosomes were incubated with the toxins for 10 min prior to determination of $^{22}Na^+$ uptake or ^{125}I-labeled scorpion toxin binding as described under Materials and Methods.

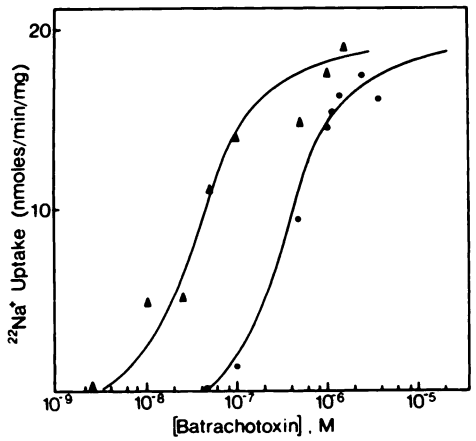


FIG. 10. *Enhancement of batrachotoxin activation by scorpion toxin in depolarized synaptosomes*

Synaptosomes were depolarized using 125 mM KCl in all incubation solutions. After a 10-min incubation with the indicated concentrations of batrachotoxin with (▲——▲) or without (●——●) 1 μM scorpion toxin, $^{22}Na^+$ uptake was measured for 5 sec as described under Materials and Methods.

of batrachotoxin action, concentration-effect curves for batrachotoxin activation of sodium channels were determined in depolarized synaptosomes in the presence and absence of a saturating concentration (1 μM) of scorpion toxin (Fig. 10). Scorpion toxin caused a 10-fold reduction in $K_{0.5}$ for batrachotoxin from 500 nM to 50 nM in depolarized synaptosomes, as previously observed (Fig. 5A) in synaptosomes at the resting membrane potential. Thus, the coupling between occupancy of the scorpion toxin receptor site and enhancement of alkaloid toxin activation is not altered by depolarization, although a marked increase in K_d for scorpion toxin binding occurs.

DISCUSSION

Our sodium flux experiments demonstrate that sodium channels in synaptosomes isolated from rat brain have essentially the same pharmacological properties as those in intact mouse neuroblastoma cells. The alkaloid toxins veratridine, batrachotoxin, and aconitine activate synaptosomal sodium channels by interaction with a common receptor site at which batrachotoxin is a full agonist and aconitine and veratridine are partial agonists. The concentration dependence of activation is very similar to that in earlier results with neuroblastoma cells (12). Sodium channels activated by alkaloid toxins are blocked by saxitoxin, with a $K_{0.5}$ of 6 nM, similar to the concentration required to block sodium flux or sodium current in peripheral nerves and neuroblastoma cells (1). Scorpion and sea anemone toxins bind at a common receptor site and enhance activation of sodium channels by the alkaloid toxins. Scorpion toxin and *Anemonia sulcata* toxin II have greater intrinsic activity in this respect than does Anthopleurin A. Scorpion toxin has 100-fold higher affinity than the two sea anemone toxins as observed in neuroblastoma cells (18). Thus, sodium channels in synaptosomes have three separate neurotoxin receptor sites with functional properties, neurotoxin binding affinities, and allosteric interactions similar to those previously described for neuroblastoma cells. Taken together, these ion flux results lead us to conclude that sodium channels in synaptosomes, a subcellular membrane preparation, retain functional properties that are identical with those in intact nerve cells. Synaptosomes therefore provide an ideal subcellular preparation for correlating the functional properties of sodium channels with structural studies of sodium channel components isolated from rat brain.

Although the results described here are essentially identical with those for neuroblastoma cells, some characteristic differences were noted. In particular, we found that the partial agonists aconitine and veratridine had greater intrinsic activity in the synaptosomal preparation, activating 18% and 54% of sodium channels at saturation as compared with 2% and 8% in neuroblastoma cells.

A major goal of this study was to correlate precisely ligand binding with sodium flux. In each case studied, we found an excellent correlation. Inhibition of sodium flux and block of ^3H-labeled saxitoxin binding by unlabeled saxitoxin gave identical concentration-effect curves (Fig. 2). Enhancement of veratridine activation of sodium channels by polypeptide toxins was precisely correlated with the block of ^{125}I-labeled scorpion toxin binding by polypeptide toxins (Figs. 6 and 7). Enhancement of ^{125}I-labeled scorpion toxin binding by alkaloid toxins was closely correlated with activation of sodium channels by alkaloid toxins (Figs. 3, 8, and 9). These data provide further strong support of the validity of these toxin binding assays as tools with which to identify, isolate, and study sodium channel components.

The results presented herein provide further insight into the mechanism of allosteric coupling between the receptor sites for alkaloid toxins and polypeptide toxins. At the resting membrane potential of synaptosomes [approximately −55 mV (5)], scorpion toxin enhances batrachotoxin action, reducing $K_{0.5}$ from 500 nM to 50 nM. Batrachotoxin has less than a 2-fold effect on the K_d for scorpion toxin binding (4). Thus, although bidirectional allosteric coupling is observed at the resting membrane potential, the effect of scorpion toxin on $K_{0.5}$ for batrachotoxin is quantitatively greater than the effect of batrachotoxin on K_d for scorpion toxin. In depolarized synaptosomes, $K_{0.5}$ for batrachotoxin remains 500 nM (Fig. 10) and saturating concentrations of scorpion toxin shift the $K_{0.5}$ to 50 nM. Depolarization therefore has no effect on batrachotoxin action under these conditions. In contrast, batrachotoxin enhancement of scorpion toxin binding is much greater at 0 mV. The 10-fold increase in binding (Fig. 9) is quantitatively similar to the 10-fold shift in $K_{0.5}$ for batrachotoxin activation caused by scorpion toxin (Fig. 10). Therefore, the effect of batrachotoxin on scorpion toxin binding, like the K_d for scorpion toxin binding itself, is voltage-dependent, whereas activation of sodium channels by batrachotoxin is not detectably voltage-dependent in this membrane potential range.

We have previously presented a two-state allosteric model which successfully describes the activation of sodium channels in neuroblastoma cells by alkaloid toxins and the enhancement by polypeptide toxins (12). We consider here the application of this model to our present results with synaptosomes. Electrophysiological studies of sodium channel function have directly detected three

functional states of sodium channels: the resting state, the active state, and the inactivated state (19). In addition, kinetic studies of activation and inactivation require the existence of multiple resting states with different probabilities of activation and at least two active and inactivated states (19, 20). The models of Hodgkin and Huxley (19) and Armstrong and Bezanilla (20) have 16 and 11 kinetically significant states, respectively. In our ion flux studies, we distinguish only conducting sodium channels from nonconducting channels after equilibrium is reached with respect to toxin action. In applying a two-state model to our data (12), we considered all nonconducting sodium channels as one state *with respect to ion transport activity*, realizing that this state undoubtedly includes substrates with different kinetics and probabilities of activation following a depolarization. At the resting membrane potential of our neuroblastoma cells [−41 mV (21)], voltage clamp data show that nearly all sodium channels are inactivated (22). Therefore, in our earlier flux studies, we measured principally the toxin-induced transition from inactivated to active sodium channels and we described the data in terms of a two-state model in which all nonconducting states (mostly inactivated states) were considered as a single state.

The allosteric model assumes that the alkaloid neurotoxins (A) bind with high affinity to the active (R) state of sodium channels and stabilize active states relative to inactive (T) states according to the law of mass action.

$$\begin{array}{ccc} A + T & \overset{M_{RT}}{\rightleftharpoons} & A + R \\ K_T \uparrow\downarrow & & \uparrow\downarrow K_R \\ TA & & RA \end{array} \qquad (1)$$

The fraction of sodium channels activated (12) is given by

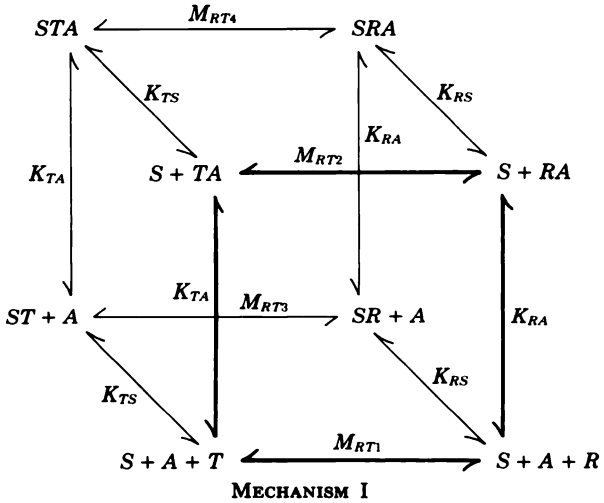
$$P_R(A) = \frac{1}{1 + M_{RT}\dfrac{1 + A/K_T}{1 + A/K_R}} \qquad (2)$$

The effect of scorpion toxin is to reduce M_{RT}. Thus, scorpion toxin reduces the energy required to cause the $T \rightleftharpoons R$ transition.

The three parameters of the model (K_T, K_R, and M_{RT}) cannot be defined uniquely unless a measurable fraction of sodium channels is active in the absence of toxins, allowing direct determination of M_{RT} (12). In synaptosomes, there is no significant effect of tetrodotoxin on $^{22}Na^+$ influx in the absence of an alkaloid toxin (Fig. 1). The data are precise enough to detect activity of 3% of the sodium channels. M_{RT} must therefore be ≥33. A self-consistent fit of the data can be derived in principle with any larger values of M_{RT} and appropriate values of K_R and K_T. To illustrate a plausible set of parameter values and to compare the data with results on neuroblastoma cells, we have taken the value of M_{RT} used to fit the neuroblastoma data [M_{RT} = 7000 (12)] and calculated representative values of K_T/K_R from equation 8 of ref. 12 (Table 1). These data show that the binding constant ratio for batrachotoxin is similar to that in neuroblastoma cells, whereas the ratios for veratridine and aconitine are each approximately 10-fold greater, reflecting their greater intrinsic activity in activating sodium channels in synaptosomes.

The effect of scorpion toxin on activation by each alkaloid toxin is best fit by an 8.9-fold reduction in M_{RT} to 788. This change in M_{RT} fits the data for all three alkaloid toxins, consistent with the conclusion that scorpion toxin causes only a change in M_{RT} and not in K_T/K_R. In our earlier work with neuroblastoma cells (12), the mechanism by which scorpion toxin reduced M_{RT} was not specified. We noted, however, that it could not simply result from selective binding of scorpion toxin to the R state because the 14-fold reduction in $K_{0.5}$ for batrachotoxin in the presence of scorpion toxin was accompanied by less than a 2-fold reduction in K_d for scorpion toxin in the presence of batrachotoxin (12). Similarly, at the resting membrane potential of synaptosomes, scorpion toxin reduced $K_{0.5}$ for batrachotoxin by 10-fold (Fig. 5A) whereas batrachotoxin reduced K_d for scorpion toxin approximately 2-fold (4). Thus, in synaptosomes at the resting membrane potential, scorpion toxin action requires a more complicated mechanism than selective binding to active (R) states.

In contrast, in depolarized synaptosomes, the increase in scorpion toxin binding caused by batrachotoxin (Fig. 9, 10-fold) is quantitatively similar to the 10-fold reduction in $K_{0.5}$ for batrachotoxin caused by scorpion toxin (Fig. 10). Thus, in the absence of membrane potential, the mechanism of scorpion toxin action is adequately described by an allosteric mechanism involving selective binding to active states of the sodium channel. The simplest such mechanism is an extension of Eq. 1 which allows complexes with scorpion toxin (S).



MECHANISM I

In Mechanism I, the equilibria corresponding to Eq. 1 are indicated with *boldface arrows*. K_{TA} and K_{RA} correspond to K_T and K_R for alkaloid toxins. K_{TS} and K_{RS} correspond to K_T and K_R for scorpion toxin. M_{RT1} replaces M_{RT} of Eq. 1 and $M_{RT2} = (K_{RA}/K_{TA}) \times M_{RT1}$. The model includes four values of the allosteric constant M_{RT} for transitions of sodium channels having no toxin (M_{RT1}), an alkaloid toxin (M_{RT2}), scorpion toxin (M_{RT3}), or both toxins (M_{RT4}) bound. For K_{TS}/K_{RS} = 10, as indicated by the data of Fig. 9, this model predicts that M_{RT1}/M_{RT3}

= 10. For a full agonist such as batrachotoxin, a 10-fold reduction in M_{RT} will cause a corresponding 10-fold reduction in $K_{0.5}$ (12). Thus, the model predicts a 10-fold shift of the batrachotoxin concentration-effect curve to the left. This predicted change in $K_{0.5}$ was observed (Fig. 10), indicating that the action of both the alkaloid toxins and scorpion toxin in depolarized synaptosomes is fit by a simple bidirectional allosteric model in which all toxins act by binding tightly to active (R) states of sodium channels.

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