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Pyrethroid Insecticides and DDT Modify Alkaloid-Dependent Sodium Channel Activation and Its Enhancement by Sea Anemone Toxin

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SUMMARY

The effects of saturating concentrations of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] and the pyrethroid insecticides cismethrin and deltamethrin on alkaloid-dependent activation of the voltage-sensitive sodium channel were studied using measurements of $^{22}Na^+$ uptake into mouse brain synaptosomes. In survey experiments, these compounds enhanced sodium uptake stimulated by veratridine and batrachotoxin, but inhibited uptake stimulated by aconitine. Concentration response curves for aconitine run in the absence and presence of 10 μM cismethrin demonstrated that the inhibition was noncompetitive. This unanticipated inhibitory effect of insecticides on aconitine-dependent sodium uptake suggests a possible overlap or negative allosteric coupling between the binding sites for insecticides and aconitine and reveals unique characteristics of the action of aconitine that are not shared by veratridine and batrachotoxin. More detailed studies of the effects of insecticides on veratridine- or batrachotoxin-stimulated uptake found small insecticide-dependent increases in the potency of these activators. In addition to this effect, DDT and deltamethrin also enhanced maximal uptake stimulated by veratridine. Possible mechanisms underlying these effects of insecticides on alkaloid-dependent uptake are discussed in light of a qualitative model formulated from these results and previous biochemical and electrophysiological studies. Additional experiments were designed to assess the interactions of insecticides and toxin II of the sea anemone *Anemonia sulcata* (ATX II) as modifiers of alkaloid-dependent uptake. DDT and ATX II acted synergistically to increase uptake stimulated by veratridine. Moreover, DDT shifted the potency of ATX II for enhancing veratridine-dependent uptake to 5-fold lower concentrations. In contrast, DDT and subsaturating concentrations of ATX II acted independently in their enhancement of sodium channel activation by batrachotoxin. Mutually exclusive effects on veratridine-dependent uptake were observed when cismethrin was co-applied with ATX II. However, independent effects of cismethrin and ATX II were found with aconitine-modified channels, in that cismethrin was able to inhibit ATX-II-enhanced aconitine-dependent sodium flux. Thus, the interactions between insecticides and ATX II as modifiers of alkaloid-dependent uptake are complex and depend on the insecticide-activator combination under study.

Pyrethroid insecticides and DDT are potent excitatory neurotoxins that cause repetitive firing and depolarization of insect motor nerve terminals (1), vertebrate sensory neurons (2), and arthropod giant axons (3). Voltage clamp studies demonstrate that the observed effects of pyrethroids and DDT on intact nerves can be ascribed to a prolongation of sodium channel currents (3). Patch clamp analysis of pyrethroid-modified sodium channels from N1E-115 neuroblastoma cells shows a marked prolongation of both the open time of modified channels and the intervals between channel openings, with no effect on single channel conductance and only a small (~15 mV) shift in the voltage dependency of channel opening (4). These findings suggest a specific interaction between pyrethroids and the sodium channel that results in fewer kinetic transitions between conducting and nonconducting channel states (4).

Interactions of pyrethroids and DDT with sodium channels have also been explored using radiosodium flux techniques. In both neuroblastoma (5, 6) and mouse brain synaptosomes (7), pyrethroids and DDT fail to stimulate sodium flux when assayed alone but enhance the stimulation of sodium uptake produced by activating neurotoxins. In N1E-115 adrenergic neuroblastoma cells, the neurotoxic pyrethroids deltamethrin and kadethrin enhance sodium uptake stimulated by VTD, BTX, and dihydrograyanotoxin II (5). Sodium channel activation and pyrethroid-dependent enhancement are also observed

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ABBREVIATIONS: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; ACN, aconitine; ATX II, toxin II isolated from the venom of *Anemona sulcata*; iso-ATX II, isoleucine isotoxin of ATX II: BSA, bovine serum albumin; BTX, batrachotoxin; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20-α-benzoate; E_{max}, maximum sodium uptake at saturating concentration of toxin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)*N,N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $K_{0.5}$, concentration of toxin giving half-maximal sodium uptake; VTD, veratridine.

in C9 neuroblastoma cells with the polypeptide toxins from the scorpion *Androctonus australis* and the sea anemone *Anemonia sulcata*, which are thought to act at a different site on the sodium channel than alkaloid activators and grayanotoxins (5). However, neuroblastoma cell sodium channels do not respond consistently to insecticides, since some neurotoxic pyrethroids fail to enhance alkaloid-dependent sodium uptake in N1E-115 cells (5), and some neurotoxic DDT analogs fail to enhance ATX II-dependent sodium uptake when assayed in C9 cells (6). Moreover, those neurotoxic pyrethroids and DDT analogs found to be ineffective in assays with neuroblastoma cells in culture enhance VTD-dependent sodium uptake by mouse brain synaptosomes (8). Thus, the insecticide recognition properties of sodium channels from mammalian brain differ from those of sodium channels in cultured neuroblastoma cells.

A broader range of studies is required to characterize the interactions of DDT and pyrethroids with mammalian brain sodium channels and to determine the extent of divergence in the responses of brain and neuroblastoma channels to these compounds. In this paper we describe the effects of DDT and two pyrethroid insecticides on sodium uptake stimulated by VTD, BTX, and ACN in mouse brain synaptosomes. We also describe the separate and combined actions of these insecticides and ATX II as modifiers of alkaloid-dependent sodium channel activation. The results of these studies provide evidence for an unanticipated inhibitory interaction between ACN and the tested insecticides and also for complex interactions between insecticides and ATX II as enhancers of alkaloid-dependent sodium uptake. A preliminary communication of some of these results has been presented (9).

Materials and Methods

Chemicals. EDTA, EGTA, BSA, and ACN were purchased from Sigma Chemical Co., St. Louis, MO. VTD was purified from veratrine (Sigma) on 0.5-mm silica gel 60 chromatoplates (EM Laboratories, Elmsford, NY) developed in cyclohexane/diethylamine (7:3, $R_f = 0.27$) as described by Zeitler (10). Carrier-free ^{22}NaCl was obtained from New England Nuclear, Boston, MA. BTX was generously provided by Dr. John Daly, National Institutes of Arthritis, Metabolism, and Digestive Disease, Bethesda, MD. Ouabain, ATX II, and iso-ATX II were purchased from Calbiochem-Behring, La Jolla, CA. ATX II and iso-ATX II were equivalently active as enhancers of alkaloid-dependent sodium uptake when present at saturating concentrations and were used interchangeably in such experiments. Only ATX II was used in experiments in which the concentration of the anemone toxin was varied. Throughout the text, the actions of both toxins are ascribed to "ATX II" for simplicity. DDT was obtained from Chem Service, West Chester, PA. Cismethrin was a gift from M. Elliott, Rothamsted Experimental Station, Harpenden, England, and was purified by preparative high resolution liquid chromatography on a LiChrosorb SI-60 column (EM Laboratories) eluted with 2% ethyl acetate in hexane at a flow rate of 4 ml/min. Deltamethrin was a gift from J. Martel, Roussel-Uclaf, Romainville, France.

Preparation of synaptosomes. Synaptosomal membrane vesicles were prepared daily from the brains of male ICR mice (Blue Spruce Farms, Altamont, NY) using the rapid method of Dodd *et al.* (11). The resulting synaptosomal pellets were coarsely dispersed with a Pasteur pipette and held on ice in a sodium-free preequilibration buffer that contained (mM): choline chloride (130), KCl (10), $MgCl_2$ (3), glucose (5.5), ouabain (3), KCN (2), HEPES-Tris (30), pH 7.4, and 1 mg/ml BSA. Just before treatment with toxins, the synaptosomes were gently resuspended by hand in a tight-fitting Teflon-glass homogenizer. The protein content of membrane preparations was determined by the method of Lowry *et al.* (12).

Sodium uptake experiments. Previous studies (13) of toxin-dependent $^{22}Na^+$ uptake have shown that the slow action of many neurotoxins requires preincubation with membranes prior to initiation of flux measurements. In our study, the lipophilic sodium channel activators BTX, VTD, and ACN were applied to incubation tubes in an ethanolic solution. The solvent was allowed to evaporate and aliquots (100 μl; ~0.4–0.6 mg of protein) of synaptosomal suspension were then added. Insecticides and anemone toxins were added to the membrane/activator mixture in 0.4 μl of ethanol and 1 μl of sodium-free preequilibration buffer, respectively. Controls received an equal volume of ethanol or buffer. Synaptosomes were allowed to equilibrate with toxins for ~18 min at room temperature. The tubes were then transferred to a water bath at 37° for 1.5–2 min and $^{22}Na^+$ flux was initiated by the addition of 100 μl of flux buffer, immediately followed by vortex mixing. The flux buffer contained (mM): choline chloride (138), NaCl (4), glucose (5.5), EGTA (0.2), $MgCL_2$ (3), ouabain (3), KCN (20), HEPES-Tris (30), pH 7.4, 1 mg/ml BSA, and 200 nCi of ^{22}NaCl. The flux buffer also contained alkaloid activators and anemone toxins at concentrations equal to those included during preincubation. Adding the insecticides to the flux buffer either as a residue or as an ethanolic solution did not enhance their effectiveness. Instead, the additional quantity of solvent introduced in the ethanolic solutions reduced activator-dependent uptake in the controls (data not shown). Therefore, the insecticides were omitted from the flux buffer. After incubation for 15 sec at 37°, uptake was terminated by the addition of 3 ml of ice-cold stopping buffer containing (mM): choline chloride (140), glucose (5.5), $MgCl_2$ (3), HEPES-Tris (30), pH 7.4. The uptake mixture was immediately filtered on type HA filters (0.45-μm pore size, Millipore Corp., Bedford, MA) and washed twice with 3 ml of ice-cold stopping buffer. Radioactivity on the filters was quantified using liquid scintillation spectrometry. Data are presented as total uptake in 15-sec incubations without consideration of initial rates of uptake. Prior studies (13, 14) have shown that the initial rate of $^{22}Na^+$ uptake in synaptosomes becomes nonlinear after 5 sec. However, we found that insecticide effects measured in 15-sec incubations permitted greater accuracy in determining low levels of stimulated flux. Except where indicated, the data have been corrected for appropriate levels of sodium uptake measured in the absence of the toxin employed as the independent variable. Each data point was replicated at least twice with three determinations in each replicate.

Results

Preliminary survey experiments (Fig. 1) explored the effects of high concentrations of DDT, cismethrin, and deltamethrin on sodium channel activation by VTD, BTX, and ACN. For each activator, the three insecticides produced qualitatively similar results. All three compounds enhanced activation by VTD and BTX. For both of these alkaloids, the enhancement by DDT was approximately equal to that by deltamethrin at the concentrations used, whereas cismethrin was less effective. In contrast to these findings, all three insecticides inhibited ACN-stimulated sodium uptake. In this case, cismethrin was the most effective inhibitor, reducing uptake by nearly 70%, whereas deltamethrin and DDT were less effective. Previous studies have shown that 20 μM deltamethrin is a saturating concentration for the enhancement of VTD-dependent sodium uptake (7), and both DDT and cismethrin approach their limits of solubility at 100 μM. Unless otherwise indicated, these concentrations were used in all subsequent experiments.

The unique inhibitory effects of these insecticides on ACN-stimulated sodium uptake prompted additional studies using cismethrin, the most effective inhibitor in our preliminary experiments. Concentration response curves for ACN-stimulated uptake in the presence or absence of 10 μM cismethrin

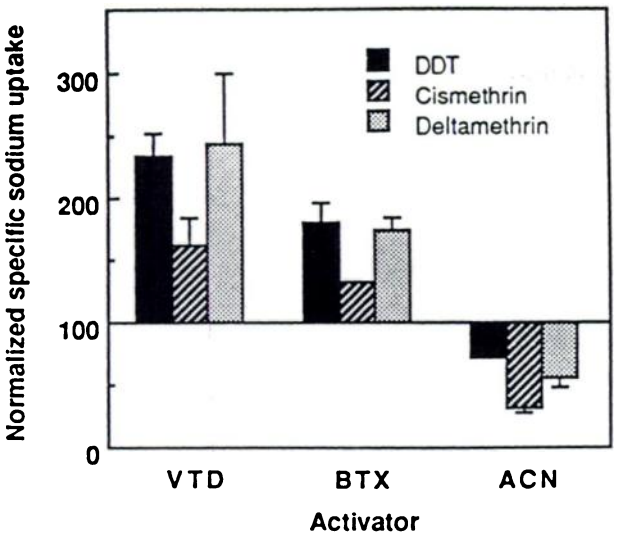

Fig. 1. Insecticide-dependent enhancement or inhibition of sodium uptake into mouse brain synaptosomes stimulated by VTD (30 μM), BTX (1 μM), or ACN (100 μM). Insecticide concentrations were 100 μM (DDT and cismethrin) or 20 μM (deltamethrin). The extent of sodium uptake stimulated by each activator in the absence of insecticides is set equal to 100. *Bars* show standard errors of three replicate experiments.

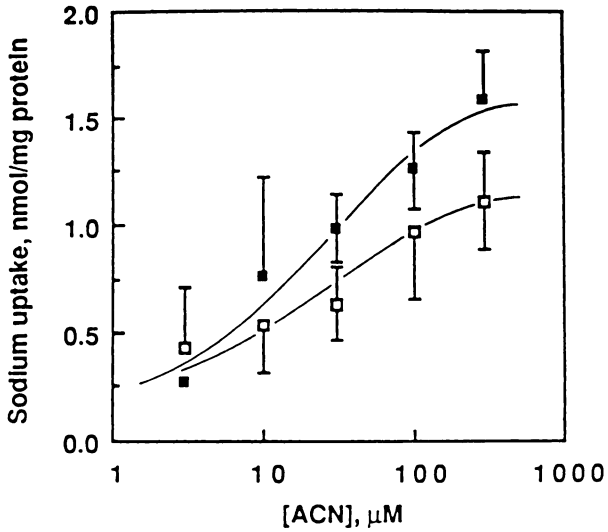

Fig. 2. Concentration dependence of ACN-stimulated sodium uptake into mouse brain synaptosomes (■) and its inhibition by cismethrin (10 μM; □). *Bars* show standard errors of three replicate experiments.

(Fig. 2) showed that cismethrin reduced specific sodium uptake stimulated by high concentrations of ACN by approximately 34% and also caused a small increase in the potency of ACN measured as the $K_{0.5}$ for the stimulation of sodium uptake (Table 1). Since the observed inhibition by this subsaturating concentration of cismethrin was not reversed at high concentrations of ACN, the antagonistic effect of cismethrin was apparently noncompetitive. Quantitation of cismethrin-dependent inhibition was complicated by the small dynamic range and variability of sodium uptake stimulated by ACN. This limitation, together with the lower efficacy of DDT and deltamethrin as inhibitors (see Fig. 1), precluded the expansion of these studies to include the other two insecticides.

Similar experiments revealed qualitative differences between the actions of the insecticides on VTD-stimulated sodium uptake (Fig. 3). DDT and deltamethrin increased the effectiveness

TABLE 1

Calculated $K_{0.5}$ and E_{max} values for sodium uptake stimulated by ACN, VTD, and BTX measured alone or in the presence of insecticides and ATX II

Mean data points for curves from Figs. 2–5 and 8 were fitted to the integrated form of the Michaelis-Menten equation according to the method of Wilkinson (15).

Activator	Modifier (μM)	$K_{0.5}$	E_{max}
		μM	nmol/mg protein
ACN	None	13.3 ± 3.6	1.5 ± 0.1
	Cismethrin (10)	7.3 ± 3.3	1.0 ± 0.2
	ATX II (1)	1.0 ± 0.3	13.3 ± 0.9
	ATX II (1) + cismethrin (100)	0.8 ± 0.3	5.4 ± 0.4
VTD	None	32.1 ± 5.5	5.3 ± 0.4
	DDT (100)	20.5 ± 6.0	9.5 ± 0.9
	Cismethrin (100)	10.8 ± 3.5	5.1 ± 0.6
	Deltamethrin (20)	15.3 ± 2.6	7.2 ± 0.4
	ATX II (1)	2.7 ± 0.6	11.0 ± 0.7
	ATX II (1) + cismethrin (100)	2.2 ± 0.7	9.8 ± 0.9
	ATX II (1) + DDT (100)	1.5 ± 0.1	16.9 ± 0.2
BTX	None	4.0 ± 1.0	21.7 ± 2.8
	DDT (100)	2.0 ± 0.6	19.3 ± 2.6

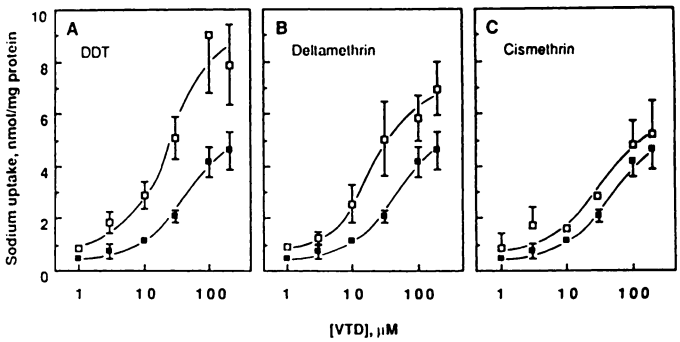

Fig. 3. Concentrated dependence of VTD-stimulated sodium uptake into mouse brain synaptosomes (■, A–C) and its enhancement by DDT (100 μM; □, A), deltamethrin (20 μM, □ B), or cismethrin (100 μM; □, C). *Bars* show standard errors for six to nine (VTD alone) or three (VTD plus insecticide) replicate experiments.

of VTD as a sodium channel activator at all VTD concentrations. Calculation of $K_{0.5}$ values from the curves shown in Fig. 3, A and B (Table 1), revealed small insecticide-dependent increases in the potency of VTD with these compounds as well. The effect of cismethrin on VTD-dependent sodium channel activation was more subtle, involving a small potency shift with no effect on efficacy (Table 1). The absence of an effect of cismethrin on the efficacy of VTD is consistent with the low levels of enhancement observed for this compound in relation to the other two insecticides in survey experiments (Fig. 1).

We also explored insecticide-BTX interactions in a more limited series of experiments. The choice of DDT for these studies was based on its effectiveness in survey experiments (Fig. 1) and on the lack of data for this compound in previous studies of the effects of insecticides on sodium channel activation by BTX in neuroblastoma cells (5). In paired experiments, DDT increased the observed potency of BTX 2-fold but had no effect on the efficacy of this activator at concentrations approaching saturation (Fig. 4, Table 1). The magnitude of the effect of DDT on the potency of BTX was similar to that observed with VTD. Preliminary experiments with cismethrin also showed a small increase in the potency of BTX with no increase in maximal uptake (data not shown).

The enhancement of VTD- and BTX-stimulated sodium uptake by insecticides led us to test for synergy between insec-

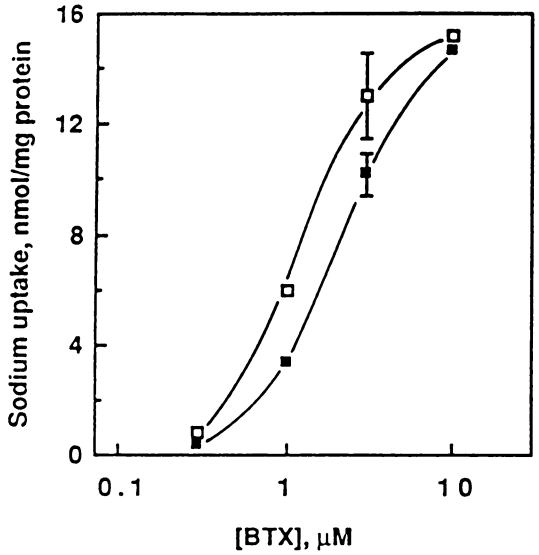

Fig. 4. Concentration dependence of BTX-stimulated sodium uptake into mouse brain synaptosomes (■) and its enhancement by DDT (100 μM; □). *Bars* show standard errors of three replicate experiments.

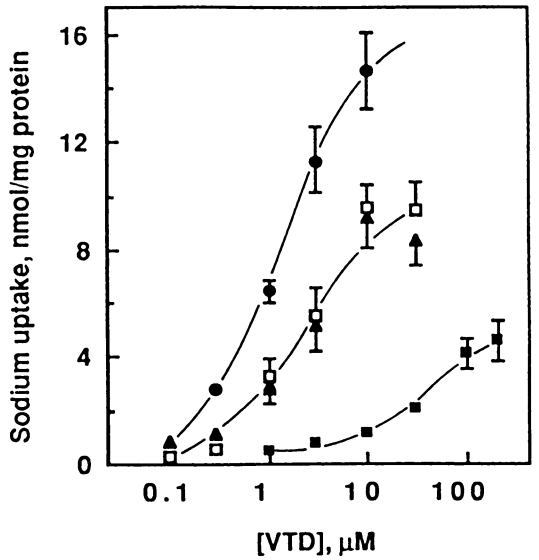

Fig. 5. Concentration dependence of VTD-stimulated sodium uptake into mouse brain synaptosomes (■) and its enhancement by ATX II (1 μM; □), ATX II (1 μM) plus cismethrin (100 μM; ▲), or ATX (1 μM) plus DDT (100 μM; ●). *Bars* show standard errors of six to nine (VTD alone), two (ATX II plus DDT), or three (all other) replicate experiments.

ticides and the polypeptide toxin ATX II, which is also known to enhance alkaloid-dependent sodium uptake in rat brain synaptosomes (13) and to activate sodium channels of neuroblastoma cells (5). Initial experiments evaluated the effects of ATX II alone and in combination with each other of the three insecticides. ATX II alone or in combination with either DDT, cismethrin, or deltamethrin did not appreciably increase sodium uptake above background levels (data not shown).

We also evaluated the independent and combined effects of ATX II and either DDT or cismethrin on VTD-dependent sodium uptake (Fig. 5). ATX II at 1 μM increased the potency of VTD approximately 12-fold and enhanced maximal uptake more than 2-fold (Table 1). DDT and ATX II in combination produced a further enhancement of VTD-dependent uptake, both increasing the potency of VTD by almost 2-fold and enhancing maximal uptake an additional 54% above the level obtained with ATX II alone. In contrast, the response curve of VTD in the presence of cismethrin and ATX II in combination was superimposable on that obtained with ATX II alone and yielded identical values for potency and maximal uptake (Table 1).

Since DDT and cismethrin differed in their ability to further enhance VTD-dependent sodium uptake in the presence of high concentrations of ATX II (Fig. 5), it was of interest to determine whether the divergent properties of these two compounds could be observed at lower ATX II concentrations. The presence of DDT enhanced ATX II-specific uptake by a similar increment at all ATX II concentrations (Fig. 6). Subtraction of the expected DDT-specific uptake under these conditions in the absence of ATX II (Fig. 6, *dashed line*) normalized the combined DDT-ATX II response curve for direct comparison with results obtained in paired experiments with ATX II alone. Comparison of these curves shows that DDT enhances the potency of ATX II 5-fold, decreasing the $K_{0.5}$ of ATX II from 0.58 to 0.12 μM. In contrast to the results obtained with DDT, we observed no effect of cismethrin on ATX-II dependent uptake at ATX-II concentrations at or above 10 nM (data not shown). At concentrations below 10 nM no ATX II-specific effect was observed, leaving only the enhancement of uptake produced by 100 μM cismethrin in the presence of 10 μM VTD.

Parallel studies were also undertaken to determine the effect of DDT on ATX II-specific uptake using BTX as the activating neurotoxin. Concentration response curves were obtained for ATX II as an enhancer of BTX-stimulated uptake either alone or in the presence of DDT (Fig. 7). Inclusion of DDT at 100 μM further enhanced sodium uptake at subsaturating concentrations of ATX II, but this effect was not observed at ATX II

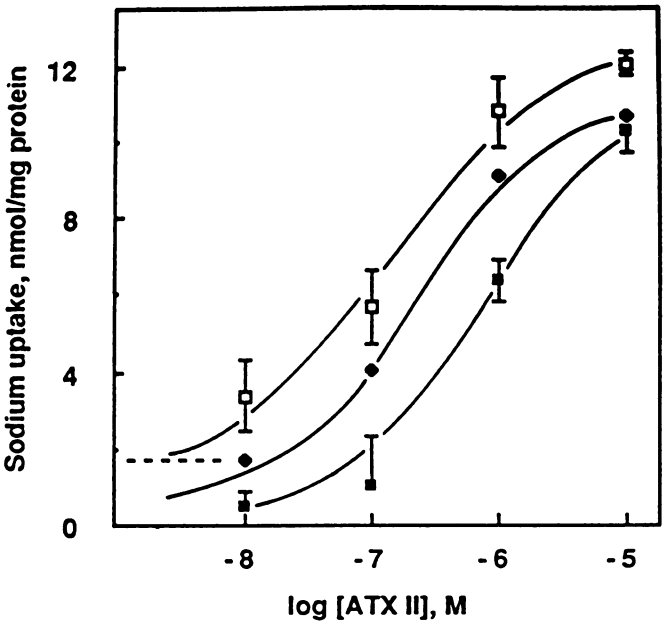

Fig. 6. Concentration dependence of the enhancement by ATX II of VTD (10 μM)-dependent sodium uptake into mouse brain synaptosomes measured alone (■) or in the presence of DDT (100 μM; □). *Bars* show standard errors of three replicate experiments. – – –, the DDT-dependent increment of specific sodium uptake obtained with 100 μM DDT in the presence of 10 μM VTD (calculated from Fig. 4A). ♦, the normalization of the curve obtained in the presence of DDT by subtraction of the DDT-dependent increment of uptake indicated by the *dashed line*.

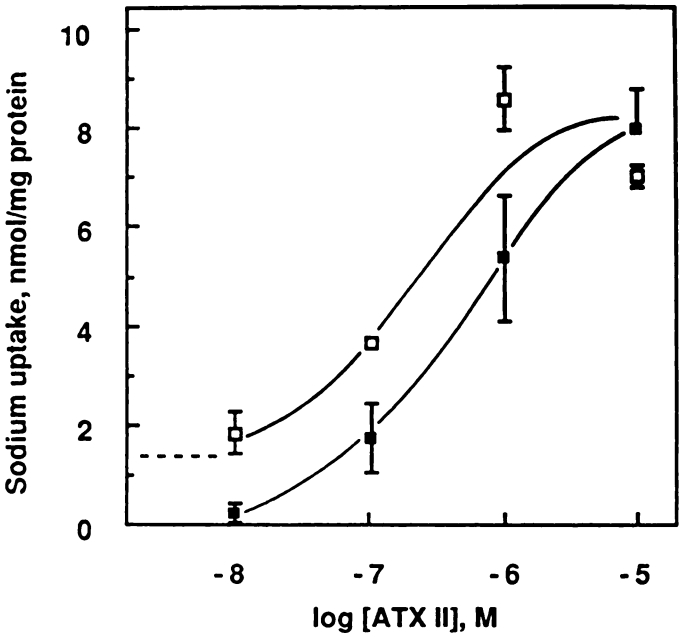

Fig. 7. Concentration dependence of the enhancement by ATX II of BTX (0.5 μM)-dependent sodium uptake into mouse brain synaptosomes measured alone (■) or in the presence of DDT (100 μM; □). *Bars* show standard errors of two replicate experiments. – – –, the expected DDT-dependent increment of specific sodium uptake obtained with 100 μM DDT in the presence of 0.5 μM BTX (interpolated from Fig. 5).

concentrations approaching saturation. Subtraction of the expected DDT-specific uptake from the combined ATX II-DDT curve yielded a normalized curve (not shown) that overlapped the concentration response curve for ATX II alone. Thus, in the presence of BTX, DDT did not exert the marked effect on the potency of ATX II observed with VTD as the activating neurotoxin. The calculation of potency and efficacy values for the normalized curve was complicated by the lack of an independent effect of DDT at saturating concentrations of ATX II.

The ineffectiveness of cismethrin as a modifier of VTD-dependent sodium uptake in the presence of ATX II (Fig. 5) led us to explore whether the inhibitory actions of this compound on ACN-dependent uptake were also suppressed in the presence of ATX II. ATX II at a high concentration greatly enhanced ACN-dependent sodium uptake (Fig. 8), increasing the potency approximately 13-fold and the calculated maximal uptake approximately 9-fold (Table 1). In the presence of 100 μM cismethrin, the effect of ATX II was strongly inhibited at all ACN concentrations (Fig. 8). The primary effect of cismethrin was a 59% reduction in E_{max}, since the potency of ACN was similar to that measured in the presence of ATX II only (Table 1). Thus, the small cismethrin-dependent increase in ACN potency we observed in the absence of ATX II (Table 1) was either not expressed in the presence of ATX II or was masked by the large 13-fold increase in the potency of ACN caused by ATX II.

We also explored the combined actions of cismethrin and ATX II on ACN-dependent sodium uptake at subsaturating ATX II concentrations (Fig. 9). ATX II produced a concentration-dependent enhancement of the uptake stimulated by 30 μM ACN. When corrected for ACN-dependent uptake in the absence of ATX II, we calculated a $K_{0.5}$ of 0.39 μM and an E_{max} value of 11.7 nmol Na uptake/mg of protein for ATX II. Inclusion of 100 μM cismethrin in parallel incubations partially

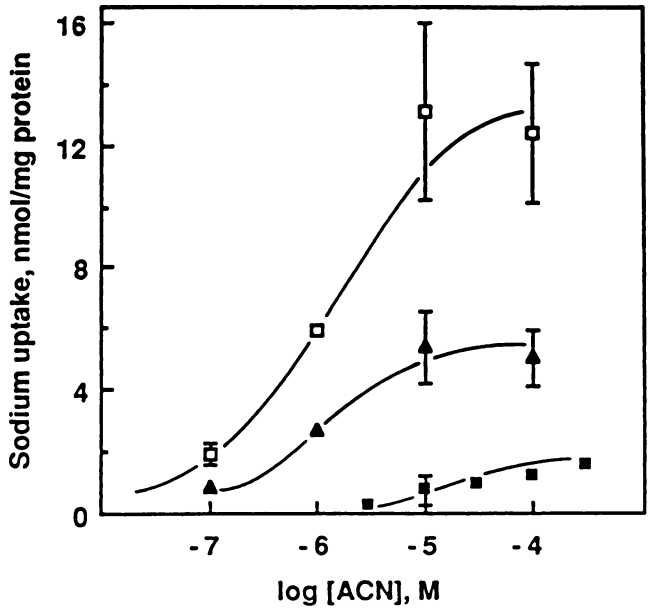

Fig. 8. Concentration dependence of ACN-stimulated sodium uptake into mouse brain synaptosomes (■) and its enhancement by ATX II (1 μM; □) or ATX II (1 μM) plus cismethrin (100 μM; ▲). *Bars* show standard errors of three replicate experiments.

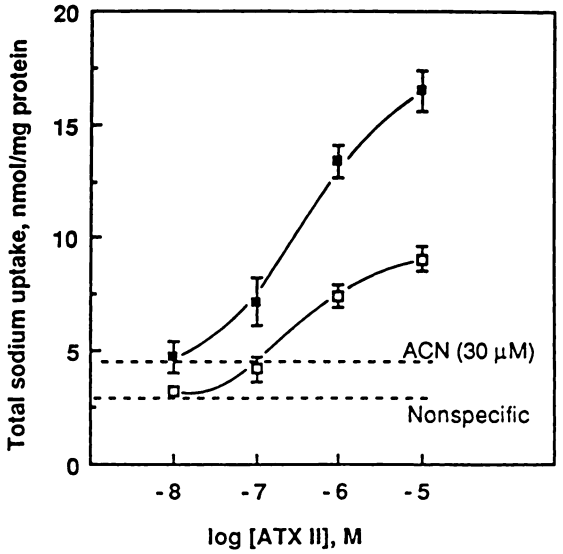

Fig. 9. Concentration dependence of the enhancement by ATX II of ACN (30 μM)-stimulated total sodium uptake into mouse brain synaptosomes measured alone (■) or in the presence of cismethrin (100 μM; □). All data points are uncorrected for nonspecific uptake. Bars show standard errors of three replicate experiments. The *upper horizontal dashed line* shows total sodium uptake obtained in the presence of 30 μM ACN (calculated from Fig. 3), and the *lower horizontal dashed line* shows a typical level of nonspecific sodium uptake obtained in the absence of toxins.

inhibited ATX II-enhanced ACN-dependent uptake at all ATX II concentrations. We calculated the ATX II-dependent component of sodium uptake in the presence of cismethrin and ACN by subtracting the estimated sodium uptake available from incubations containing only 30 μM ACN and 100 μM cismethrin. Parameters from a computer fit of the normalized curve show that cismethrin caused a slight reduction in the calculated potency of ATX II ($K_{0.5}$ = 0.53 μM) and a 48% reduction in calculated maximal ATX II-enhanced uptake (E_{max} = 6.1 nmol Na uptake/mg of protein).

Discussion

In mouse synaptosomal sodium uptake assays, DDT and pyrethroids do not activate sodium flux and their effects on sodium channel function are observed only in conjunction with activating neurotoxins. These findings are consistent with previous ion flux studies on neuroblastoma cells (5) and with voltage clamp studies demonstrating that these insecticides prolong voltage-activated sodium currents but induce only small (10–20 mV) shifts in the voltage dependency of sodium channel activation (4, 16–18). The effects of insecticides on sodium channel activation are much smaller in magnitude than those of alkaloid activators, which induce large (50–90 mV) shifts in the voltage dependency of activation in addition to their effects on inactivation (19–21).

The DDT- and deltamethrin-dependent increases in the efficacy of VTD as an agonist at the activator recognition site (Fig. 3) are consistent with previous studies of deltamethrin-VTD interactions in mouse brain synaptosomes (8) and with pyrethroid effects on sodium channels activated by both VTD and dihydrograyanotoxin II in neuroblastoma cells (5). However, these previous investigations did not observe the increased potency of VTD induced by deltamethrin in the present study. The cismethrin-dependent increase in the potency of VTD without a concomitant increase in efficacy suggests that there are two distinct effects of insecticides on VTD-dependent activation that may vary independently with the structure of the insecticide ligand. For the three insecticides in this study, there is no apparent correlation between the type of effect on VTD-dependent activation and either chemical structure or the lifetime of insecticide-modified sodium currents, as measured by decay constants for sodium tail currents under voltage clamp conditions (3). In contrast to its effect on VTD-dependent activation, DDT increased the potency of BTX as an activator without altering efficacy. The different actions of insecticides on activation by VTD and BTX are analogous to the interactions of α-toxins with these activators in assays with rat brain synaptosomes (13).

The inhibition of ACN-stimulated sodium uptake by DDT and pyrethroids is unprecedented and represents a unique action of these compounds that differentiates them from α-polypeptide toxins and brevetoxins, which act uniformly as allosteric enhancers of alkaloid-dependent sodium channel activation (13, 22). Our characterization of this effect was limited by the low levels of sodium uptake stimulated by ACN in this assay and by the incomplete inhibition afforded by high concentrations of DDT and deltamethrin. However, the interactions between cismethrin and ACN measured both in the absence and in the presence of ATX II demonstrate a noncompetitive inhibition of ACN-dependent activation. Although ACN displaces [^{3}H]BTX-B binding to the activator site (23) and functions as a partial agonist at this site in sodium uptake assays (13), the insecticide effects observed with this compound suggest that either the interactions of ACN with the activator site or the properties of the ACN-modified channel differ from those of BTX and VTD. The unique properties of ACN-modified channels in relation to insecticide-sodium channel interactions are also evident in the resistance profile of mutant strains of the housefly that exhibit reduced neuronal sensitivity to DDT and pyrethroids. These strains are also resistant to ACN both in toxicity bioassays (24, 25) and at the level of the nerve (26), but this resistance does not appear to extend to VTD (24).

A qualitative conceptual model describing the interactions of insecticides and alkaloid activators with the voltage-sensitive sodium channel can be formulated from our data and results of previous biochemical and neurophysiological studies (Fig. 10). Although evidence for multiple channel states has been observed in detailed kinetic studies (27), in the simplest case the sodium channel exists in three functional states: resting (R), open (O), and inactivated (I). Since voltage clamp studies found that DDT and pyrethroids (P) interact with sodium channels in both the R (28, 29) and O states (20, 28), we show the formation of the RP and OP states to be modification pathways for these compounds. The alkaloid activators (A) are thought to require the presence of the open configuration in order to modify the channel-gating mechanism. This conclusion is predicated on the observation that repetitive stimulation of nerve fibers (30) or repetitive delivery of depolarizing voltage clamp steps (21, 31) increases the rate of sodium channel modification by VTD and BTX. Thus, we assume that alkaloid-dependent modification of sodium channels in synaptosomes occurs principally in the open state. This activator-modified open state (OA) can undergo transition to a closed, activator-modified state (CA) (30, 32). We hypothesize that the activator-modified open channels can bind insecticide to form an activator-ion channel-insecticide ternary complex (OAP). Alternatively, formation of this state could result from activator binding to open channels modified by insecticide. We also hypothesize that the ternary complex may undergo relaxation to a closed modified state (CAP).

This model does not consider removal of inactivation by insecticides and ATX II to be a critical step in enhancing activator-dependent flux by providing a larger pool of open channels available for activator modification (31, 33). We would expect the existence of a large pool of insecticide-modified or α-toxin-modified open channels to permit measurable sodium uptake into synaptosomes. However, when assayed alone or in combination, insecticides and ATX II showed no increase in sodium uptake above background levels. Thus, the insecticide-modified or α-toxin-modified open state must be a small fraction of the total sodium channel complement, even at high ligand concentrations. We therefore defined the OP state in our model as functionally nonconducting, leaving only the OA and OAP channel states as conducting from the perspective of

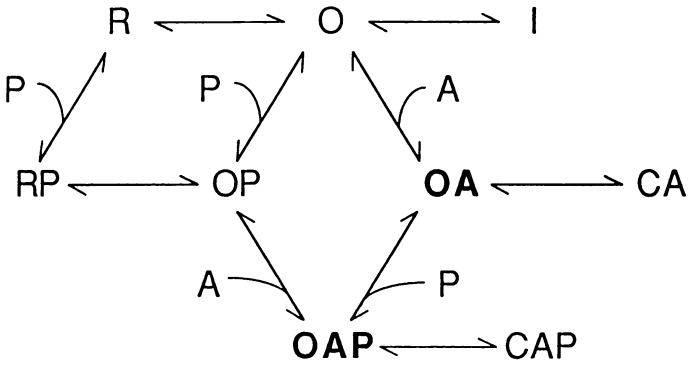

Fig. 10. Model of the interactions of insecticides and alkaloid activators with the voltage-sensitive sodium channel. Conducting states of toxin- or insecticide-modified channels detectable by increased sodium flux above background are shown in *boldface lettering*. See the text for explanation.

radiosodium uptake. As a consequence, the recruitment of open channels by alkaloid activators into a long-lived open state (OA) available for insecticide binding would be consistent with the observed requirement for chemical activation and would probably be the dominant pathway to the doubly modified OAP state.

Given the restrictions placed on the initial channel modification steps, the model suggests some possible kinetic transmission to explain the dual actions of insecticides on alkaloid-dependent activation. For VTD-dependent sodium flux, the major effect of deltamethrin and DDT was to increase maximal uptake. This effect would be observed if insecticides stabilized the conducting ternary complex by slowing or preventing transition to the closed (CAP) state. This interpretation is consistent with a recent study of the action of deltamethrin on neuroblastoma cell sodium channels under whole cell and patch clamp conditions, in which deltamethrin stabilized a variety of channel states by drastically slowing the transitions between them (4). Alternatively, deltamethrin and DDT could increase maximal VTD-dependent uptake by increasing the single channel conductance above that displayed by channels modified with VTD alone. The lack of increase in the efficacy of BTX by DDT is consistent with the action of BTX as a full agonist at the activator site. At saturating concentrations BTX modifies virtually all of the sodium channels (13) and, unlike VTD, only rarely allows reversion to a closed state (32). Moreover, the lack of increase in the efficacy of BTX suggests that the insecticides do not increase the single channel conductance of BTX-modified channels. The insecticide-dependent reduction in maximal uptake stimulated by ACN probably reflects a negative allosteric interaction that is unique to this activator. This interaction is less obviously related to the known properties of insecticides and activators at the single channel level than the positive allosteric effects of insecticides on the efficacy and potency of VTD. However, it may involve reduced conductance of channels modified by both insecticide and ACN (OAP) or longer residence times in nonconducting states (CAP). Alternatively, channels in the OP state may not bind ACN or may be refractory to ACN-dependent activation. Further insight into the mechanism by which insecticides inhibit ACN-dependent uptake may be obtained from single-channel electrophysiological studies comparing the properties of activator-modified channels in the absence and presence of insecticides.

The second action of insecticides was a small but consistent increase in the potency of the sodium channel activators (Table 1). This increase in potency may reflect the greater affinity of insecticide-modified open channels for activators. Stabilization of alkaloid binding in the OAP state by pyrethroids and DDT could also contribute to an increase in affinity, possibly by reducing the rate of activator dissociation from the OAP complex. Additional evidence supporting increased binding affinity comes from studies that showed stimulation of [^{3}H]BTX-B binding to brain microsac preparations by deltamethrin and the toxic isomers of cypermethrin (34). However, no information is available on the effects of these insecticides on the binding rate constants. Further studies of the insecticide-dependent modification of activator binding may be addressed by determining the effects of insecticides on VTD- and ACN-dependent displacement of [^{3}H]BTX-B binding.

Experiments exploring the action of insecticides and ATX II in combination as modifiers of alkaloid-stimulated sodium flux show that the nature of this interaction depends on both the activator and the insecticide used. DDT and ATX II acted synergistically to enhance uptake stimulated by VTD, as shown by the 5-fold increase in the potency of ATX II (Fig. 6). In contrast, DDT and subsaturating concentrations of ATX II acted independently in their enhancement of sodium channel activation by BTX (Fig. 7). The failure of DDT to enhance BTX-dependent uptake measured at saturating concentrations of ATX II may be related to the inability of DDT to increase the efficacy of BTX. Moreover, there was no DDT-dependent increase in ATX II affinity when BTX served as the activating neurotoxin. Another type of interaction of insecticides with α-toxins was observed in the mutually exclusive effects of cismethrin and ATX II on VTD-activated channels. Independent effects of cismethrin and ATX II are observed, however, with ACN-modified channels, in that cismethrin is able to inhibit ATX II-enhanced ACN-dependent activation.

The DDT-dependent shift in the potency of ATX II as an enhancer of VTD-stimulated sodium uptake (Fig. 6) provides evidence for an allosteric effect of this insecticide on α-polypeptide toxin binding. This effect is apparently not a general property of the action of insecticides in this system, however, since DDT had no discernable effect on the potency of ATX II as an enhancer of BTX-dependent sodium channel activation (Fig. 7) and cismethrin did not increase the potency of ATX II as an enhancer of VTD- or ACN-dependent activation. Moreover, the antagonism of ATX II-enhanced, ACN-stimulated sodium uptake by cismethrin (Fig. 9) appears to be mediated by a reduction of ACN-dependent activation rather than by an effect on the interaction of ATX II with the sodium channel. It will be of interest to clarify these relationships by measuring the effect of insecticides on the binding of labeled α-toxin derivatives both alone and in the presence of different activating toxins.

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