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Pyrethroid Insecticides: Stereospecific Allosteric Interaction with the Batrachotoxinin-A Benzoate Binding Site of Mammalian Voltage-Sensitive Sodium Channels

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SUMMARY

Pyrethroid insecticides are synthetic neurotoxins patterned after the naturally occurring pyrethrins. Their mechanism of action is thought to involve effects primarily at the voltage-sensitive sodium channel of both insect and mammalian neurons, although recent studies have raised the possibility that these compounds may also act at the γ-aminobutyric acid receptor-chloride ionophore complex. Here we show that active pyrethroids of the α-cyano-3-phenoxybenzyl class allosterically enhance the binding of [^{3}H]batrachotoxinin-A 20-α-benzoate to voltage-sensitive sodium channels of rat brain in a dose-dependent and stereospecific manner. Comparison of the rank order of potency for enhancement of [^{3}H]batrachotoxinin-A 20-α-benzoate binding and insecticidal activity in a series of toxic stereoisomers of cypermethrin, representative of the class, reveals a correlation between the two measures. These results support a sodium channel site model for pyrethroid action and suggest a useful and practical method to help evaluate the relationship between effects at the sodium channel and insecticidal potency for members of this class of compounds.

Synthetic pyrethroids, structurally based upon esters of the naturally occurring chrysanthemic acid (2,2-dimethyl-3-(2-methylpropenyl)cyclopropane carboxylic acid), have found widespread use as potent insecticides (1, 2). These compounds are also known to have profound neurotoxic effects in mammals (3, 4). Behaviorally, these effects have been classified as either a "T" (tremor) syndrome or "CS" (convulsions/salivation) syndrome (5). The early synthetic pyrethroids, or type I pyrethroids, of which permethrin is prototypical (Fig.1B), generally elicit a T response. The CS syndrome is typically induced upon intoxication with a newer class of derivatives, or type II pyrethroids, represented by deltamethrin and cypermethrin (Fig. 1, A and C). As seen in Fig. 1, the major difference in these two types of compounds rests in the presence or absence of the α-cyano group on the 3-phenoxybenzyl alcohol portion. Structure-activity studies in a number of laboratories have also revealed strict stereochemical requirements for pyrethroid toxicity (6–8). Within the cyclopropane carboxylate portion for all pyrethroids, only compounds having the cyclopropane-C1 in the *R*-configuration are active, whereas the α-cyano-bearing carbon *S*-configuration yields a more toxic enantiomer than the *R*-configuration for type II pyrethroids.

Electrophysiological studies, carried out primarily in non-mammalian systems, have provided strong evidence for an effect of both type I and type II pyrethroids at low micromolar concentrations on the voltage-sensitive sodium channel (9–12). Voltage clamp experiments reveal the presence of a depolarizing afterpotential in pyrethroid-treated nerve fibers, resulting from a decrease in the rate of sodium channel inactivation. The distinction between T and CS syndromes may well be explained by differences in the kinetics of this effect for type I and II pyrethroids (11, 13, 14). Additional data supporting a sodium channel site as a primary target for pyrethroids in mammalian neuronal tissue have been reported. Jacques *et al.* (15) observed a synergistic enhancement by some pyrethroids of $^{22}Na^+$ uptake mediated by the sodium channel activators veratridine, batrachotoxin, scorpion toxin, and sea anemone toxin in mouse neuroblastoma cells. Similar results were reported by Ghiasuddin and Soderlund (16) using a rat brain synaptosomal preparation. These workers also found that the pyrethroid-induced enhancement was stereospecific in that the nontoxic cyclopropane C1-*S*-isomers were without effect. Further, Bloomquist and Miller (17) reported that houseflies resistant to pyrethroids and DDT (*kdr* strain) were also resistant to the plant alkaloid aconitine, a batrachotoxin and veratridine analogue.

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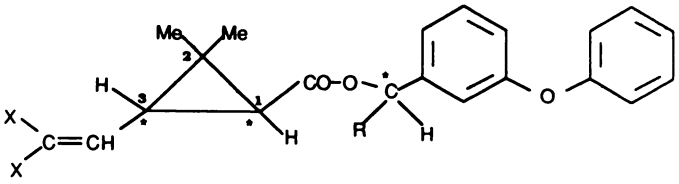
ABBREVIATIONS: DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane; BTX-B, batrachotoxinin-A 20-α-benzoate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GABA, γ-aminobutyric acid.



		X	R
A.	DELTAMETHRIN	Br	CN
B.	PERMETHRIN	Cl	H
C.	CYPERMETHRIN	Cl	CN

Fig. 1. Structures of pyrethroid insecticides. The symbol * represents asymmetric centers.

Batrachotoxin, a steroidal alkaloid isolated from the Colombian arrow poison frog *Phyllobates aurotaenia* (18), and its congeners have in recent years found utility as specific probes for the voltage-sensitive sodium channel (19–21). As discussed below, a number of other sodium channel neurotoxins are now known to interact allosterically with the batrachotoxin binding site, and in these cases the effect may be quantitatively analyzed by monitoring the binding of the tritiated batrachotoxin analog, [^{3}H]BTX-B (22–27). In view of the synergistic effect of the 3-phenoxybenzyl pyrethroids on batrachotoxin-mediated $^{22}Na^+$ flux referred to above (15, 16), the present experiments were designed to determine whether this effect would be reflected in the binding of [^{3}H]BTX-B and, if so, to examine the correlation with insecticidal potency.

Experimental Procedures

Materials. Deltamethrin, permethrin, and the chlorinated hydrocarbon insecticides were a kind gift of T. Miller, University of California, Riverside. The cypermethrin stereoisomers were generously provided by L. Gsell of CIBA-GEIGY, Basel, Switzerland. Veratridine was purified from a mixture of veratrum alkaloids (Sigma Chemical Co., St. Louis, MO) as described by Blount (28). BTX-B and [^{3}H]BTX-B were prepared as previously reported (22). [^{3}H]BTX-B had a specific activity of 14 Ci/mmol (1 Ci = 37 GBq) and a radiochemical purity of >95%. ([^{3}H]BTX-B is currently available from New England Nuclear, Boston, MA, at a specific activity of 30–50 Ci/mmol.) All other materials were obtained from commercial sources.

Synaptoneurosomes. This vesicular preparation of Sprague-Dawley rat brain cerebral cortex was prepared by the method of Chasin *et al.* (29) as modified by Creveling *et al.* (25). The biochemical and morphological properties of synaptoneurosomes have been recently described (30). The preparation consists primarily of resealed postsynaptic membrane with attached presynaptic endings. The vesicles maintain a nominal membrane potential and behave similarly to synaptosomes with respect to sodium channel neurotoxin binding (27, 31).

[^{3}H]BTX-B binding assays. Measurement of specific [^{3}H]BTX-B binding was performed as reported previously (23). Briefly, binding reactions were initiated by addition of 150 μl of synaptoneurosome suspension containing approximately 1.0 mg of protein to a solution in standard incubation buffer of [^{3}H]BTX-B, 50 μg of *Leiurus quinquestriatus quinquestriatus* scorpion venom, and various unlabeled insecticides as indicated. The concentration of labeled toxin was generally 2–10 nM, and the total assay volume was 335 μl. Standard incubation buffer contained 130 mM choline chloride, 50 mM HEPES buffer adjusted to pH 7.4 with Tris base, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl. The insecticides were added from concentrated stock solutions in methanol (pyrethroids) or dimethylsulfoxide (chlorinated hydrocarbons) so that the final concentration of organic vehicle in the assay was less than 1% (v/v). Control experiments demonstrated that this concentration of methanol or dimethylsulfoxide had negligible effects on BTX-B binding. In some cases, greater volumes of methanol stock solutions were added to assay tubes and concentrated under a gentle stream of nitrogen before the addition of the other assay components in order to attain the higher concentrations of effectors without increasing the percentage of methanol in the assay. Incubations were carried out for 60 min at room temperature and were then terminated by addition of 3 ml of cold wash buffer. The tissue was immediately collected on Whatman GF/C glass fiber filters and washed three more times with 3 ml of cold wash buffer. The wash buffer contained 163 mM choline chloride, 5 mM HEPES (pH 7.4), 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$. Radioactivity associated with the tissue was determined by liquid scintillation counting of the filters suspended in 10 ml of scintillation cocktail (3a70B, RPI). Nonspecific binding was determined from parallel incubations containing 250 μM veratridine and has been subtracted from the data.

Data analysis. All data points were determined in duplicate or triplicate and are presented as the mean of these determinations. The figures show the results of one experiment that is representative of two or more separate determinations. Curvilinear fits to the data have been drawn freehand, whereas linear fits are the result of regression analysis.

Results

Deltamethrin enhances BTX-B binding. Specific binding of [^{3}H]BTX-B to rat brain synaptoneurosomes was increased by the presence of deltamethrin (Fig. 1A) in the assay in a dose-dependent manner. As indicated in Fig. 2, maximal stimulation of binding was approximately 2-fold and occurred at a deltamethrin concentration of 20 μM, whereas the half-maximal response could be found at about 3 μM, closely approximating the $K_{0.5}$ (2 μM) for deltamethrin enhancement of

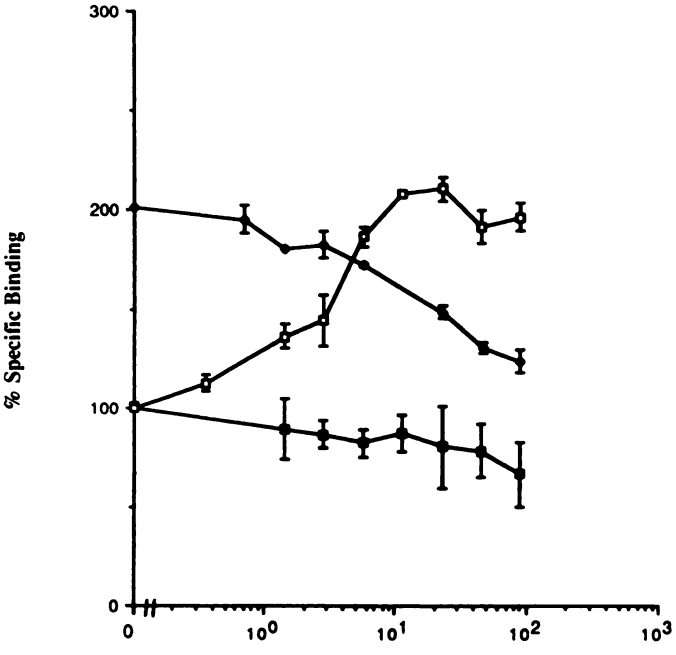


Fig. 2. The effect of type I and type II phenoxybenzyl pyrethroids on the specific binding of [^{3}H]BTX-B to rat brain synaptoneurosomes. Equilibrium binding of 7.2 nM [^{3}H]BTX-B was measured in the presence of increasing concentrations of deltamethrin (□) or permethrin (■). The effect of permethrin on deltamethrin-enhanced BTX-B binding (◆) was determined by incubation of 7.2 nM [^{3}H]BTX-B and 50 μM deltamethrin with the indicated concentrations of permethrin. Nonspecific binding was determined in parallel assay tubes containing 250 μM veratridine and has been subtracted from the data, which are presented as the means ± standard deviation for triplicate determinations.

alkaloid toxin-mediated $^{22}Na^+$ flux in neuroblastoma cells reported by Jacques *et al.* (15). Interestingly, note that in the present experiment, concentrations of deltamethrin higher than 30 μM produced somewhat less than maximal enhancement.

In contrast to the effect of deltamethrin, the noncyano type I pyrethroid *trans*-permethrin (Fig. 1B) produced a weak dose-dependent inhibition of specific BTX-B binding (Fig. 2). When [^{3}H]BTX-B binding in the presence of a saturating concentration of deltamethrin was measured as a function of increasing concentrations of *trans*-permethrin, the enhancement produced by deltamethrin was similarly blocked with an apparent EC_{50} of 30 μM (Fig. 2).

In order to assess the mechanism of deltamethrin-induced enhancement of BTX-B binding, complete binding isotherms for [^{3}H]BTX-B in the presence and absence of 50 μM deltamethrin were determined by displacement assay. Specific binding for a constant concentration of [^{3}H]BTX-B was measured as a function of increasing concentrations of unlabeled BTX-B. The resultant inhibition curves are shown in Fig. 3A, indicating a shift to the left for [^{3}H]BTX-B displacement in the presence of deltamethrin. For further analysis, these data were examined in the form of a Scatchard plot as illustrated in Fig. 3B. From the slopes of the lines, the apparent dissociation constants for BTX-B binding in the absence and presence of deltamethrin were calculated to be 95 nM and 35 nM, respectively. Because there is little effect of deltamethrin on B_{max}, the enhancement of BTX-B binding by deltamethrin may be accounted for by the approximately 3-fold increase in BTX-B binding affinity induced by the pyrethroid.

Pyrethroid enhancement of BTX-B binding is stereospecific and correlated with insecticidal activity. As mentioned, stereochemistry is an important determinant of toxicity for synthetic pyrethroid insecticides. For type II pyrethroids, the three asymmetric centers (marked with an asterisk) in Fig. 1 result in a total of eight possible stereoisomers. In the context of a recent work, Ackermann *et al.* (32) have examined the insecticidal activity of all eight stereoisomers of cypermethrin (Fig. 1C). In these studies, cypermethrin stereoisomers having the C1-*R* configuration were found to be most active and relative molar potencies for contact activity against fourth instar larvae of *Heliothis virescens* were established from LD_{50} determinations. The remaining four stereoisomers with the C1-*S* configuration were too weakly toxic to determine an accurate LD_{50}. For these "inactive" compounds, crude estimates of relative molar potencies were nonetheless made from the per cent mortality after application of a single high dose. As shown in Fig. 4, this treatment resulted in a range of relative potencies covering 5 orders of magnitude for the eight stereoisomers. In essence, however, the analysis defined two groups, relatively toxic and relatively nontoxic cypermethrins, that are structurally differentiated by the absolute configuration at C1. Within the toxic group of cypermethrins, the rank order of potency reflected the following stereochemistries (C3, C1, α-cyano C): *R,R,S* > *S,R,S* > *R,R,R* > *S,R,R*.

Against this background, it was of interest to test the effects of these eight stereoisomers in the BTX-B binding assay. Given the many differences between an assay involving topical application to insects and one examining *in vitro* binding to rat brain membranes, there is no *a priori* expectation of observing quantitative agreement between the two measures. On the other hand, if the effects of these pyrethroids in both systems are ultimately mediated by binding to and alteration of sodium channels, one might reasonably expect that the stereochemical determinants for pyrethroid insect toxicity would be qualitatively mirrored in effects on BTX-B binding. Fig. 4 shows the results of experiments in which specific binding for a constant concentration of [^{3}H]BTX-B was determined as a function of concentration of cypermethrin stereoisomers in the range 1–150 μM. An increase in specific binding is noted for four isomers, each of which has the cyclopropane C1 in the *R*-configuration. Of these, the two isomers having the α-cyano-bearing carbon in the *S*-configuration are the most active. The nontoxic cypermethrin isomers with the C1-*S*-configuration are ineffective at enhancing the affinity of BTX-B binding. As in the insect bioassay, these results similarly divide the eight stereoisomers into two groups. The four cypermethrins with C1-*R*-stereochemistry all enhance BTX-B binding whereas those with C1-

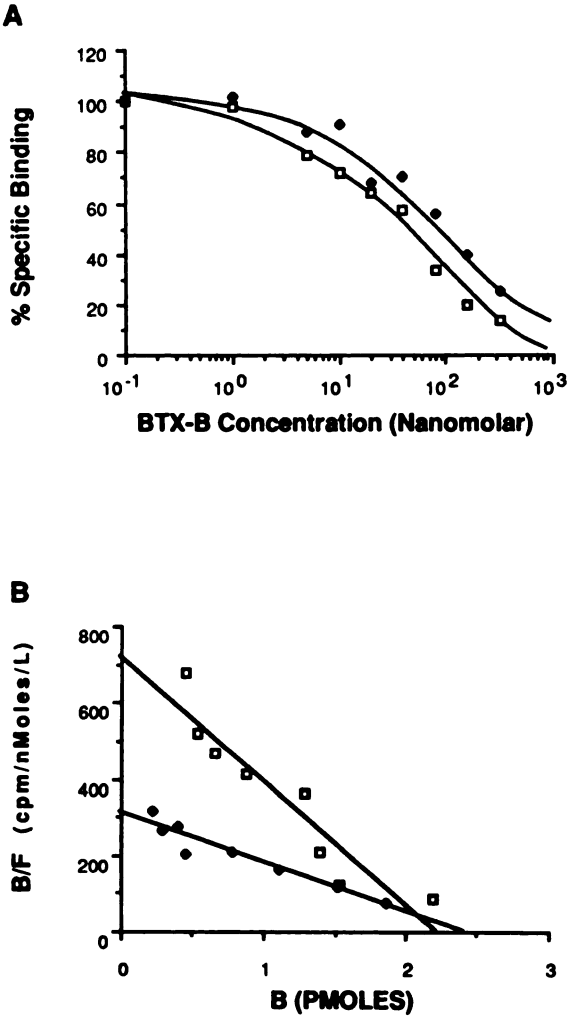


Fig. 3. Deltamethrin enhancement of BTX-B binding. A, Displacement curves for specific BTX-B binding are illustrated in the absence (♦) and presence (□) of 50 μM deltamethrin. The concentration of [^{3}H]BTX-B in the assays was held constant at 10.4 nM. Each *point* is the mean of triplicate determinations. Error bars representing the standard deviation have been omitted for clarity, but in no case did the SD exceed 6%. B, The data from A are presented in the form of a Scatchard plot. The apparent K_d values for BTX-B binding in the absence and presence of deltamethrin were calculated from the slopes of the lines to be 95 and 35 nM, respectively. There is little effect of deltamethrin on B_{max}.

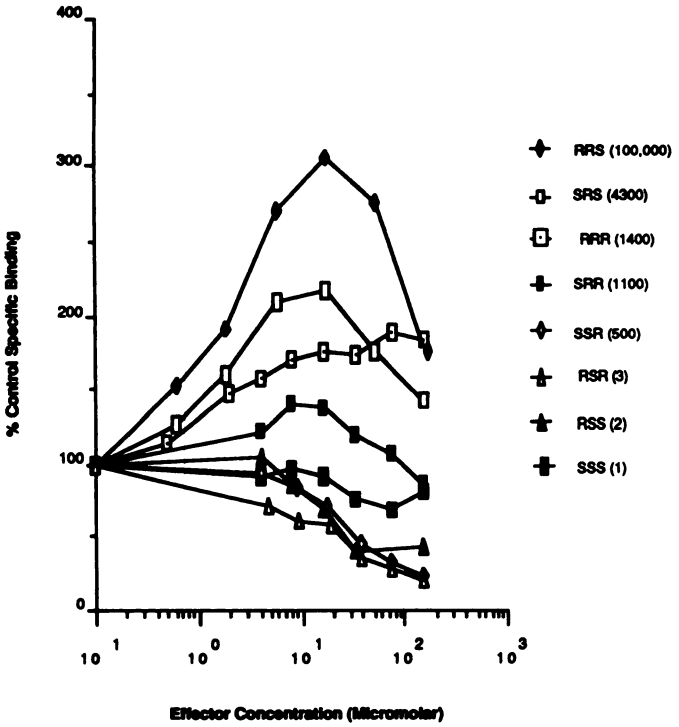


Fig. 4. Effects of cypermethrin stereoisomers on BTX-B binding. Varying concentrations of the eight possible stereoisomers of cypermethrin ranging from 0.5 to 150 μM were included in the standard BTX-B binding assay and the resulting specific binding was recorded as a percentage of that in control assays, which contained no pyrethroid. The *right margin* indicates the stereochemistry for each compound (with reference to Fig. 1; asymmetric centers are listed left to right) and the corresponding relative insecticidal molar potency derived from assay of contact activity against fourth instar larvae of *Heliothis virescens* (31).

S-stereochemistry do not. Among the "active" cypermethrins, the rank order of potency for enhancement of BTX-B binding is identical to that found for insect toxicity.

It is interesting that the EC_{50} values for enhancement of BTX-B binding by the cypermethrins are all approximately the same (2–3 μM) regardless of their maximal stimulation, or relative toxicity, suggesting that insecticidal potency may be in part a function of efficacy rather than affinity. It can also be seen in Fig. 4 that, with the apparent exception of cypermethrin with *R,R,R*-stereochemistry, high concentrations of the insecticides lead to diminished BTX-B binding. For the active compounds, this results in a characteristic bell-shaped dose-response curve, whereas the inactive compounds show a moderate inhibition with apparent EC_{50} values of 20–40 μM.

Chlorinated hydrocarbon insecticides inhibit BTX-B binding. Chlorinated hydrocarbons of the lindane or aldrin/dieldrin type represent a separate class of insecticides that are thought to act by a different mechanism than pyrethroids (33). Recent studies with this class have focussed attention on a probable interaction with the GABA receptor complex (34, 35). As a test of the specificity of pyrethroid effects in the BTX-B binding assay, we elected to screen several of these chlorinated hydrocarbon insecticides, using concentrations of 15 and 150 μM. The results in Fig. 5 show a dose-dependent inhibition of BTX-B binding for all of the chlorinated hydrocarbons tested. Aldrin is the most potent inhibitor, almost completely eliminating specific BTX-B binding at a concentration of 150 μM. These results, however, are not correlated with the insecticidal potency of the compounds, nor with their apparent affinities at the GABA receptor complex (34).

Also included in this screening assay were tetramethrin and DDT. Tetramethrin is a type I pyrethroid and DDT, although not related structurally, has often been compared with type I pyrethroids in its mechanism of action (36). These two compounds make an interesting comparison, because tetramethrin is a potent inhibitor and DDT, an enhancer of BTX-B binding.

Discussion

Binding of the labeled batrachotoxin derivative [^{3}H]BTX-B to its specific site on the voltage-sensitive sodium channel has been demonstrated to be allosterically modulated by a wide variety of other sodium channel neurotoxins, including α-polypeptide toxins from scorpion and sea anemone (23), tetrodotoxin and saxitoxin (27), local anesthetics (25, 26), and selected anticonvulsants (24). The BTX-B site thus appears to be in an area that is particularly responsive to conformational alterations induced at sites on the channel that may even be molecularly remote. Following the work of Jacques *et al.* (15) and Ghiasuddin and Soderlund (16), we have tested the hypothesis that binding of certain phenoxybenzyl pyrethroid insecticides at the mammalian neuronal sodium channel might be similarly reflected in an effect on the binding of [^{3}H]BTX-B.

From the results of the experiments shown in Figs. 2, 3, and 4, we conclude that binding of active pyrethroids of the α-cyano phenoxybenzyl class (type II) can result in an increase of BTX-B binding affinity, presumably through a direct allosteric interaction. An alternative possibility is that these pyrethroids secondarily alter the binding of BTX-B by a primary effect on scorpion toxin-sodium channel interactions. Scorpion toxin is included in all of the assays to increase the affinity of specific BTX-B binding and facilitate the filtration assay (23). Although we have not tested this possibility directly, it does seem unlikely because scorpion toxin is present at saturating concentrations in the assay. We therefore favor the interpretation of Jacques *et al.* (15), based upon $^{22}Na^+$ flux measurements, that the effects of the pyrethroids and scorpion toxin are additive and result from binding of the ligands at distinctive sites.

As shown in Fig. 4, the rank order of potency for the enhancement of BTX-B binding is identical to that for the relative insecticidal potency of the four active cypermethrin stereoisomers; *R,R,S* > *S,R,S* > *R,R,R* > *S,R,R*. Both of these measures depend critically upon the absolute configuration, particularly at the cyclopropane C1 carbon and α-cyano-bearing carbon. This concordance strengthens the hypothesis that pyrethroid insecticides exert their actions primarily through an effect on the voltage-sensitive sodium channel. In this context it should be noted that Lawrence and Casida (37) have reported similar correlations for the effects of type II pyrethroids at the GABA receptor-chloride ionophore complex (i.e., displacement of specifically-bound *t*-butylbicyclophosphorothionate). In those studies, the apparent EC_{50} values were approximately 5 times higher than those found in the present experiments but still within the range typically found for pyrethroid effects on vertebrate or mammalian systems. Because pyrethroids appear to be much more potent in insects, with many showing activity at low nanomolar concentrations, it has generally been difficult to reinforce correlations concerning mechanism of action by comparison of dose-response data between insect and *in vitro* mammalian preparations (38). Some of the discrepancies may

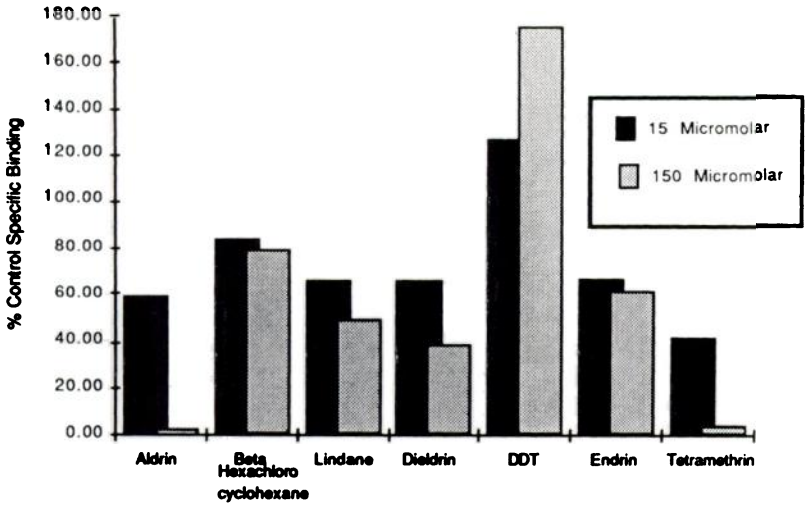


Fig. 5. Effect of chlorinated hydrocarbon insecticides on BTX-B binding. The specific binding of [^{3}H]BTX-B to rat brain synaptoneurosomes in the presence of various chlorinated hydrocarbon insecticides and tetramethrin at concentrations of 15 and 150 μM are shown as a percentage of control specific binding in the absence of insecticides.

be related to drug pharmacodynamics in insects and/or the low solubility of these compounds in aqueous solutions and the attendant problems of assessing the true free concentration of pyrethroids in the presence of significant amounts of hydrophobic membranous material. In view of this we are inclined to weigh the rank order correlation and stereospecificity observed in our experiments more heavily than the absolute dose-response data.

Type I pyrethroids, and inactive stereoisomers of type II, were found to inhibit the specific binding of BTX-B in our experiments (Figs. 2 and 4). On the other hand, Ghiasuddin and Soderlund (16) determined that examples of both type I and type II pyrethroids in the phenoxybenzyl class were able to enhance veratridine-stimulated $^{22}Na^+$ uptake into mouse brain synaptosomes. In an electroencephalogram study, Staatz and Hosko (39) likewise found that both deltamethrin and *cis*-permethrin produced similar preseizure and seizure electroencephalogram patterns and related the greater potency of deltamethrin to a greater efficacy of cyanopyrethroids. To reconcile these observations, it is useful to note that, in the present BTX-B binding assays, even the most potent enhancers yielded less than maximal stimulation at higher concentrations. Stated another way, almost all pyrethroids tested, regardless of type, produced inhibition of BTX-B binding at high concentrations. This effect was not stereospecific nor correlated with potency, thus suggesting that inhibition results from interaction of the insecticides at a different site and in a nonspecific fashion. This concept is further supported by the observations in Fig. 5 that chlorinated hydrocarbon insecticides, thought not to interact with the sodium channel, also produced inhibition of BTX-B binding at high concentrations in a manner that did not correlate with potency. DDT, which functionally seems to resemble type I pyrethroids, apparently does not share this property of nonspecific inhibition, and its stimulatory effect on BTX-B binding is consequently manifested. Taken together, these observations lead us to suggest that pyrethroids as a group bind to a specific sodium channel site resulting in potentiation of BTX-B binding. However, they also share a nonspecific interaction, either with the channel or the membrane environment, leading to inhibition of BTX-B binding. Whether or not enhancement of binding is seen then becomes a function of the relative difference in magnitude between the specific and nonspecific binding constants or efficacy. Electrophysiological studies have shown that active pyrethroids at relatively low concentrations induce a depolarizing afterpotential and hyperexcitability due to slowing of sodium channel inactivation, whereas at higher concentrations, conduction block can occur concomitant with a reduction in the spike amplitude and membrane depolarization (40). Additional studies are required to determine whether inhibition of BTX-B binding at high pyrethroid concentrations is related to this conduction block.

Although the data presented here do not unequivocally establish a cause and effect relationship between pyrethroid binding at voltage-sensitive sodium channels and insecticidal potency, they do lend additional credence to this concept. The [^{3}H]BTX-B binding assay as described should facilitate further studies of relevance to neurotoxic mechanisms by providing a convenient method to quantitatively assess pyrethroid interactions at the sodium channel. Further, the assay may also have practical utility as a mechanism to screen new synthetic pyrethroids as they are developed.

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