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NEUROTOXIC INSECTICIDES INHIBIT GABA-DEPENDENT CHLORIDE UPTAKE BY MOUSE BRAIN VESICLES

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The neurotoxic insecticides endrin, dieldrin, aldrin, lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) and deltamethrin inhibited γ-aminobutyric acid-dependent $^{36}Cl^-$ uptake by mouse brain vesicles. Of the insecticides examined, the chlorinated cyclodienes endrin and dieldrin were the most potent, producing 50% inhibition at 2.8 and 13.9 μM, respectively. Lindane and deltamethrin were less effective, and with deltamethrin the effect was incompletely stereospecific. These results demonstrate the disruption of γ-aminobutyric acid receptor-chloride ionophore function in mammalian brain by neurotoxic insecticides and provide evidence that this complex is the principal site of cyclodiene action.

The GABA receptor-chloride ionophore complex of the vertebrate central nervous system is the site of action of several classes of neuroactive drugs and toxins, including benzodiazepine anxiolytics, barbiturates, and convulsants such as picrotoxinin and the bicyclic phosphates (1). Chlorinated cyclodiene insecticides, γ-HCH, and pyrethroid insecticides having an α-cyano substituent inhibit the binding of [^{3}H]dihydropicrotoxinin and [^{35}S]TBPS to rat brain membranes (2-7), thereby implicating the GABA receptor-ionophore complex as the site of action of these important environmental toxicants as well. However, ligand displacement studies alone are unable to demonstrate the functional modification of this putative target. Recently, a method was described for studying the coupling of GABA receptor

Abbreviations: BSA, bovine serum albumin; DTM, deltamethrin; 1S-DTM, the 1S,cis,αR isomer of deltamethrin; GABA, γ-aminobutyric acid; β-HCH, 1,2,3,4,5,6-hexachlorocyclohexane, β isomer; γ-HCH, 1,2,3,4,5,6-hexachlorocyclohexane, γ isomer; I_{50}, concentration of toxin which gives 50% inhibition of GABA-stimulated chloride uptake; PTX, picrotoxinin; TBPS, t-butylbicyclophosphorothionate.

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binding sites to their associated chloride ionophore by measuring GABA-stimulated $^{36}Cl^-$ uptake into brain vesicles (8). We now report the use of this method to explore the inhibition of GABA-dependent chloride uptake by neurotoxic insecticides.

METHODS

Chemicals. GABA and PTX were purchased from Sigma Chemical Co., St. Louis, MO. Endrin, β-HCH, and γ-HCH were purchased from Chem Service, West Chester, PA. Aldrin and dieldrin were obtained from Shell Development Co., Modesto, CA. TBPS and [^{36}Cl]HCl were purchased from NEN Research Products, Boston, MA. DTM was a gift from Roussel-Uclaf, Romainville, France, and 1S-DTM was previously synthesized in this laboratory (9).

Chloride flux assays. Assays of $^{36}Cl^-$ uptake by mouse brain vesicles followed the procedure of Harris and Allan (8) with minor modifications. Brains of male ICR mice (20-30 g; Blue Spruce Farms, Altamont, NY) were homogenized in 20 mM HEPES-Tris buffer, pH 7.4, containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $MgSO_4$, and 54 mM glucose (1.25 ml/brain). Homogenates were diluted with 5 volumes of the same buffer, filtered through 3 layers of nylon mesh, and centrifuged at 1000 g for 15 min. The resulting pellets were resuspended in the same buffer (2.5 ml/brain equivalent) and again centrifuged at 1000 g for 15 min. The final pellets were resuspended in the same buffer containing 1 mg/ml BSA (1.25 ml/brain equivalent) and used immediately. Aliquots of this suspension (200 µl, 2.16 ± 0.05 mg protein; mean ± S.E., n=28) were preincubated for 15-30 min at 30°C either with ethanol (0.5-1 µl) or with toxins in ethanol. Preliminary experiments established that ethanol at concentrations up to 0.5% did not affect GABA-dependent chloride uptake. Assays were initiated by the addition of $^{36}Cl^-$ (0.1 µCi) in 200 µl of uptake buffer (20 mM HEPES-Tris, pH 7.4, containing 145 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $MgSO_4$, and 27 mM glucose) containing 1 mg/ml BSA and 200 µM GABA (assays of stimulated uptake only). After incubation for 4 sec, uptake was terminated by the addition of ice cold buffer (4 ml) and rapid vacuum filtration on Whatman GF/C filters. Filters were washed with 8 ml of uptake buffer, and the $^{36}Cl^-$ content of recovered membrane vesicles was determined by liquid scintillation counting. Specific GABA-stimulated uptake was calculated as the difference between basal uptake and total uptake in the presence of GABA (final concentration, 100 µM) for each membrane preparation. Inhibition of stimulated uptake was determined by comparing $^{36}Cl^-$ uptake in the presence of GABA plus toxin to that obtained with GABA alone in the same membrane preparation. All results are the means ± S.E. of three replicate experiments using different membrane preparations with three determinations in each replicate. Initial surveys of inhibitory activity employed one or two arbitrarily chosen toxin concentrations, whereas I_{50} values were calculated by probit analysis of data from 6 toxin concentrations that gave 10-90% inhibition.

RESULTS

In the absence of other toxins, GABA (100 µM) increased $^{36}Cl^-$ uptake by mouse brain vesicles 2.5-fold when compared to control levels (Table 1). GABA-stimulated uptake was inhibited by PTX (Figs. 1, 2A) and TBPS (Fig.1), both of which are established antagonists of GABA-stimulated chloride flux

Table 1. Basal and GABA-stimulated $^{36}Cl^-$ uptake by mouse brain vesicles

Condition	$^{36}Cl^-$ uptake, nmol/mg protein[a]
Basal	26.43 ± 0.97
Stimulated (+100 μM GABA)	65.78 ± 2.07
Net stimulated[b]	39.35 ± 1.61[c]

[a] Mean ± S.E. (n=28) for 4 sec incubations.

[b] Calculated from the net stimulated uptake in each replicate.

[c] P<0.0001, paired t-test, 27 d.f.

(1). PTX inhibition of GABA-stimulated uptake was concentration dependent (Fig. 2A) with an I_{50} of 11.2 μM.

The cyclodiene insecticides endrin and dieldrin were potent inhibitors of GABA-stimulated chloride uptake, with I_{50} values of 2.8 μM and 13.9 μM, respectively (Figs. 1, 2B). Aldrin, which differs from dieldrin only in the absence of an epoxide moiety, was much less potent in this assay, giving slightly greater than 50% inhibition at 100 μM (Fig. 1). γ-HCH was less

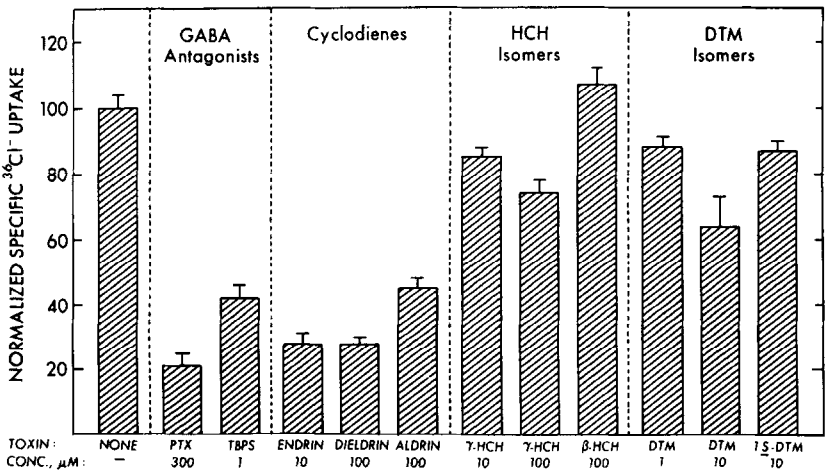

Fig. 1. The effect of preincubation with various toxins on subsequent GABA-stimulated $^{36}Cl^-$ uptake by mouse brain vesicles. Data are normalized so that GABA-stimulated uptake in the absence of toxins is set equal to 100. Bars show standard errors for 21 determinations (GABA without toxins) or 3 determinations (GABA plus toxins). Reductions in specific uptake were significant at P<0.05 (paired t-test, 2 d.f.) for all compounds except DTM at 1 μM (P<0.10) and β-HCH (NS).

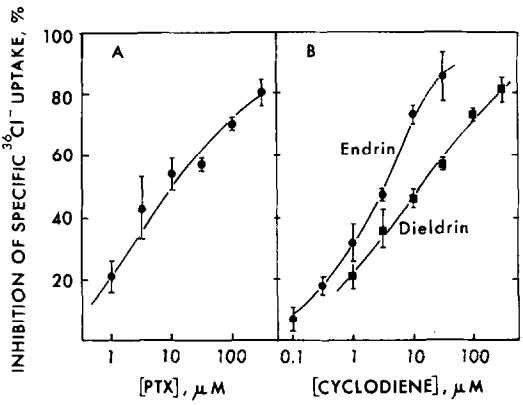

Fig. 2. Concentration dependence of the inhibition of GABA-stimulated $^{36}Cl^-$ uptake by (A) PTX and (B) endrin and dieldrin. Bars show standard errors (n=3).

potent than aldrin, giving less than 30% inhibition at 100 μM (Fig. 1). This effect was stereospecific, however, since the nontoxic β isomer had no effect on specific chloride uptake at the same concentration (Fig. 1).

The potent pyrethroid insecticide DTM was slightly less effective than dieldrin as an inhibitor of GABA-gated chloride flux, producing marginal inhibition at 1 μM and less than 40% inhibition at 10 μM (Fig. 1). The enantiomer of DTM, which has no demonstrable toxicity when administered intracerebrally to mice (9), was less effective than DTM but nevertheless produced statistically significant inhibition of specific chloride uptake at 10 μM (Fig. 1).

DISCUSSION

The stimulation of $^{36}Cl^-$ uptake by GABA and its inhibition by PTX and TBPS confirm that this assay permits investigation of the GABA receptor-chloride ionophore complex as a functional unit (8). Because our results with this system reflect the ability of neurotoxic insecticides to alter GABA receptor-ionophore function, they are particularly relevant for evaluating the significance of this complex as a site of toxic action. We found that cyclodiene insecticides are potent and effective inhibitors of GABA-gated chloride uptake. Moreover, the potencies of these compounds in

this assay correlate well with their acute mammalian toxicities (13). Our data therefore provide further evidence in support of the hypothesis (2-4) that this target is the principal site of cyclodiene action. γ-HCH, which is also postulated to act at this site (2-4), is a much less effective inhibitor of specific chloride uptake than would be expected from its acute toxicity (13) or its potency as an inhibitor of TBPS binding (3,4). This result raises the possibility that other more sensitive pharmacological targets for γ-HCH may exist.

In the case of DTM and other cyano-substituted pyrethroids, a primary action on the GABA receptor-ionophore complex appears unlikely. The effects of DTM on specific chloride uptake are observed only at concentrations above 1 μM and are incompletely stereospecific. However, DTM enhances voltage-dependent sodium channel activation in mouse brain synaptosomes at nanomolar concentrations, and this effect exhibits the high degree of stereospecificity expected on the basis of acute toxicity determinations (9). Thus, the sodium channel is the most sensitive target identified to date for pyrethroids, and the GABA receptor-ionophore complex is likely to be involved in pyrethroid intoxication only at extremely high doses.

Our studies also provide the basis for assessing the fidelity of radioligand displacement assays for predicting neurotoxic potency at the GABA receptor-ionophore complex. Although the qualitative agreement between our results and those obtained in binding assays (2-4,6,10,11) is high, we found that radioligand binding assays overestimated potency in the chloride flux assay by 10- to >667-fold for all compounds in this study except DTM (Table 2). The range of I_{50} ratios for PTX, TBPS, and the cyclodienes were very similar, suggesting a common relationship between binding site occupancy and functional potency among these compounds. The lower potencies found in assays of chloride uptake may result from the presence of endogenous GABA in this system; GABA is known to inhibit TBPS binding when present at micromolar concentrations (10), but its effects on cyclodiene binding are not known.

Table 2. Comparison of the potencies of GABA antagonists and insecticides as inhibitors of specific $^{36}Cl^-$ uptake and [^{35}S]TBPS binding

Compound	I_{50}, μM		I_{50} ratio[b]
	$^{36}Cl^-$ uptake	[^{35}S]TBPS binding[a]	
PTX	11.2[c]	0.19; 0.55 (10,11)	20; 59
TBPS	1[d]	0.017; 0.066 (10,3)	15; 59
Endrin	2.8[c]	0.03; 0.22 (4,3)	13; 93
Dieldrin	13.9[c]	0.1; 1.4 (4,3)	10; 139
Aldrin	100[d]	0.5; 8.7 (4,3)	11; 200
γ-HCH	>100[d]	0.15; 1.7 (4,3)	>59; >667
DTM	>10[d]	5.6 (10)	>1.8

[a]Highest and lowest values shown where two or more determinations are reported; references in parentheses.

[b]Calculated as I_{50} ($^{36}Cl^-$ uptake)/I_{50} ([^{35}S]TBPS binding); highest and lowest ratios are calculated where different I_{50} values for [^{35}S]TBPS binding are reported.

[c]Calculated from Figs. 2A and 2B.

[d]Estimated from Fig. 1.

The range of I_{50} ratios for γ-HCH (Table 2) was considerably greater than those for GABA antagonists and cyclodienes. Although γ-HCH and cyclodienes have been considered to have similar pharmacological actions on the basis of binding data (2-4), the functional modification of the GABA receptor-ionophore complex by γ-HCH is less well correlated with binding than in the case of the cyclodienes. In contrast, there is close quantitative correlation between the inhibition of chloride uptake and inhibition of TBPS binding for DTM (Table 2). Moreover, the weak inhibition of specific chloride uptake by 1S-DTM is in close agreement with a recent report of the effect of the nontoxic 1S,cis,αR enantiomer of cypermethrin, a closely related pyrethroid, on TBPS binding (22% inhibition at 5 μM; 12). These differences in I_{50} ratios suggest that cyclodienes and pyrethroids inhibit specific chloride uptake by interacting with different binding domains on the GABA receptor-ionophore complex. This conclusion is consistent with the different types of inhibition of TBPS binding observed for these insecticide classes (3,6) and the differential effects of detergent solubilization on their interactions with this complex (12).

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