

# Pyrethroid Insecticide-Induced Alterations in Mammalian Synaptic Membrane Potential<sup>1</sup>

JANIS T. EELLS, PETER A. BANDETTINI, PATRICIA A. HOLMAN and JENNIFER M. PROPP

Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin

Accepted for publication May 18, 1992

## ABSTRACT

The neuroexcitatory actions of two toxicologically distinct classes of pyrethroid insecticides were characterized in rat brain synaptosomes using [<sup>3</sup>H]tetraphenylphosphonium to measure changes in synaptosomal membrane potential and by measuring the release of [<sup>3</sup>H]acetylcholine. Both type I (permethrin) and type II (deltamethrin, cypermethrin and fenvalerate) pyrethroids produced a concentration-dependent tetrodotoxin-sensitive membrane depolarization which was stereospecific for the neurotoxic isomer of each pyrethroid. Deltamethrin was the most potent and efficacious pyrethroid in these studies, with an EC<sub>50</sub> of 30 nM and a maximal estimated membrane depolarization of 27 mV, followed by cypermethrin, fenvalerate and permethrin. The phenoxybenzyl pyrethroids also increased the spontaneous

release of [<sup>3</sup>H]acetylcholine from rat brain synaptosomes, further supporting a depolarizing action of these insecticides on nerve terminal membranes. Pyrethroid-induced release of [<sup>3</sup>H]acetylcholine was tetrodotoxin-sensitive and occurred over the same concentration range as membrane depolarization. These data indicate that type I and type II phenoxybenzyl pyrethroids act potently and stereoselectively on the voltage-sensitive sodium channel to increase sodium influx into synaptic terminals producing membrane depolarization and neurotransmitter release. Furthermore, they show that pyrethroid-induced alterations in synaptosomal membrane potential is a sensitive measure of pyrethroid action on the sodium channel and of pyrethroid toxicity.

The synthetic pyrethroids are potent and widely used insecticides derived from natural pyrethrins found in the flowers of the genus *Chrysanthemum*. Pyrethroid insecticides modify the ionic permeability of nerve membranes, producing a neuroexcitatory toxicity in all species thus far studied (Casida *et al.*, 1983; Vijverberg and van den Bercken, 1990). Pyrethroids have been subdivided into two classes based on structural differences and on toxicological and neurophysiological actions. Structurally, the type II pyrethroids, including deltamethrin, cypermethrin and fenvalerate, contain an  $\alpha$ -cyanophenoxybenzyl moiety, whereas the type I pyrethroids such as permethrin lack this  $\alpha$ -cyano component (fig. 1). The presence or absence of the  $\alpha$ -cyano substituent determines the type of poisoning syndrome observed in pyrethroid-intoxicated mammals and insects (Gammon *et al.*, 1981; Gray 1985). Noncyano (type I) compounds produce symptoms in rodents that progress from increased reactivity to whole body tremor (Verschoyle and Aldridge, 1980; Ray, 1982). Cyano pyrethroids (type II) produce a syndrome consisting of salivation, choreoathetosis and clonic

seizures (Verschoyle and Aldridge, 1980; Ray, 1982). The toxicity of the  $\alpha$ -cyano pyrethroids is generally 5 to 10 times greater than their noncyano analogs, which may be an indication of a greater intrinsic activity conferred by the cyano substituent (Gray, 1985). Type I and type II pyrethroids also produce different actions in isolated invertebrate nerve preparations. In general, the type I pyrethroids induce repetitive firing in isolated nerves, whereas the type II pyrethroids produce a long-lasting depolarization after potential and nerve conduction block (Vijverberg and de Weille, 1985).

Pyrethroids have well-defined stereochemical requirements for insecticidal activity and mammalian toxicity (Elliott *et al.*, 1974; Soderlund, 1979; Lawrence and Casida, 1982, 1983). For permethrin, cypermethrin and deltamethrin, an *R* configuration at the cyclopropane C-1 position is essential for neurotoxicity, and the corresponding 1-*S* enantiomers are nontoxic (Lawrence and Casida, 1982; Gray, 1985). In the case of fenvalerate, the 2-*S* isomer is toxic to mammals and the 2-*R* isomer has no measurable mammalian toxicity (Lawrence and Casida, 1982). In the type II pyrethroids, the configuration of the  $\alpha$ -cyano group also influences toxicity. Type II pyrethroids with an *S* configuration of the  $\alpha$ -cyano carbon are potent mammalian toxicants, whereas the  $\alpha$ -*R* enantiomers are essentially nontoxic (Lawrence and Casida, 1982, 1983; Gray 1985).

Received for publication December 27, 1991.

<sup>1</sup>This work was supported by United States Public Health Service Grant R29-ES05006 to J. T. E. A preliminary report of the findings presented in this paper was made at the August 1989 meeting of the American Society for Pharmacology and Experimental Therapeutics, Salt Lake City, Utah.

ABBREVIATIONS: ACh, acetylcholine; TPP<sup>+</sup>, tetraphenylphosphonium; TTX, tetrodotoxin; DMSO, dimethylsulfoxide.

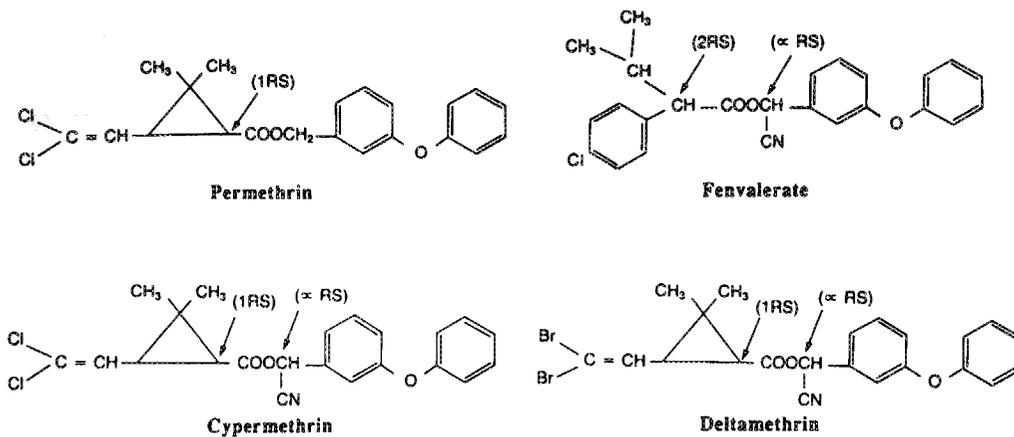


Fig. 1. Structures of pyrethroid insecticides. Asymmetric carbons are indicated by arrows. The following pyrethroid stereoisomers were used in these investigations: permethrin (1*R*-*cis* and 1*S*-*cis*); fenvalerate (2*S*- $\alpha$ *S* and 2*S*- $\alpha$ *R*); cypermethrin (1*R*-*cis*- $\alpha$ *S* and 1*S*-*cis*- $\alpha$ *R*) and deltamethrin (1*R*-*cis*- $\alpha$ *S*) and its 1*R*-*cis*- $\alpha$ *R* enantiomer.

The molecular basis of the neuroexcitatory actions of pyrethroids has been attributed to their actions on voltage-dependent sodium channels (Narahashi, 1985; Vijverberg and de Weille, 1985) and on receptor-regulated channels (Staatz *et al.*, 1982; Abbassy *et al.*, 1983; Lawrence and Casida, 1983). Electrophysiological investigations have shown that both type I and type II pyrethroids prolong the opening of the voltage-dependent sodium channel (Jacques *et al.*, 1980; Lund and Narahashi, 1983). Pyrethroid action on the ion channels associated with the nicotinic acetylcholine receptor (Abbassy *et al.*, 1983; Sherby *et al.*, 1986), excitatory amino acid receptors (Staatz *et al.*, 1982) and  $\gamma$ -aminobutyric acid<sub>A</sub> receptor (Lawrence and Casida, 1983) have been proposed on the basis of ion flux and ligand displacement studies.

The toxicity of pyrethroids in insects has been directly related to their depolarizing action on nerve membranes (Miller and Salgado, 1985; Salgado *et al.*, 1983a,b). However, little information is available on the ability of pyrethroids to depolarize mammalian nerve membranes and on the relationship between membrane depolarization and toxicity in mammals. We have previously shown that type II pyrethroids stimulate calcium-dependent neurotransmitter release in mammalian brain slices indicative of a direct depolarizing action of these agents on presynaptic nerve terminals (Eells and Dubocovich, 1988). In contrast, the actions of pyrethroids on radiolabeled sodium influx and on neurotransmitter release in mammalian nerve terminal preparations have been reported to be dependent upon sodium channel activation (Ghiasuddin and Soderlund, 1985; Bloomquist and Soderlund, 1988) or on potassium-induced membrane depolarization (Brooks and Clark, 1987). The present studies were undertaken in part to resolve this conflict by directly measuring the effect of pyrethroids on nerve membrane potential using the membrane permeant cation TPP<sup>+</sup> to estimate membrane potential in rat brain synaptosomes (Ramos *et al.*, 1979; Pauwels and Laduron, 1986; Aiuchi *et al.*, 1989). The effects of both type I and type II pyrethroids on membrane potential and neurotransmitter release were characterized, and the site of pyrethroid action on the nerve membrane was determined. The investigations reported here establish that both type I and type II phenoxybenzyl pyrethroids act stereospecifically on voltage-sensitive sodium channels to directly depolarize mammalian synaptosomal plasma membranes and elicit neurotransmitter release.

## Methods

**Materials.** Deltamethrin [*S*]- $\alpha$ -cyano-3-phenoxybenzyl-*cis*(1*R*,3*R*)-2,2-dimethyl-3-(2,2-dibromovinyl)cyclopropane carboxylate; 1*R*-*cis*- $\alpha$ *S*) and its 1*R*-*cis*- $\alpha$ *R* enantiomer were provided by Dr. J. P. Demoute at Roussel Uclaf (Romainville, France). The 1*R*-*cis*- $\alpha$ *S* and the 1*S*-*cis*- $\alpha$ *R* isomers of cypermethrin ( $\alpha$ -cyano-3-phenoxybenzyl-*cis*,3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate), and the 1*R*-*cis* and 1*S*-*cis* isomers of permethrin (3-phenoxybenzyl-*cis*,3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate) were provided Dr. A. A. Ramsey at FMC Corporation (Princeton, NJ). The 2*S*- $\alpha$ *S* and the 2*R*- $\alpha$ *R* isomers of fenvalerate ( $\alpha$ -cyano-3-phenoxybenzyl-2-[4-chlorophenyl]-3-methylbutyrate) were provided by Dr. A. E. Lund of E.I. DuPont De Nemours & Co., Inc. (Wilmington, DE). The purity of the pyrethroid stereoisomers was reported by each source to be greater than 98%. TTX, veratridine, aconitine and hemicholinium-3 were obtained from Sigma Chemical Co. (St. Louis, MO.) Tetra[<sup>3</sup>H]phenylphosphonium bromide, [<sup>3</sup>H]H<sub>2</sub>O, [<sup>14</sup>C]sorbitol and [<sup>3</sup>H]choline were purchased from Amersham Corporation (Arlington Heights, IL). Preblend 3a70B scintillation cocktail was obtained from Research Products International Corp. (Mount Pleasant, IL). All other reagents were of the highest available purity and purchased from standard commercial sources.

**Animals.** Adult, male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) that weighed 250 to 300 g were used in these experiments. All animals were supplied with food and water *ad libitum* and maintained on a 12-hr light/dark schedule in a temperature- and humidity-controlled environment before sacrifice. Animals were handled in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

**Preparation of Synaptosomes.** Rats were sacrificed by decapitation, brains were removed and the forebrain was rapidly dissected. Brain tissue was homogenized using a Teflon-glass homogenizer in 12.5 volumes of ice-cold 0.32 M sucrose containing 3.0 mM HEPES (pH 7.5 with tris base) and 1.0 mM EDTA. The homogenate was centrifuged at 1000  $\times$  g for 10 min to separate the nuclear fraction. The resulting supernatant was centrifuged at 12,000  $\times$  g for 20 min to obtain the P2 synaptosomal pellet. The P2 pellet was resuspended to a final protein concentration of 0.25 to 0.35 mg/ml in incubation buffer that contained 135 mM NaCl, 50 mM HEPES buffer adjusted to pH 7.4 with tris base, 1.3 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub> and 5.5 mM glucose. In some instances, a more purified preparation was obtained from the crude synaptosomal fraction using discontinuous sucrose-Ficoll density gradient centrifugation as described by Cottrill (1974). The effects of the alkaloid neurotoxins and the pyrethroids were similar in both synaptosomal

preparations. The protein content of synaptosomal preparations was determined by the method of Bradford (1976).

**TPP<sup>+</sup> accumulation assay.** [<sup>3</sup>H]TPP<sup>+</sup> accumulation was measured using a modification of the method of Pauwels and Laduron (1986). Synaptosomes (50–75 μg of protein) were incubated in 0.4 ml of incubation buffer containing 2 nM (phenyl-<sup>3</sup>H)TPP<sup>+</sup> (28 Ci/mmol) at 37°C in a shaking waterbath for 15 min to allow TPP<sup>+</sup> equilibration. TPP<sup>+</sup> uptake was directly proportional to synaptosomal protein concentration from 20 to 100 μg of protein. Pyrethroids, toxins and/or other drugs were then added and the mixture was incubated for an additional 10 min. The pyrethroid isomer used in any experiment was the neurotoxic isomer unless otherwise indicated. The neurotoxic isomers of deltamethrin and cypermethrin have a 1*R*-*cis*- $\alpha$ S configuration, the neurotoxic isomer of fenvalerate has a 2*S*- $\alpha$ S configuration and the neurotoxic isomer of permethrin is the 1*R*-*cis* isomer (Elliott *et al.*, 1974; Soderlund, 1979; Lawrence and Casida, 1982, 1983). Pyrethroid insecticides, veratridine and aconitine were added (1–2 μl) from concentrated stock solutions in dimethyl sulfoxide (DMSO) so that the final concentration of organic vehicle in the assay was less than 0.1%. Control experiments demonstrated that this concentration of DMSO had negligible effects on TPP<sup>+</sup> accumulation. In most experiments, ouabain (1 mM) was added with the pyrethroids or alkaloid neurotoxins to block the ATP-dependent extrusion of sodium *via* the electrogenic sodium pump (Ghiasuddin and Soderlund, 1984). The reaction was terminated by the addition of ice-cold stopping buffer containing (mM): Choline chloride (163), HEPES-tris (50), MgCl<sub>2</sub> (0.8) and CaCl<sub>2</sub> (1.8), pH 7.4, followed by rapid vacuum filtration through glass fiber filters (#32, Schleicher and Schuell, Keene, NH). Synaptosomal [<sup>3</sup>H]TPP<sup>+</sup> accumulation was determined by liquid scintillation counting of the filters suspended in 4 ml of scintillation cocktail (3a70B, RPI). In some experiments, valinomycin (5 μM) was added to eliminate the accumulation of [<sup>3</sup>H]TPP<sup>+</sup> by intrasynaptosomal mitochondria (Scott and Nicholls, 1980). Nonspecific accumulation was determined from parallel incubations of boiled synaptosomes and has been subtracted from the data. Unless stated otherwise, the sodium concentration was decreased in incubates that contained potassium concentrations greater than 5 mM to maintain isosmolarity. Results were calculated as the percent of the vehicle (0.1% DMSO) control value for TPP<sup>+</sup> accumulation. Values for half-maximal (EC<sub>50</sub>) effects on TPP<sup>+</sup> accumulation were determined by fitting data at several pyrethroid or neurotoxin concentrations to a modified form of the Michaelis-Menten equation (Tamkun and Catterall, 1981; Ghiasuddin and Soderlund, 1985; Bloomquist and Soderlund, 1988) by the method of Wilkinson (1961).

**Determination of intrasynaptosomal volume.** The internal volume of the synaptosomes was determined by a modification of the method described by Lichtshtein *et al.* (1979). Synaptosomes were prepared as described above, and a 50-μl aliquot (1.0 mg of protein) was added to 450 μl of incubation buffer containing [<sup>3</sup>H]H<sub>2</sub>O (10<sup>6</sup> dpm) and [<sup>14</sup>D]sorbitol (275,000 dpm). Synaptosomes were allowed to equilibrate for 10 min at 30°C, followed by centrifugation and washing of the synaptosomal pellet. The total volume of the synaptosomal pellet was determined from the [<sup>3</sup>H]H<sub>2</sub>O content and the extracellular space from the [<sup>14</sup>C]sorbitol content. The intrasynaptosomal volume was taken as the difference between total and extrasynaptosomal volumes. An average intrasynaptosomal volume of 3.4 μl/mg protein was calculated from the data. Similar values were obtained when synaptosomes were treated with veratridine (100 μM), aconitine (100 μM) or the pyrethroids (100 μM).

**Calculation of TPP<sup>+</sup> concentration gradients and membrane potential.** The intrasynaptosomal concentration of TPP<sup>+</sup> was calculated from the accumulation of TPP<sup>+</sup> per milligram of protein and the mean intrasynaptosomal volume (3.4 μl/mg protein). Subtraction of values obtained for TPP<sup>+</sup> accumulation in 130 mM external K concentration from values obtained at 5 mM K yielded the TPP<sup>+</sup> concentration in potassium-sensitive compartments (*i.e.*, across the plasma membrane). Concentration gradients were calculated as TPP<sup>+</sup><sub>in</sub>/TPP<sup>+</sup><sub>out</sub>, and membrane potential was estimated by applying this ratio to the Nernst equation as described by Lichtshtein *et al.* (1979).

**ACh release from synaptosomes.** Synaptosomes suspended in 2 ml of TPP<sup>+</sup> incubation buffer at a protein concentration of 2.5 mg/ml were incubated for 15 min at 37°C in the presence of 0.1 μM [<sup>3</sup>H] choline to allow for the incorporation of radiolabel into the intrasynaptosomal pool of ACh (Eells and Dubocovich, 1988). After incubation, synaptosomes were diluted in 35 ml of cold unlabeled incubation buffer and washed four times by centrifugation at 3000 × *g* (5 min/wash) to remove the unincorporated label. After the final wash, the synaptosomal pellet was resuspended at a protein concentration of 0.1 mg/ml in incubation buffer containing hemicholinium-3 (10 μM) to inhibit the high-affinity uptake system for choline, equilibrated at 37°C for 15 min, and then incubated for 15 min in the presence of pyrethroid insecticide (neurotoxic isomer), veratridine (100 μM) or potassium (20 mM). In some experiments, TTX (1 μM) was added to the incubation mixture before the addition of pyrethroid or veratridine. The reaction was terminated by vacuum filtration through glass fiber filters and the radioactivity in the filtrate and filters determined by liquid scintillation counting. The stimulation-evoked release of tritium was calculated by subtracting the efflux values of vehicle (0.1% DMSO)-treated synaptosomes from the efflux values of synaptosomes exposed to releasing agents and was expressed as a percentage of the total amount of tritium present in the synaptosomes before stimulation (dpm in filtrate × 100/dpm in filtrate + dpm remaining on filter) (Rowell and Winkler, 1984; Minnema *et al.*, 1991; Pearce *et al.*, 1991). Previous studies in rat brain synaptosomes have shown that approximately 90% of the total tritium released after loading with [<sup>3</sup>H]choline is ACh (Minnema *et al.*, 1988).

**Statistical analysis.** Results are presented as means ± S.E.M. Statistical comparisons of group means were made using a Student's *t* test if only one comparison was made between two groups. In all cases in which several comparisons were required, analysis of variance was performed followed by a Dunnett's test procedure for multiple comparisons with a single control (Winer, 1972). In all cases, the minimum level of significance was taken as *P* < .05.

## Results

**TPP<sup>+</sup> accumulation as a probe of synaptosomal membrane potential.** The accumulation of the lipophilic cation TPP<sup>+</sup> was used to estimate membrane potential in rat forebrain synaptosomes. Synaptosome preparations, suspended in a medium containing physiological concentrations of Na and K (135 mM Na and 5 mM K), accumulated TPP<sup>+</sup>, reaching a steady-state level of accumulation within 5 to 10 min that was maintained for at least 30 min (fig. 2A). This steady-state level of synaptosomal TPP<sup>+</sup> accumulation has been shown to reflect both the equilibration of this lipophilic cation across the plasma membrane and accumulation into intrasynaptosomal mitochondria (Scott and Nicholls, 1980; Ramos *et al.*, 1979; Aiuchi *et al.*, 1989). Extrasynaptosomal mitochondria present in synaptosomal preparations have been reported to exert a negligible effect on TPP accumulation (Ramos *et al.*, 1979; Komulainen and Bondy, 1987). This has been attributed to damage of free mitochondria in preparative media, which is not designed for mitochondrial stabilization. With increasing external K concentrations, TPP<sup>+</sup> accumulation established at the steady state decreased exponentially from 3.2 pmol/mg protein at an external K concentration of 5 mM to 0.8 pmol/mg of protein at 130 mM K, indicating, as reported by others, that the membrane potential across the synaptosomal membrane is due primarily to a K diffusion gradient (fig. 2B). The difference in accumulation of TPP<sup>+</sup> in the presence of a depolarizing concentration of KCl (130 mM) relative to that in standard buffer (5 mM) was taken as being due to the potential across the plasma membrane. After correction for potassium-insensitive accumulation of TPP<sup>+</sup>, values for transmembrane potential ranging

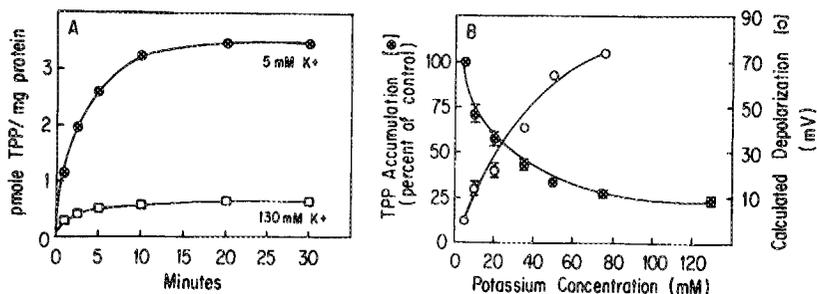


Fig. 2. A) Time course of  $[^3\text{H}]\text{TPP}^+$  accumulation in rat brain synaptosomes under physiological (5 mM) (●) and depolarizing (130 mM) (□) potassium conditions. Synaptosomes were incubated in the presence of 2 nM  $[^3\text{H}]\text{TPP}^+$  at 37°C in buffer containing 5 mM  $\text{K}^+$  or 130 mM  $\text{K}^+$ . Samples were removed at the times indicated and measured as described under "Methods." Data are expressed as picomoles of  $\text{TPP}^+$  accumulated per milligram of synaptosomal protein as a function of time. Shown are the mean values from two experiments. B)  $[^3\text{H}]\text{TPP}^+$  accumulation and calculated membrane depolarization as a function of external potassium concentration. Synaptosomes were incubated for 20 min at the potassium concentrations indicated in the presence of 2 nM  $[^3\text{H}]\text{TPP}^+$ . Iso-osmolarity was maintained by decreasing sodium concentrations as potassium concentrations increased. Data are expressed as percent of control (5 mM  $\text{K}^+$ )  $\text{TPP}^+$  accumulation (●) and calculated membrane depolarization (mV) (○) as a function of potassium concentration. Membrane potential was determined by application of the Nernst equation to the equilibrium distribution of  $\text{TPP}^+$  as described under "Methods." Shown are the mean values  $\pm$  S.E.M. from four experiments.

-65 to -75 mV were estimated. These values are in good agreement with previously estimated values for synaptosomal membrane potential (Ramos *et al.*, 1979; Mayor *et al.*, 1986; Komulainen and Bondy, 1987).

**Membrane depolarization produced by alkaloid neurotoxins.** The alkaloid neurotoxins veratridine and aconitine produced concentration-dependent decreases in  $\text{TPP}^+$  accumulation in rat brain synaptosomes (fig. 3). Maximal decreases in  $\text{TPP}^+$  accumulation were observed at concentrations of 100  $\mu\text{M}$  for both veratridine and aconitine. Both neurotoxins exhibited similar efficacy, producing approximately 70% decreases in  $\text{TPP}^+$  accumulation that corresponded to an estimated maximal membrane depolarization between 65 and 75 mV. The half-maximal response ( $\text{EC}_{50}$ ) values for veratridine and aconitine-induced decreases in  $\text{TPP}^+$  accumulation were 0.6 and 1.5  $\mu\text{M}$ , respectively. Measurements of  $\text{TPP}^+$  accumulation were standardly conducted in the presence of ouabain (1 mM) to inhibit the Na/K ATPase-dependent extrusion of

intrasyntosomal sodium and resulting synaptosomal repolarization (Lichtshtein *et al.*, 1979). In the absence of ouabain, veratridine and aconitine decreased  $\text{TPP}^+$  accumulation with diminished efficacy and potency. The maximal estimated depolarization was reduced from 65 to 75 mV for both neurotoxins to 44 mV for veratridine and 17 mV for aconitine, and the  $\text{EC}_{50}$  values were increased from 0.6 to 5  $\mu\text{M}$  for veratridine and from 1.5 to 3  $\mu\text{M}$  for aconitine. The depolarizing actions of veratridine and aconitine were antagonized by the sodium channel blocker TTX (1  $\mu\text{M}$ ). TTX alone did not alter  $\text{TPP}^+$  accumulation. In addition, veratridine and aconitine had no effect on  $\text{TPP}^+$  accumulation in concentrations of extracellular potassium (130 mM) which abolish the plasma membrane potential. The actions of veratridine and aconitine on  $\text{TPP}^+$  accumulation in rat brain synaptosomes and their ability to activate the voltage-sensitive sodium channel, producing an increase in sodium influx and membrane depolarization in other neuronal preparations (Narahashi, 1977; Lichtshtein *et al.*, 1979; Tamkin and Catterall, 1981).

**Membrane depolarization produced by pyrethroid insecticides.** Both type I (permethrin) and type II (deltamethrin, cypermethrin and fenvalerate) pyrethroids produced concentration-dependent decreases in  $\text{TPP}^+$  accumulation indicative of membrane depolarization (fig. 4). The depolarizing actions of the pyrethroids were apparent over a concentration range from 10 nM to 100  $\mu\text{M}$ , with maximal effects observed between 10 and 100  $\mu\text{M}$  for each pyrethroid. Reductions in  $\text{TPP}^+$  accumulation and calculated synaptosomal membrane potential changes produced by the type I and type II pyrethroids (100  $\mu\text{M}$ ) are presented in tables 1 and 2. Deltamethrin was the most efficacious and potent pyrethroid examined, producing a 44% decrease in  $\text{TPP}^+$  accumulation at 100  $\mu\text{M}$  that corresponded to an estimated membrane depolarization of  $27 \pm 1$  mV. The apparent  $\text{EC}_{50}$  for deltamethrin induced synaptosomal membrane depolarization was 30 nM. Cypermethrin produced a maximal depolarization of  $18 \pm 1$  mV with an apparent  $\text{EC}_{50}$  of 80 nM followed by fenvalerate, which produced a  $10 \pm 1$  mV maximal depolarization with an  $\text{EC}_{50}$  of 200 nM. The type I pyrethroid, permethrin, was the least active pyrethroid studied, producing a maximal depolarization of  $6 \pm 1$  mV with an  $\text{EC}_{50}$  of 850 nM. Pyrethroids produced nearly identical effects on  $\text{TPP}^+$  accumulation in purified synaptosomes (table 2), providing evidence that the effect of pyrethroids in the crude synap-

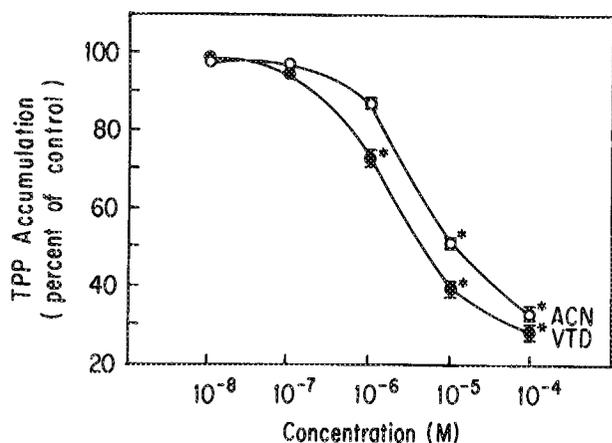


Fig. 3. Alkaloid neurotoxin-induced decreases in  $[^3\text{H}]\text{TPP}^+$  accumulation in rat brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM  $[^3\text{H}]\text{TPP}^+$  at 37°C for 15 min to allow  $\text{TPP}^+$  equilibration. Veratridine (VTD) or aconitine (ACN) were then added at the concentrations indicated in the presence of ouabain (1 mM) and the mixture was incubated for 10 additional min. Results are expressed as percent of vehicle (0.1% DMSO, 1 mM ouabain) control value for  $\text{TPP}^+$  accumulation. Shown are the mean values  $\pm$  S.E.M. from six experiments. An asterisk indicates  $P < .05$  compared to accumulation in the absence of neurotoxins using Dunnett's modification of the  $t$  test.

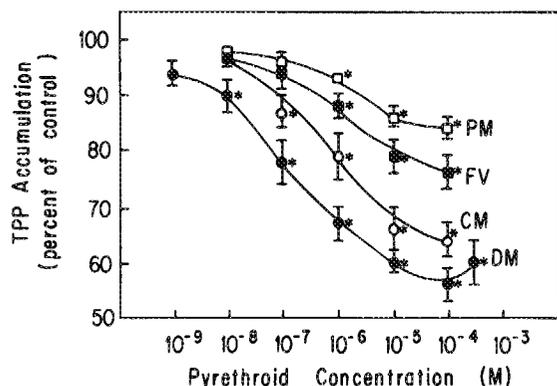


Fig. 4. Pyrethroid insecticide-induced decreases in [ $^3\text{H}$ ]TPP $^+$  accumulation in rat brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM [ $^3\text{H}$ ]TPP $^+$  at 37°C for 15 min to allow TPP $^+$  equilibration. 1*R*-*cis* Permethrin (PM), 2*S*- $\alpha$ S fenvalerate (FV), 1*R*-*cis*- $\alpha$ S cypermethrin (CM) or deltamethrin (DM) was then added at the concentrations indicated in the presence of 1 mM ouabain and the mixture was incubated for 10 additional min. Results are expressed as percent of vehicle (0.1% DMSO; 1 mM ouabain) control value for TPP $^+$  accumulation. Shown are the means  $\pm$  S.E.M. from six experiments. An asterisk indicates  $P < .05$  compared to accumulation in the absence of pyrethroid using Dunnett's modification of the  $t$  test.

TABLE 1

**Stereospecificity and TTX sensitivity of pyrethroid insecticide-induced decreases in [ $^3\text{H}$ ]TPP $^+$  accumulation in rat brain synaptosomes**

Synaptosomes equilibrated with [ $^3\text{H}$ ]TPP $^+$  as described under "Methods" were incubated for 10 min at 37°C in the presence of ouabain (1 mM) and the indicated pyrethroid stereoisomer (100  $\mu\text{M}$ ) and in the presence or absence of TTX (1  $\mu\text{M}$ ). Results are expressed as percent of vehicle (0.1% DMSO; 1 mM ouabain) control value for [ $^3\text{H}$ ]TPP $^+$  accumulation. Shown are the mean values  $\pm$  S.E.M. from four experiments. An asterisk indicates  $P < .05$  compared to accumulation in the absence of pyrethroids using Dunnett's modification of the  $t$  test.

Pyrethroid	Percent of Control TPP $^+$ Accumulation		
	Active Isomer <sup>a</sup>	Active Isomer <sup>a</sup> + TTX	Inactive Isomer <sup>b</sup>
Permethrin	84 $\pm$ 2*	94 $\pm$ 7	103 $\pm$ 2
Fenvalerate	76 $\pm$ 3*	96 $\pm$ 2	100 $\pm$ 5
Cypermethrin	64 $\pm$ 3*	97 $\pm$ 3	104 $\pm$ 2
Deltamethrin	56 $\pm$ 3*	97 $\pm$ 3	103 $\pm$ 2

<sup>a</sup> Active isomers: 1*R*-*cis* permethrin, 2*S*- $\alpha$ S fenvalerate, 1*R*-*cis*- $\alpha$ S cypermethrin and deltamethrin (1*R*-*cis*- $\alpha$ S).

<sup>b</sup> Inactive isomers: 1*S*-*cis* permethrin, 2*R*- $\alpha$ R fenvalerate, 1*S*-*cis*- $\alpha$ R cypermethrin and the 1*R*-*cis*- $\alpha$ R enantiomer of deltamethrin.

tosomal preparation results from an effect on the synaptosomes present in the preparation.

As mentioned, stereochemistry is an important determinant of both insecticidal activity and mammalian toxicity for synthetic pyrethroid insecticides (Elliott *et al.*, 1974; Soderlund, 1979; Lawrence and Casida, 1982, 1983). Reductions in TPP $^+$  accumulation were specific for the neurotoxic pyrethroid isomers: 1*R*-*cis* permethrin, 2*S*- $\alpha$ S fenvalerate, 1*R*-*cis*- $\alpha$ S cypermethrin and deltamethrin (1*R*-*cis*- $\alpha$ S) (fig. 4 and table 1). No detectable effects on TPP accumulation were produced by 1*S*-*cis* permethrin, 2*R*- $\alpha$ R fenvalerate, 1*S*-*cis*- $\alpha$ R cypermethrin or the 1*R*-*cis*- $\alpha$ R enantiomer of deltamethrin at concentrations up to and including 100  $\mu\text{M}$  (table 1).

TPP $^+$  accumulation in synaptosomes reflects the equilibration of the lipophilic cation across both the plasma membrane and accumulation into intrasynaptosomal mitochondria (Scott and Nicholls, 1980; Ramos *et al.*, 1979; Aiuchi *et al.*, 1989). To determine if pyrethroids were acting to depolarize the synap-

TABLE 2

**Effect of pyrethroid insecticides on synaptosomal membrane potential in P2 synaptosomes, valinomycin-treated P2 synaptosomes and purified synaptosomes**

Synaptosomes equilibrated with [ $^3\text{H}$ ]TPP $^+$  as described under "Methods" were incubated for 10 min at 37°C in the presence of ouabain (1 mM) and the neurotoxic pyrethroid isomer (100  $\mu\text{M}$ ). Membrane potential was determined by applying the Nernst equation to the equilibrium distribution of TPP $^+$  as described under "Methods." Results are expressed as the difference (mV depolarization) between the membrane potential values determined for pyrethroid-treated synaptosomes and their respective vehicle-treated (0.1% DMSO; 1 mM ouabain;  $\pm$  5  $\mu\text{M}$  valinomycin) controls. Shown are the mean values  $\pm$  S.E.M. from four to six experiments.

Pyrethroid	Calculated Synaptosomal Depolarization		
	P <sub>2</sub> Synaptosomes	P <sub>2</sub> Synaptosomes + Valinomycin <sup>a</sup>	Purified Synaptosomes <sup>b</sup>
	mV		
Permethrin (1 <i>R</i> - <i>cis</i> )	6 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 2
Fenvalerate (2 <i>S</i> - $\alpha$ S)	10 $\pm$ 2	9 $\pm$ 1	13 $\pm$ 2
Cypermethrin (1 <i>R</i> - <i>cis</i> - $\alpha$ S)	18 $\pm$ 2	16 $\pm$ 3	19 $\pm$ 2
Deltamethrin	27 $\pm$ 1	20 $\pm$ 3	21 $\pm$ 2

<sup>a</sup> Incubations were conducted in the presence of 5  $\mu\text{M}$  valinomycin.

<sup>b</sup> Purified synaptosomes were obtained from the P2 synaptosomal fraction using discontinuous Ficoll density gradient centrifugation as described by Cottman (1974).

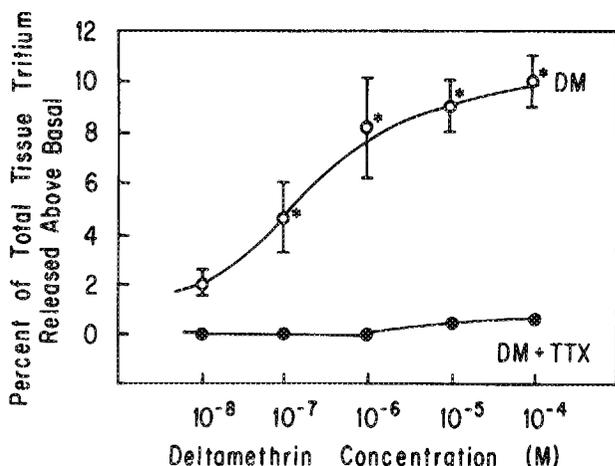
tosomal plasma membrane or the intrasynaptosomal mitochondrial membrane or both, we examined pyrethroid actions on TPP $^+$  accumulation under conditions which selectively eliminated the plasma membrane potential difference or the mitochondrial membrane potential difference. Under conditions which selectively depolarize the plasma membrane (130 mM K), the pyrethroids (100  $\mu\text{M}$ ) had no effect on the TPP $^+$  accumulation, indicating that they do not influence TPP accumulation by intrasynaptosomal mitochondria (data not shown). However, when the mitochondrial membrane potential was selectively depressed by the potassium ionophore, valinomycin, pyrethroids decreased TPP $^+$  accumulation. As shown in table 2, similar degrees of membrane depolarization were produced by the pyrethroids (100  $\mu\text{M}$ ) in the presence and in the absence of valinomycin, indicating that these agents act to depolarize the synaptosomal plasma membrane. To determine if interaction with the voltage-dependent sodium channel in the synaptosomal plasma membrane was required for pyrethroid-induced synaptosomal membrane depolarization, pyrethroids were added to synaptosomal preparations in the presence of the sodium channel blocker TTX (1  $\mu\text{M}$ ). TTX completely antagonized the decrease in TPP $^+$  accumulation produced by the pyrethroid insecticides (table 1).

Inhibition of Na/K ATPase activity was required for the measurement of pyrethroid-induced synaptosomal depolarization. In the absence of ouabain, both type I and type II pyrethroids produced 10 to 20% increases in TPP $^+$  accumulation, indicative of membrane hyperpolarization. The observed increases in TPP $^+$  accumulation were stereospecific for the neurotoxic pyrethroid isomers and were blocked by TTX (1  $\mu\text{M}$ ), suggesting that the pyrethroids were acting on the voltage-sensitive sodium channel to increase sodium influx and thus activating the pump.

Inhibition of the Na/K ATPase by ouabain (1 mM) produced a 9  $\pm$  1% decrease in TPP $^+$  accumulation that corresponded to an estimated membrane depolarization of 3.7  $\pm$  0.7 mV. Some studies have suggested that pyrethroids can enhance sodium channel activity or stimulate neurotransmitter release only under depolarizing conditions (Jacques *et al.*, 1980; Ghiasuddin and Soderlund, 1985; Brooks and Clark, 1987). To determine

if an initial membrane depolarizing stimulus is required for pyrethroids to reduce TPP<sup>+</sup> accumulation, we examined the actions of deltamethrin in the presence of a concentration of potassium that produced a decrease in TPP<sup>+</sup> accumulation equivalent to that observed in the presence of 1 mM ouabain. The decrease in TPP<sup>+</sup> accumulation produced by 8 mM KCl (8 ± 2% decrease; 3.7 ± 4 mV estimated depolarization) was not enhanced by the addition of 100 μM deltamethrin (10 ± 2% decrease; 4.0 ± 0.9 mV), indicating that the depolarizing actions of the pyrethroids are not dependent upon an initial depolarizing stimulus. These results indicate that measurement of pyrethroid-induced membrane depolarization is not dependent upon the depolarizing actions of ouabain, but upon the ability of ouabain to inhibit sodium efflux *via* the Na/K ATPase.

**Pyrethroid-evoked release of ACh from rat brain synaptosomes.** Neurotransmitter release occurs secondary to the activation of voltage-sensitive calcium channels after synaptic membrane depolarization (Kostyuk, 1980). We examined the ability of type I and type II pyrethroids to stimulate the release of [<sup>3</sup>H]ACh from rat forebrain synaptosomes as another functional parameter of synaptic membrane depolarization. Synaptosomes were incubated *in vitro* with [<sup>3</sup>H]choline to label the intrasynaptosomal pool of ACh, and then exposed to pyrethroids (1–100 μM), 20 mM potassium or 10 μM veratridine for 15 min. These experiments were conducted in parallel with the TPP<sup>+</sup> accumulation experiments, utilizing the same synaptosome preparations and incubation buffer to enable a direct comparison of the effects of pyrethroids on membrane potential and neurotransmitter release. A concentration-dependent increase in [<sup>3</sup>H]ACh release was observed after exposure to the type II pyrethroid deltamethrin (0.01–100 μM) (fig. 5). ACh release evoked by deltamethrin was detectable at a concentration of 0.01 μM (15 fmol/mg protein) and reached a plateau between 10 and 100 μM. At a concentration of 100 μM, deltamethrin elicited an increase in ACh release of 35 ± 2 fmol/mg



**Fig. 5.** Concentration dependence and TTX sensitivity of deltamethrin-evoked release of [<sup>3</sup>H]ACh from rat brain synaptosomes. Synaptosomes were labeled *in vitro* with [<sup>3</sup>H]choline as described under "Methods" and then incubated for 15 min at 37°C in the presence of deltamethrin or deltamethrin plus TTX (1 μM). Values for pyrethroid-evoked release are expressed as the percent of total tissue tritium released above basal levels (0.1% DMSO) during the 15-min incubation period with pyrethroid or pyrethroid plus TTX. Shown are the mean values ± S.E.M. from four experiments. An asterisk indicates P < .05 compared to [<sup>3</sup>H]ACh release in the absence of pyrethroid using Dunnett's modification of the *t* test.

protein (10 ± 1% total tissue tritium released above basal release). The EC<sub>50</sub> for deltamethrin-stimulated ACh release was calculated to be 30 nM, identical to the EC<sub>50</sub> for deltamethrin-induced membrane depolarization. Deltamethrin-evoked release of [<sup>3</sup>H]ACh was abolished by the addition of the sodium channel blocking agent TTX (1 μM). TTX-sensitive increases in ACh release were also observed in synaptosome preparations treated with permethrin, cypermethrin and fenvalerate (table 3). Pyrethroid-evoked release of ACh was compared with that induced by two known nerve membrane depolarizing agents, elevated extracellular potassium and veratridine. Potassium (20 mM) depolarization elicited a calcium-dependent increase in ACh release which was 7.3 ± 1% of the initial intrasynaptosomal ACh content. Veratridine (100 μM) elicited a TTX-sensitive increase in ACh release which was 12.8 ± 1% of the initial intrasynaptosomal [<sup>3</sup>H]ACh content. These data further support a direct depolarizing action of pyrethroid insecticides on isolated nerve terminals.

## Discussion

The neuroexcitatory actions of two toxicologically distinct classes of pyrethroid insecticides were characterized in rat brain synaptosomes using the permeant lipophilic cation TPP<sup>+</sup> to measure changes in synaptosomal membrane potential and the release of [<sup>3</sup>H]ACh. These investigations show that type I and type II phenoxybenzyl pyrethroids interact potently and stereoselectively with the voltage-sensitive sodium channel in rat brain synaptosomes, producing membrane depolarization and neurotransmitter release. Furthermore, they show that pyrethroid-induced reductions in TPP<sup>+</sup> accumulation are a sensitive measure of the neuroexcitatory actions of these insecticides.

The results presented in this article show that the effects of pyrethroid insecticides and other sodium channel-specific neurotoxins on synaptosomal membrane potential can be estimated by measuring changes in the accumulation of the permeant lipophilic cation TPP<sup>+</sup> in P2 synaptosomal preparations. Experiments conducted in purified synaptosomes and in synaptosomal preparations incubated in the presence of the mitochondrial depolarizing agent valinomycin indicate that the pyrethroids are acting on the synaptosomal plasma membrane and do not affect the mitochondrial component of TPP<sup>+</sup> accumulation. Moreover, pyrethroid actions were fully antagonized

**TABLE 3**

### TTX sensitivity of pyrethroid insecticide-induced release of [<sup>3</sup>H]ACh from rat brain synaptosomes

Synaptosomes were incubated *in vitro* with [<sup>3</sup>H]choline to label the intrasynaptosomal pool of acetylcholine as described under "Methods," and then were incubated for 15 min at 37°C in the presence of the neuroactive pyrethroid isomer (10 μM) with or without TTX (1 μM). Values for pyrethroid-evoked release are expressed as the percent of total tissue tritium released above basal levels (0.1% DMSO) during the 15-min incubation period with pyrethroid or pyrethroid plus TTX. Shown are the mean values ± S.E.M. from four experiments.

Pyrethroid	Percent of Total Tissue Tritium Released Above Basal Levels	
	Pyrethroid-Evoked Release of [ <sup>3</sup> H]ACh	Pyrethroid-Evoked Release of [ <sup>3</sup> H]ACh in the Presence of TTX
Permethrin (1 <i>R</i> - <i>cis</i> )	5.2 ± 1.3*	0.9 ± 0.1
Fenvalerate (2 <i>S</i> -α <i>S</i> )	9.0 ± 0.4*	0.5 ± 0.4
Cypermethrin (1 <i>R</i> - <i>cis</i> -α <i>S</i> )	10.2 ± 0.6*	0.7 ± 0.2
Deltamethrin	9.0 ± 1.0*	0.4 ± 0.3

\* P < .05 compared to [<sup>3</sup>H]ACh release in the absence of pyrethroid using Dunnett's modification of the *t* test.

by the sodium channel blocker TTX, indicating that these insecticides depolarize the plasma membrane by interacting with the voltage-sensitive sodium channel.

The depolarizing actions of the pyrethroid insecticides in rat brain synaptosomes were concentration dependent, with  $EC_{50}$  values in the nanomolar range for both type I and type II pyrethroids. In this same concentration range, pyrethroids have been shown to delay the inactivation of voltage-sensitive sodium channels in neuroblastoma cells (Vijverberg *et al.*, 1986), to enhance veratridine-stimulated sodium influx into mouse brain synaptosomes (Ghiasuddin and Soderlund, 1985) and to stimulate spontaneous neurotransmitter release from brain slices (Eells and Dubocovich, 1988) and synaptosomes (Nicholson *et al.*, 1983; Doherty *et al.*, 1987). Deltamethrin was the most potent and efficacious pyrethroid in our studies, followed in potency and efficacy by cypermethrin, fenvalerate and permethrin. The same relative relationship has been shown to hold in electrophysiological measurements of pyrethroid-induced prolongation of the inward sodium current during excitation in frog nerve fibers, with tail currents of 1770, 1115, 600 and 28 msec reported for deltamethrin, cypermethrin, fenvalerate and permethrin, respectively (Vijverberg and de Weille, 1985). There also appears to be a strong relationship between pyrethroid-induced membrane depolarization and enhancement of sodium uptake in isolated mammalian nerve terminals. The half-maximal concentration for membrane depolarization for deltamethrin was 30 nM in our studies, compared with a value of 25 nM for half-maximal enhancement of sodium uptake in mouse brain synaptosomes (Ghiasuddin and Soderlund, 1985). We also measured a 10-fold difference in the depolarizing potency of cypermethrin and its descyano analog permethrin (cypermethrin > permethrin), consistent with the 10-fold difference in toxicity and sodium flux-enhancing potency described by Ghiasuddin and Soderlund (1985) for deltamethrin and its descyano analog NRDC 157.

The membrane-depolarizing action of the pyrethroids studied paralleled their mammalian toxicity profiles and was specific for the neuroactive isomer of each pyrethroid. Among the pyrethroids characterized in this study, the rank potency for membrane depolarization was identical to that found for pyrethroid toxicity in the rat after i.v. pyrethroid administration (Verschoyle and Aldridge, 1980). Furthermore, the  $EC_{50}$  values for membrane depolarization in rat brain synaptosomes for permethrin, cypermethrin and deltamethrin paralleled the concentrations of these pyrethroids in rodent brain associated with the onset of tremor or convulsions (Glickman and Lech, 1982; Rickard and Brodie, 1985; Edwards *et al.*, 1986). The stereoselective nature of pyrethroid actions on membrane potential also strongly supports the toxicological relevance of this effect, because both insecticidal activity and mammalian toxicity are only observed with the neuroactive enantiomer of these insecticides (Elliott *et al.*, 1974; Lawrence and Casida, 1982, 1983).

The depolarizing actions of veratridine and aconitine were significantly enhanced by blocking sodium efflux, but were evident even in the absence of ouabain. In contrast, inhibition of the electrogenic sodium pump was necessary for measurement of pyrethroid-induced membrane depolarization. The differences in the membrane-depolarizing actions of pyrethroid insecticides and alkaloid neurotoxins are indicative of fundamental differences in the mechanism of action of these two classes of neurotoxins on the sodium channel. Electrophysiological studies have shown that veratridine and aconitine in-

duce persistent activation of the sodium channel (Narahashi, 1977), whereas pyrethroid insecticides prolong the open time of the sodium channel by delaying inactivation (Vijverberg *et al.*, 1986; Lund and Narahashi, 1983; Salgado *et al.*, 1989). Persistent sodium channel activation is likely to stimulate a greater amount of sodium influx, overwhelming the ability of the Na/K ATPase to pump out the sodium and resulting in a measurable decrease in  $TPP^+$  accumulation. Conversely, the pyrethroid insecticides act to delay inactivation of open sodium channels, inducing a small influx of sodium which is effectively removed by the electrogenic sodium pump. Consistent with this prediction, both veratridine and aconitine produce measurable sodium influx in neuroblastoma cells and in mouse brain synaptosomes (Jacques *et al.*, 1980; Ghiasuddin and Soderlund, 1984; Bloomquist and Soderlund, 1988). However, measurements of  $^{22}Na$  influx are not sufficiently sensitive to demonstrate an interaction of pyrethroids in these neuronal preparations in the absence of sodium channel-activating neurotoxins (Jacques *et al.*, 1980; Ghiasuddin and Soderlund, 1985), although electrophysiological studies in neuroblastoma cells show that pyrethroids by themselves are active on the sodium channel (Jacques *et al.*, 1980). Our studies indicate that measurements of membrane depolarization as determined by  $TPP^+$  accumulation may provide a more sensitive and direct index of the actions of pyrethroids on the voltage-sensitive channel.

We interpret the depolarizing action of the pyrethroids shown in these studies to indicate that there are a finite number of open sodium channels at any given time in synaptosomal preparations. Pyrethroids prolong the open time of these channels, producing an increase in sodium influx which then acts to depolarize the membrane generating more open sodium channels for pyrethroids act upon. This interpretation is consistent with kinetic models of voltage-sensitive sodium channel function (Lund, 1984; Lund and Narahashi, 1983) and with neurochemical (Eells and Dubocovich, 1988; Nicholson *et al.*, 1983; Gusovsky *et al.*, 1989) and electrophysiological (Salgado *et al.*, 1983a, b) studies of pyrethroid action on mammalian and insect neuronal preparations. Moreover, microelectrode and voltage clamp studies in crayfish giant axon directly support our interpretation, showing that both type I and type II pyrethroids depolarize the nerve membrane at resting potential by increasing steady-state sodium conductance (Lund and Narahashi, 1983; Salgado *et al.*, 1989).

The pyrethroids increased the spontaneous release of [ $^3H$ ] ACh from rat brain synaptosomes as further evidence of the depolarizing actions of these insecticides on nerve terminal membranes. Pyrethroid-induced release of ACh was TTX sensitive and occurred over the same concentration range as membrane depolarization. The estimated  $EC_{50}$  for deltamethrin-induced release of ACh (30 nM) was identical to that for membrane depolarization, supporting the coupling of membrane excitation and neurotransmitter secretion in this synaptosomal preparation. Comparison of ACh release from rat brain synaptosomes with ACh release from rabbit brain slices (Eells and Dubocovich, 1988) revealed a remarkable similarity in pyrethroid actions in two different *in vitro* neuropreparations. In both cases, the percent of total transmitter released above nonstimulated conditions was about 8 to 12% for the cyanopyrethroids at a concentration of 10  $\mu M$  and was similar to the amount of transmitter released by veratridine (10  $\mu M$ ) or potassium-evoked (20 mM) depolarization. Pyrethroid-evoked neurotransmitter release has been reported in other

mammalian and nonmammalian nerve terminal preparations exposed to cyanopyrethroids (Doherty *et al.*, 1986; Schouest *et al.*, 1986; Nicholson *et al.*, 1983). However, Brooks and Clark (1987) have reported that cyanopyrethroids enhance potassium-induced norepinephrine release in superfused rat brain synaptosomes, but have no effect on neurotransmitter release in the absence of potassium-induced membrane depolarization. Our studies not only show pyrethroid-induced membrane depolarization and neurotransmitter release, but also, clearly show no enhancement of potassium-induced membrane depolarization by pyrethroids.

#### Acknowledgments

The authors thank Dr. C. J. Hillard and Dr. S. G. Frackman for helpful discussions and critical comments on the manuscript. The secretarial assistance of Ms. C. Knapp is acknowledged.

#### References

- ABBASSY, M. A., ELDEFRAWI, M. E. AND ELDEFRAWI, A. T.: Pyrethroid action on the nicotinic acetylcholine receptor channel. *Pestic. Biochem. Physiol.* **19**: 299-308, 1983.
- AIUCHI, T., MATSUNAGA, M., NAKAYA, K. AND NAKAMURA, Y.: Calculation of membrane potential in synaptosomes with use of a lipophilic cation (tetraphenylphosphonium). *Chem. Pharm. Bull.* **37**: 3333-3337, 1989.
- BLOOMQUIST, J. R. AND SODERLUND, D. M.: Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. *Mol. Pharmacol.* **23**: 543-550, 1988.
- BRADFORD, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-252, 1976.
- BROOKS, M. W. AND CLARK, J. M.: Enhancement of norepinephrine release from rat brain synaptosomes by alpha cyano pyrethroids. *Pestic. Biochem. Physiol.* **28**: 127-139, 1987.
- CASIDA, J. E., GAMMON, D. W., GLICKMAN, A. H. AND LAWRENCE, L. J.: Mechanism of selective action of pyrethroid insecticides. *Annu. Rev. Pharmacol. Toxicol.* **23**: 413-418, 1983.
- COTMAN, C. W.: Isolation of synaptosomal and synaptic plasma membrane fractions. In *Methods in Enzymology*, ed. by S. Fleischer and L. Packer, pp. 445-452, Academic Press, New York, 1974.
- DOHERTY, J. D., NISHIMURA, K., KURIHARA, N. AND FUJITA, T.: Promotion of norepinephrine release and inhibition of calcium uptake by pyrethroids in rat brain synaptosomes. *Pestic. Biochem. Physiol.* **29**: 187-191, 1987.
- EDWARDS, R., MILLBURN, P. AND HUTSON, D. H.: Comparative toxicity of cypermethrin in rainbow trout, frog, mouse and quail. *Toxicol. Appl. Pharmacol.* **84**: 512-522, 1986.
- ELLS, J. T. AND DUBOCOVICH, M. L.: Pyrethroid insecticides evoke neurotransmitter release from rabbit striatal slices. *J. Pharmacol. Exp. Ther.* **246**: 514-521, 1988.
- ELLIOTT, M., FARNHAM, A. W., JANES, N. F., NEEDHAM, P. H. AND PULMAN, D. A.: Insecticidally active conformations of pyrethroids. In *Mechanism of Pesticide Action*, ed. by G. K. Kohn, pp. 80-91, American Chemistry Society, Washington, 1974.
- GAMMON, D. W., BROWN, M. A. AND CASIDA, J. E.: Two classes of pyrethroid action in the cockroach. *Pestic. Biochem. Physiol.* **15**: 181-186, 1981.
- GHIASUDDIN, S. M. AND SODERLUND, D. M.: Mouse brain synaptosomal sodium channels: Activation by aconitine, batrachotoxin and veratridine, and inhibition by tetrodotoxin. *Comp. Biochem. Physiol. C* **77**: 267-271, 1984.
- GHIASUDDIN, S. M. AND SODERLUND, D. M.: Pyrethroid insecticides: Potent, stereospecific enhancers of mouse brain sodium channel activation. *Pestic. Biochem. Physiol.* **24**: 200-206, 1985.
- GLICKMAN, A. H. AND LECH, J. J.: Differential toxicity of trans-permethrin in rainbow trout and mice. *Toxicol. Appl. Pharmacol.* **66**: 162-171, 1982.
- GRAY, A. J.: Pyrethroid structure-toxicity relationships in mammals. *Neurotoxicology* **6**: 127-138, 1985.
- GUSOVSKY, F., SECUNDA, S. I. AND DALY, J. W.: Pyrethroids: involvement of sodium channels in effects on inositol phosphate formation in guinea pig synaptoneuroosomes. *Brain Res.* **492**: 72-78, 1989.
- JACQUES, Y., ROMÉY, G., CAVEY, M. T., KARTALOVSKI, B. AND LAZDUNSKI, M.: Interaction of pyrethroids with the Na<sup>+</sup> channel in mammalian neuronal cells in culture. *Biochem. Biophys. Acta* **600**: 882-897, 1980.
- KOMULAINEN, H. AND BONDY, S. C.: Modulation of levels of free calcium within synaptosomes by organochlorine insecticides. *J. Pharmacol. Exp. Ther.* **241**: 575-581, 1987.
- KOSTYUK, P. G.: Calcium ionic channels in electrically excitable membranes. *Neuroscience* **5**: 945-959, 1980.
- LAWRENCE, L. J. AND CASIDA, J. E.: Pyrethroid toxicology: Mouse intracerebral structure-toxicity relationships. *Pestic. Biochem. Physiol.* **18**: 9-14, 1982.
- LAWRENCE, L. J. AND CASIDA, J. E.: Stereospecific action of pyrethroid insecticides on the  $\lambda$ -amino butyric acid receptor ionophore complex. *Science (Wash. DC)* **221**: 1399-1401, 1983.
- LICHTSSTEIN, D., KABACK, H. R. AND BLUME, A. J.: Use of a lipophilic cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. *Proc. Natl. Acad. Sci. USA* **76**: 650-654, 1979.
- LUND, A. E.: Pyrethroid modification of sodium channel: current concepts. *Pestic. Biochem. Physiol.* **22**: 161-168, 1984.
- LUND, A. E. AND NARAHASHI, T.: Kinetics of sodium channel modification as the basis for the variation in the nerve membrane effects of pyrethroids and DDT analogs. *Pestic. Biochem. Physiol.* **20**: 203-216, 1983.
- MAYOR JR., F., DIEZ-GUERRA, J., VALDIVIESO, F. AND MAYOR, F.: Effect of bilirubin on the membrane potential of rat brain synaptosomes. *J. Neurochem.* **47**: 363-369, 1986.
- MILLER, T. A. AND SALGADO, V. L.: The mode of action of pyrethroids on insects. In *The Pyrethroid Insecticides*, ed. by J. P. Leahey, pp. 43-56, Taylor and Francis, London, 1985.
- MINNEMA, D. J., COOPER, G. P., AND SCHAMER, M. M.: Differential effect of triethyllead on synaptosomal [<sup>3</sup>H]-dopamine vs. [<sup>3</sup>H]-acetylcholine and [<sup>3</sup>H]-gamma amino butyric acid release. *Neurotoxicol. Teratol.* **13**: 257-265, 1991.
- MINNEMA, D., MICHAELSON, I. A., AND COOPER, G. P.: Calcium efflux and neurotransmitter release from rat hippocampal synaptosomes exposed to lead. *Toxicol. Appl. Pharmacol.* **92**: 351-357, 1988.
- NARAHASHI, T.: Toxic chemicals as probes of nerve membrane function. *Adv. Exp. Med. Biol.* **84**: 407-445, 1977.
- NARAHASHI, T.: Nerve membrane ionic channels as the primary target of pyrethroids. *Neurotoxicology* **6**: 3-22, 1985.
- NICHOLSON, R. A., WILSON, R. G., POTTER, C. AND BLACK, H.: Pyrethroid- and DDT-evoked release of GABA from the nervous system *in vivo*. In *Pesticide Chemistry. Mode of Action, Metabolism, Toxicology*, ed. by J. Miyamoto and P. C. Kearney, vol. 3, pp. 75-78, Pergamon Press, New York, 1983.
- PAUWELS, P. J. AND LADURON, P. M.: TPP<sup>+</sup> accumulation in rat brain synaptosomes as a probe for Na<sup>+</sup> channels. *Eur. J. Pharmacol.* **132**: 289-293, 1986.
- PEARCE, L. B., BUCK, T. AND ADAMEC, E.: Rapid kinetics of potassium-evoked release of acetylcholine from rat brain synaptosomes: analysis by rapid superfusion. *J. Neurochem.* **57**: 636-647, 1991.
- RAMOS, S., GROLLMAN, E. F., LAZO, P. S., DYER, S. A., HABIG, W. H., HARDER-GREE, M. C., KABACK, H. R. AND KOHN, L. D.: Effect of tetanus toxin on the accumulation of the permanent lipophilic cation tetraphenylphosphonium by guinea pig brain synaptosomes. *Proc. Natl. Acad. Sci. USA* **76**: 4783-4787, 1979.
- RAY, D. E.: The contrasting actions of two pyrethroids (deltamethrin and cis-methrin) in the rat. *Neurobehav. Toxicol. Teratol.* **4**: 801-804, 1982.
- RICKARD, J. AND BRODIE, M. E.: Correlation of blood and brain levels of the neurotoxic pyrethroid deltamethrin with the onset of symptoms in rats. *Pestic. Biochem. Physiol.* **23**: 143-156, 1985.
- ROWELL, P. P. AND WINKLER, D. L.: Nicotinic stimulation of [<sup>3</sup>H]acetylcholine release from mouse cerebral cortical synaptosomes. *J. Neurochem.* **43**: 1593-1598, 1984.
- SALGADO, V. L., HERMAN, M. D. AND NARAHASHI, T.: Interactions of the pyrethroid fenvalerate with nerve membrane sodium channels: temperature dependence and mechanism of depolarization. *Neurotoxicology* **10**: 1-14, 1989.
- SALGADO, V. L., IRVING, S. N. AND MILLER, T. A.: Depolarization of motor nerve terminals by pyrethroids in susceptible and kdr resistant house flies. *Pestic. Biochem. Physiol.* **20**: 100-114, 1983a.
- SALGADO, V. L., IRVING, S. N. AND MILLER, T. A.: The importance of nerve terminal depolarization in pyrethroid poisoning of insects. *Pestic. Biochem. Physiol.* **20**: 169-182, 1983b.
- SCHOUEST JR. L. P., SALGADO, V. L. AND MILLER, T. A.: Synaptic vesicles are depleted from motor nerve terminals of deltamethrin-treated house fly larvae, *Musca domestica*. *Pestic. Biochem. Physiol.* **25**: 381-386, 1986.
- SCOTT, I. D. AND NICHOLLS, D. G.: Energy transduction in intact synaptosomes. *Biochem. J.* **186**: 21-33, 1980.
- SHERBY, S. M., ELDEFRAWI, A. T., DESHPANDE, S. S., ALBURQUERQUE, E. X. AND ELDEFRAWI, M. E.: Effects of pyrethroids on nicotinic acetylcholine receptor binding and function. *Pestic. Biochem. Physiol.* **26**: 107-115, 1986.
- SODERLUND, D. M.: Pharmacokinetic behavior of enantiometric pyrethroid esters in the cockroach, *Periplaneta americana* L. *Pestic. Biochem. Physiol.* **12**: 38-48, 1979.
- STAATZ, C. G., BLOOM, A. S. AND LECH, J. J.: Effects of pyrethroid on [<sup>3</sup>H]-kainic acid binding to mouse forebrain membranes. *Toxicol. Appl. Pharmacol.* **64**: 566-569, 1982.
- TAMKUN, M. M. AND CATTERALL, W. A.: Ion flux studies of voltage-sensitive sodium channels in synaptic nerve ending particles. *Mol. Pharmacol.* **19**: 78-86, 1981.
- VERSCHOYLE R. D. AND ALDRIDGE, W. N.: Structure-activity relationships of some pyrethroids in rats. *Arch. Toxicol.* **45**: 325-332, 1980.

- VIJVERBERG, H. P. M. AND DE WEILLE, J. R.: Ther interaction of pyrethroids with voltage-dependent  $\text{Na}^+$  channels. *Neurotoxicology* **6**: 23-34, 1985.
- VIJVERBERG, H. P. M., DE WEILLE, J. R., RUGHT, G. S. F. AND VAN DEN BERCKEN, J.: The effect of pyrethroid structure on the interaction with the sodium channel in the nerve membrane. *In Neuropharmacology and Pesticide Action*, ed by M. G. Ford, G. G. Lunt, R. C. Reay and P. N. R. Usherwood, pp. 267-285, Ellis Horwood, England, 1986.
- VIJVERBERG, H. P. M. AND VAN DEN BERKEN, J.: Neurotoxicological effects and the model of action of pyrethroid insecticides. *Crit. Rev. Toxicol.* **21**: 105-126, 1990.

WILKINSON, G. N.: Statistical estimations in enzyme kinetics. *Biochem. J.* **80**: 324-332, 1961.

WINER, B.: *Statistical Principles in Experimental Design*, pp. 201-204, McGraw Hill, New York, 1972.

---

Send reprint requests to: Janis T. Eells, Ph.D., Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226.

---