

Differences in the Neuroexcitatory Actions of Pyrethroid Insecticides and Sodium Channel-Specific Neurotoxins in Rat and Trout Brain Synaptosomes¹

JANIS T. EELLS,² JENNIFER L. RASMUSSEN, PETER A. BANDETTINI, AND JENNIFER M. PROPP

Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

Received August 10, 1992; accepted June 23, 1993

Differences in the Neuroexcitatory Actions of Pyrethroid Insecticides and Sodium Channel-Specific Neurotoxins in Rat and Trout Brain Synaptosomes. EELLS, J. T., RASMUSSEN, J. L., BANDETTINI, P. A., AND PROPP, J. M. (1993). *Toxicol. Appl. Pharmacol.* 123, 107-119.

The effects of pyrethroid insecticides and other sodium channel-specific neurotoxins on synaptosomal membrane potential were investigated in rat and trout brain synaptosomes using the membrane-permeant lipophilic cation [³H]tetraphenylphosphonium (TPP⁺). Concentration-dependent and tetrodotoxin-insensitive decreases in TPP⁺ accumulation, indicative of membrane depolarization, were produced by veratridine, aconitine, scorpion (*Leiurus quinquestriatus*) venom, and type I and type II pyrethroids, in both species. Veratridine, aconitine, and *Leiurus* venom were more potent and efficacious membrane-depolarizing agents in rat synaptosomes than in trout synaptosomes. Type II (deltamethrin, cypermethrin) pyrethroids produced similar depolarizing responses in rat and trout synaptosomes; however, the *1R-cis-αR* isomer of deltamethrin, which had no effect on membrane potential in rat synaptosomes, depolarized trout synaptosomes. This isomer of deltamethrin was also shown to produce toxicity in trout, but not in rats. The type I pyrethroids, permethrin and NRDC 157, exhibited significantly greater intrinsic activity in trout brain synaptosomes, producing maximal membrane depolarizations that were three times greater than those observed in rat brain synaptosomes. These results provide evidence of species-specific differences in the membrane-depolarizing properties of pyrethroid insecticides and sodium channel-specific neurotoxins. They also suggest that some of the neurotoxin binding domains of the voltage-sensitive sodium channel in trout brain differ from those in mammalian brain. The hypersensitivity of fish to the neurotoxic actions of pyrethroid insecticides may be related to these differences. © 1993 Academic Press, Inc.

The synthetic pyrethroids are among the most potent and effective insecticides available (Casida *et al.*, 1983; Vij-

verberg and van den Bercken, 1990), accounting for more than 30% of the world market in insecticides. The low toxicity of these insecticides to mammals and birds and their limited soil persistence has encouraged the widespread and increasing use of pyrethroids in agriculture. Fish, however, are extremely sensitive to the neurotoxic effects of these insecticides. Pyrethroids have been shown to be neurotoxic and lethal to fish at concentrations 10-1000 times lower than corresponding values for mammals and birds (Glickman *et al.*, 1982; Glickman and Lech, 1982; Edwards *et al.*, 1986; Bradbury and Coats, 1989).

The hypersensitivity of fish to pyrethroid intoxication is due to species-specific differences in pyrethroid metabolism and to an increased sensitivity of the piscine nervous system to these agents. Comparative *in vivo* and *in vitro* metabolic studies have shown that rainbow trout have a lower capacity than rodents and birds to metabolize and eliminate pyrethroid insecticides (Glickman and Lech, 1981, 1982; Glickman *et al.*, 1982). However, metabolic differences do not entirely account for the hypersensitivity of fish to pyrethroid intoxication. Glickman and Lech (1982) reported that some pyrethroid isomers are still 60 times more toxic to trout than to mice when pyrethroid metabolism is inhibited in both species. These investigators also showed that pyrethroids produce profound toxicity in rainbow trout at CNS concentrations 3-20 times lower than those required to produce the same signs of toxicity in mammals. Other laboratories have corroborated these findings, reporting 10- to 20-fold differences in toxic brain concentrations of pyrethroid insecticides in trout compared to rodents (Edwards *et al.*, 1986). These findings are indicative of a high intrinsic sensitivity of the trout central nervous system to pyrethroid insecticides.

The voltage-sensitive sodium channel is a primary molecular target of the pyrethroid insecticides (Narahashi, 1985; Vijverberg and van den Bercken, 1990). Pyrethroid action on the voltage-sensitive sodium channel is supported by electrophysiological studies in neuronal cells or tissues (Lund and Narahashi, 1983; Vijverberg *et al.*, 1986), by ²²Na flux studies in cultured neuronal cells (Jacques *et al.*,

¹ This research was presented in part at the Annual Meeting of the Society of Toxicology, Miami Beach, FL (February 1990) and at Neurotox'91, International Symposium on the Molecular Basis of Drug and Pesticide Action at the University of Southampton, England (April 1991).

² To whom reprint requests should be addressed.

1980; Roche *et al.*, 1985) and synaptosomes (Ghiasuddin and Soderlund, 1985; Bloomquist and Soderlund, 1988) and by binding studies using mammalian brain membranes (Brown *et al.*, 1988; Lombet *et al.*, 1988). Pyrethroid insecticides prolong the opening of the voltage-dependent sodium channel, causing an extended depolarization of the nerve membrane and a prolonged depolarization tail current which is responsible for a repetitive sodium impulse activity (Narahashi, 1985; Vijverberg and De Weille, 1985).

The kinetics and sodium conductance of voltage-sensitive sodium channels are modified by a variety of neurotoxins in addition to the pyrethroid insecticides. These neurotoxins act at distinct receptor sites and have been used as molecular probes of channel structure and function (Catterall, 1988). At least five distinct binding sites on the mammalian channel have been identified. Saxitoxin and tetrodotoxin bind to neurotoxin site 1 and block sodium ion translocation. The lipid-soluble toxins, batrachotoxin, veratridine, and aconitine, bind at neurotoxin receptor site 2, producing persistent channel activation. Certain scorpion α -toxins and sea anemone toxins bind at neurotoxin receptor site 3 and suppress channel inactivation. The β scorpion toxins bind at site 4 to alter sodium channel activation, and ciguatoxins and brevetoxins act at site 5. Pyrethroids bind stereoselectively at a distinct, but incompletely characterized, domain on the sodium channel and modulate the binding and the activity of toxins acting at sites 2–4 (Lombet *et al.*, 1988; Brown *et al.*, 1988).

The pharmacological and functional properties of voltage-sensitive sodium channels in cultured mammalian neuronal cells and mammalian nerve terminal preparations have been well characterized; however, considerably less is known about the function and pharmacology of these ion channels in fish brain. Radiolabeled sodium flux studies in trout brain synaptosome (Stuart *et al.*, 1987) and synaptoneurosome (Rubin and Soderlund, 1992) preparations have provided evidence for functional sodium channels in these preparations, but have yielded conflicting results with respect to the pharmacologic properties of these channels. In addition, measurement of neurotoxin-stimulated radiolabeled sodium flux was not sensitive enough to assess pyrethroid potencies in fish brain preparations (Rubin and Soderlund, 1992).

Recent studies by our laboratory have shown 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 membrane-permeant lipophilic cation tetraphenylphosphonium (TPP^+)³ in rat brain P2 synaptosomal preparations (Eells *et al.*, 1992). In the present study, we

have employed this biochemical probe to compare the effects of pyrethroid insecticides and other neurotoxins in rat and trout brain synaptosomal preparations. The investigations reported here show that TPP^+ accumulation is a direct and sensitive measure of membrane depolarization secondary to sodium channel activation in trout nerve terminal preparations. In addition, these studies present pharmacological evidence for differences between mammalian and piscine sodium channel modulation by pyrethroid insecticides and other sodium channel-specific neurotoxins.

METHODS

Materials

Deltamethrin ((*S*)- α -cyano-3-phenoxybenzyl-*cis*-(1*R*,3*R*))-2,2-dimethyl-3-(2,2-dibromovinyl)cyclopropane carboxylate; 1*R*-*cis*- α S), the descyano analog of deltamethrin, NRDC 157 (1*R*-*cis*), and the 1*R*-*cis*- α R enantiomer of deltamethrin were provided by Dr. J. P. Demoute at Roussel Uclaf (Romainville, France). The 1*R*-*cis*- α S and the 1*S*-*cis*- α R isomers of cypermethrin (α -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-dimethylcyclopropane carboxylate) were provided by Dr. A. A. Ramsey at FMC Corporation (Princeton, NJ). The purity of the pyrethroid stereoisomers was reported by each source to be greater than 98%. Tetrodotoxin, veratridine, aconitine, and *Leiurus quinquestriatus* (North African) venom were obtained from Sigma Chemical Co. (St. Louis, MO). [³H]Tetraphenylphosphonium bromide, [³H]H₂O, and [¹⁴C]sorbitol were purchased from Amersham Corp. (Arlington Heights, IL). Sorbent 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 250–300 g and rainbow trout (*Oncorhynchus mykiss*), 16–25 cm in length, were used in these experiments. Rats were supplied with food and water *ad libitum* and maintained on a 12-h light/dark schedule in a temperature- and humidity-controlled environment prior to euthanization. Trout obtained from the Department of the Interior fish hatcheries were fed trout pellets (Murray Elevators, Murray, UT) and maintained in flow-through conditions in dechlorinated tap water at 10–15°C on a 12-h light/dark schedule prior to euthanization. All 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.

Pyrethroid Toxicity Studies

Solutions of the 1*R*-*cis*- α R enantiomer of deltamethrin and deltamethrin (1*R*-*cis*- α S) were prepared in DMSO/saline (1/1). Pyrethroids were administered by intraperitoneal injection at a dose of 5 mg/kg (trout; $n = 3$) or 100 mg/kg (rats; $n = 3$) in a volume of 0.1–0.3 ml, and animals were placed in aquaria or cages and observed for symptoms of toxicity for 3 hr. An equivalent volume of the vehicle was administered to control animals. Pyrethroid doses were selected on the basis of pyrethroid toxicity studies conducted in trout (Glickman and Lech, 1982; Bradbury and Coats, 1989) and rats (Verschoyle and Aldridge, 1980; Gray, 1985). The limited amount of the 1*R*-*cis*- α R enantiomer of deltamethrin available to our laboratory precluded LD₅₀ determinations. Animals exhibiting signs of toxicity were observed for 5–10 min to provide a description of the toxic

³ Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPP^+ , tetraphenylphosphonium; TTX, tetrodotoxin.

symptoms then euthanized by cervical dislocation (trout) or lethal dose of pentobarbital (rats). No differences were observed in the behavior of vehicle-treated animals relative to untreated controls.

Preparation of Synaptosomes

Rats and trout were euthanized 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes; pH 7.5 with Tris base) and 1.0 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 1000g for 10 min to separate the nuclear fraction. The resulting supernatant was centrifuged at 12,000g for 20 min (rat) or 17,000g for 30 min (trout) to obtain the P2 synaptosomal pellet. The increase in centrifugation force and time for the trout preparation was required to obtain a packed and reproducible P2 pellet. The P2 pellet was resuspended to a final protein concentration of 0.25–0.35 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 CaCl₂, 0.8 mM MgSO₄, and 5.5 mM glucose. The protein content of synaptosomal preparations was determined by the method of Bradford (1976).

TPP⁺ Accumulation Assay

[³H]TPP⁺ 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*-³H]TPP⁺ (28 Ci/mmol) at 37°C (rat) or 15°C (trout) in a shaking waterbath for 15 min to allow TPP⁺ equilibration. TPP⁺ uptake was directly proportional to synaptosomal protein concentration from 20 to 100 μg of protein. In some experiments, synaptosomes from both species were incubated at 25°C. 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 *1R-cis-αS* configuration and the neurotoxic isomer of permethrin is the *1R-cis* isomer. Pyrethroid insecticides, veratridine and aconitine, were added (1–2 μl) from concentrated stock solutions in dimethylsulfoxide (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⁺ accumulation. In most experiments, ouabain (1 mM) was added with the pyrethroids or 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 (in mM) choline chloride (163), Hepes-Tris (50), MgCl₂ (0.8), and CaCl₂ (1.8), pH 7.4, followed by rapid vacuum filtration through glass fiber filters (#32, Schleicher and Schuell, Keene, NH). Synaptosomal [³H]TPP⁺ 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 [³H]TPP⁺ by intrasynaptosomal mitochondria (Scott and Nicholls, 1980). Nonspecific accumulation was determined from parallel incubations of boiled synaptosomes and has been subtracted from the data. Synaptosomal TPP⁺ accumulation in the presence of valinomycin (5 μM) and potassium (80 mM) was comparable to nonspecific accumulation indicating that these treatments abolished both mitochondrial and plasma membrane accumulation of TPP⁺. Unless stated otherwise, the sodium concentration was decreased in incubates that contained potassium concentrations greater than 5 mM to maintain iso-osmolality. Values for half-maximal (EC₅₀) effects on TPP⁺ accumulation were determined by fitting data from individual experiments to the logistic equation $e = e_{\max}/1 + [k/x]^n$, where e is the calculated change in membrane potential at a given

concentration of agonist (x), k is the EC₅₀ value for the agonist, and n is the slope factor, using the Sigma-Plot scientific graphics program.

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 protein) was added to 450 μl of incubation buffer containing [³H]H₂O (10⁶ dpm) and [¹⁴C]sorbitol (275,000 dpm). Synaptosomes were allowed to equilibrate for 10 min at 30°C (rat) or 15°C (trout), followed by centrifugation and washing of the synaptosomal pellet. The total volume of the synaptosomal pellet was determined from the [³H]H₂O content and the extracellular space from the [¹⁴C]sorbitol content. The intrasynaptosomal volume was taken as the difference between total and extrasynaptosomal volumes. Average intrasynaptosomal volumes of 3.6 (rat) and 6.4 μl per milligram of protein (trout) were calculated from the data. Similar values were obtained in both species when synaptosomes were treated with DMSO (0.1%), veratridine (100 μM), aconitine (100 μM), *Leiurus* venom (100 μg), or the pyrethroids (100 μM).

Calculation of TPP⁺ Concentration Gradients and Membrane Potential

The intrasynaptosomal concentration of TPP⁺, [TPP⁺]_{in}, was calculated from the accumulation of TPP⁺ per milligram of protein and the mean intrasynaptosomal volume (3.6 μl/mg of protein for rat and 6.4 μl/mg protein for trout). Subtraction of values obtained for TPP⁺ accumulation in 80 mM external potassium concentration from values obtained at 5 mM potassium yielded the TPP⁺ concentration in potassium-sensitive compartments (i.e., across the plasma membrane). External TPP⁺ concentration, [TPP⁺]_{out}, was calculated by subtracting the amount taken up from the concentration of TPP⁺ originally present in the reaction mixture. Concentration gradients were calculated as [TPP⁺]_{in}/[TPP⁺]_{out}, and membrane potential was estimated by applying this ratio to the Nernst equation as described by Lichtshtein *et al.* (1979).

Statistical Analysis

Results are presented as means ± SE. 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, one-way or two-way analyses of variance were performed. For some comparisons, these were 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 $p < 0.05$.

RESULTS

Estimation of Synaptosomal Membrane Potential in Trout Brain Synaptosomes

The accumulation of [³H]tetraphenylphosphonium was used to estimate membrane potential in rat and trout brain synaptosomes. TPP⁺ is a lipophilic cation that passes through the hydrophobic core of biological membranes and passively distributes across the membrane according to the Nernst equation. This probe has been utilized to estimate membrane potential in cultured cells (Lichtshtein *et al.*, 1979) and in mammalian synaptosomes (Ramos *et al.*,

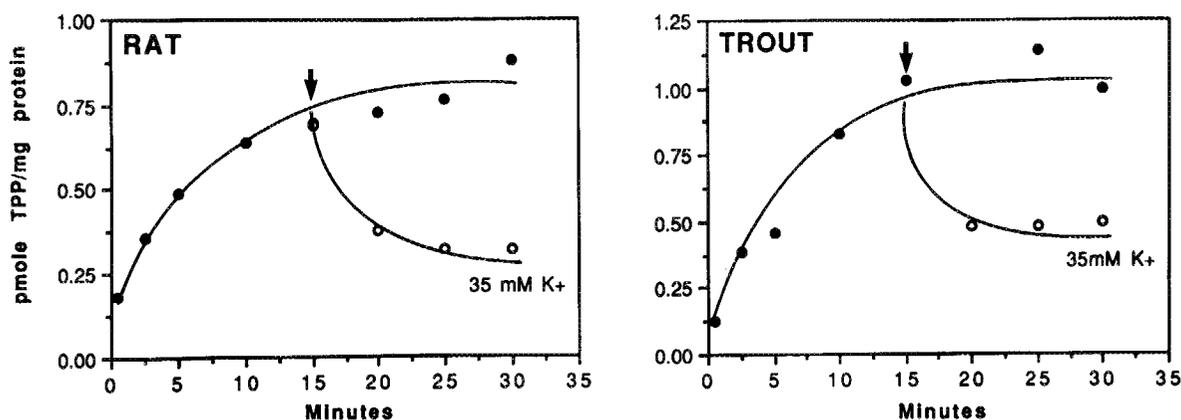


FIG. 1. Time course of $[^3\text{H}]\text{TPP}^+$ accumulation in rat and trout brain P2 synaptosomes. Synaptosomes (0.075 mg protein) were added to incubation buffer containing 2 nM $[^3\text{H}]\text{TPP}^+$ and incubated at 37°C (rat) or 15°C (trout) for the times shown (closed circles). At the arrow, high potassium buffer (final potassium concentration, 35 mM, open circles) was added and the incubation was continued for the times indicated. Separate incubates were filtered at the times indicated and TPP accumulation was measured as described under Methods. Data are expressed as pmol of TPP accumulated per milligram of synaptosomal protein as a function of time. Results shown are from a single experiment that was repeated three times with similar results.

1979; Pauwels and Laduron, 1986; Aiuchi *et al.*, 1989; Eells *et al.*, 1992). Rat and trout brain synaptosome preparations suspended in a medium containing physiological concentrations of sodium and potassium (135 mM Na^+ and 5 mM K^+) accumulated TPP^+ , reaching comparable steady state levels of accumulation within 10–15 min, which were maintained for at least 30 min, as shown in Fig. 1. This steady state level of synaptosomal TPP^+ accumulation reflects equilibration of this cation across the plasma membrane and accumulation into intrasynaptosomal mitochondria (Scott and Nicholls, 1980; Ramos *et al.*, 1979; Aiuchi *et al.*, 1989). In both species, TPP^+ accumulation was similarly decreased by increasing extracellular potassium concentrations to 35 mM (Fig. 1). Moreover, the equilibrium level of TPP^+ in synaptosomes from both species decreased in an exponential manner to a limiting value of approximately 20% when the external potassium concentration was increased from 5 to 80 mM, indicating that the membrane potential across the synaptosomal membrane is due primarily to a potassium diffusion gradient (Fig. 2). Subtraction of values obtained for TPP^+ accumulation in 80 mM external potassium concentration from values obtained at 5 mM potassium yielded the TPP^+ concentration in potassium-sensitive compartments (i.e., across the plasma membrane). Concentration gradients were calculated as $[\text{TPP}^+]_{\text{in}}/[\text{TPP}^+]_{\text{out}}$, and membrane potential was estimated by applying this ratio to the Nernst equation as described by Lichtshtein *et al.* (1979). When these determinations were done in the presence of valinomycin to eliminate the mitochondrial contribution to membrane potential, it was possible to estimate the resting plasma membrane potential in rat and trout synaptosomes. Resting membrane potential values of -80 ± 3 ($n = 6$) and -78 ± 2 mV ($n = 6$)

were estimated for rat and trout brain synaptosomes, respectively. The similarity in the resting membrane potential, the time course for TPP^+ accumulation, and the profiles of potassium-dependent membrane depolarization observed in rat and trout brain synaptosomes suggests that TPP^+ is a useful probe for neurotoxin-induced membrane potential changes in trout brain synaptosomes, as has been previously reported for rat brain synaptosomes (Eells *et al.*, 1992).

Effects of Alkaloid Neurotoxins on Membrane Potential

The alkaloid neurotoxins, veratridine and aconitine, interact with voltage-sensitive sodium channels to induce persistent channel activation (Catterall, 1988). As shown in Fig. 3, veratridine and aconitine (0.01–300 μM) produced concentration-dependent synaptosomal membrane depolarization in both species with maximal depolarizing responses observed at concentrations of 100 μM . We have previously shown in rat brain synaptosomes that veratridine and aconitine exhibit similar efficacy and potency. Both agents reduced TPP^+ accumulation by nearly 70% at concentrations of 100 μM , corresponding to an estimated membrane depolarization of 60–70 mV (Eells *et al.*, 1992). In contrast, both neurotoxins were less effective and less potent membrane-depolarizing agents in trout brain synaptosomes. As shown in Table 1, veratridine and aconitine produced maximal estimated membrane depolarizations of 31 ± 1 and 23 ± 3 mV, respectively, in trout brain synaptosomes. The half-maximal response (EC_{50}) values for veratridine- and aconitine-induced membrane depolarization in trout synaptosomes were 5.0 ± 0.8 and 10 ± 1 μM ,

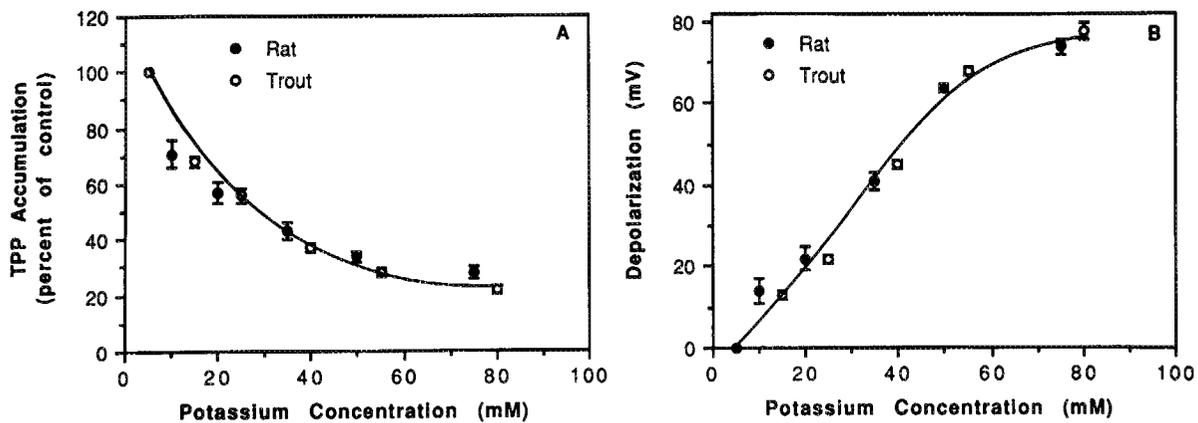


FIG. 2. $[^3\text{H}]\text{TPP}^+$ accumulation and calculated membrane depolarization in rat and trout P2 synaptosomes as a function of external potassium concentration. Synaptosomes were incubated at 37°C (rat) or 15°C (trout) for 20 min, at the potassium concentrations indicated, in the presence of 2 nM $[^3\text{H}]\text{TPP}^+$. Iso-osmolarity was maintained by decreasing sodium concentrations as potassium concentrations increased. Data are expressed as percentage of control (5 mM K) TPP^+ accumulation (A) and calculated membrane depolarization (mV) (B) as a function of potassium concentration. Membrane potential was determined by applying the Nernst equation to the equilibrium distribution of TPP^+ as described under Methods. Shown are the mean values \pm SE from 4 experiments in each species.

respectively, compared with EC_{50} values of 2.3 ± 1.0 and $5.0 \pm 0.5 \mu\text{M}$ in rat brain synaptosomes. Measurements of the effects of sodium channel-specific neurotoxins on TPP^+ accumulation were conducted in the presence of ouabain (1 mM) to inhibit the Na/K ATPase-dependent extrusion of intrasynaptosomal sodium and resulting synaptosomal repolarization (Lichtshtein *et al.*, 1979; Eells *et al.*, 1992). The presence of ouabain in the incubation medium reduced TPP^+ accumulation by approximately 10% in both rat and trout brain synaptosomes, corresponding to a 3- to 4-mV depolarization.

Effects of Scorpion Venom (*L. quinquestratus*) on Membrane Potential

The α -polypeptide toxin present in the venom of the North American scorpion *L. quinquestratus* delays inactivation of voltage-sensitive sodium channels (Catterall, 1988). *Leiurus* venom produced concentration-dependent synaptosomal depolarization in rat and trout brain synaptosomes as shown in Fig. 4. *Leiurus* venom also demonstrated greater efficacy and potency in rat brain synaptosomes than in trout brain synaptosomes. The maximal esti-

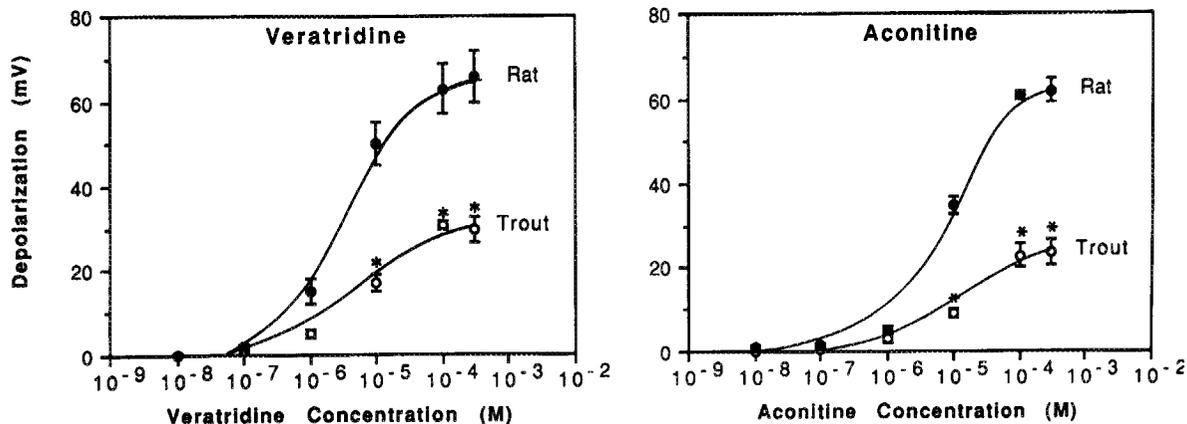


FIG. 3. Alkaloid neurotoxin-induced synaptosomal depolarization in rat and trout brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM $[^3\text{H}]\text{TPP}^+$ at 37°C (rat) or 15°C (trout) for 15 min to allow for TPP^+ equilibration. Veratridine or aconitine were then added at the concentrations indicated, in the presence of ouabain (1 mM), and the mixture was incubated for 10 additional minutes. 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 neurotoxin-treated synaptosomes and their respective vehicle-treated (0.1% DMSO; 1 mM ouabain) controls. Shown are the mean values \pm SE from 5 experiments. An asterisk indicates $p < 0.05$ compared to depolarization measured in rat brain synaptosomes using Dunnett's modification of the *t* test.

TABLE 1
Calculated EC_{50} and E_{max} Values and Tetrodotoxin Sensitivity for Synaptosomal Membrane Depolarization Induced by Sodium Channel-Specific Neurotoxins and Pyrethroid Insecticides^a

Agent	Rat			Trout		
	EC_{50} (μM)	E_{max} (mV)	$E_{max} + TTX$ (mV)	EC_{50} (μM)	E_{max} (mV)	$E_{max} + TTX$ (mV)
Veratridine	2.3 \pm 1.0	+63 \pm 6	-2 \pm 1	5.0 \pm 0.8 ^b	+31 \pm 1 ^b	+1 \pm 1
Aconitine	5.0 \pm 0.5	+61 \pm 1	-1 \pm 1	10.0 \pm 1.0 ^b	+23 \pm 3 ^b	0 \pm 1
<i>Leiurus</i> venom	0.15 \pm 0.02	+45 \pm 3	+3 \pm 1	0.3 \pm 0.04 ^b	+20 \pm 3 ^b	+1 \pm 1
Permethrin (<i>IR-cis</i>)	3.5 \pm 0.9 ^c	+5 \pm 1 ^c	0 \pm 1	3.8 \pm 0.9	+15 \pm 2 ^b	+1 \pm 1
NRDC 157 (<i>IR-cis</i>)	5.4 \pm 1.0 ^d	+4 \pm 1 ^d	+1 \pm 1	5.0 \pm 0.7	+11 \pm 2 ^{b,d}	+1 \pm 1
Cypermethrin (<i>IR-cis-\alpha S</i>)	0.6 \pm 0.3	+20 \pm 3	+2 \pm 1	2.0 \pm 0.4 ^b	+20 \pm 1	+1 \pm 1
Deltamethrin (<i>IR-cis-\alpha S</i>)	0.4 \pm 0.1	+25 \pm 3	-1 \pm 1	2.0 \pm 0.3 ^b	+22 \pm 3	-2 \pm 1

Note. Values represent the means \pm SE from 4 to 6 experiments.

^a Values for half-maximal (EC_{50}) effects on TPP^+ accumulation were determined by fitting data from individual experiments to the logistic equation $e = e_{max}/1 + [k/x]^n$ as described under Methods. E_{max} values are expressed as the difference (mV change) between the membrane potential values determined for pyrethroid (100 μM) \pm TTX (1 μM) or neurotoxin (veratridine and aconitine, 100 μM ; *Leiurus* venom, 10 μM) \pm TTX (1 μM) treated synaptosomes and their respective vehicle-treated (0.1% DMSO) controls.

^b Significantly different from same treatment in rat brain synaptosomes with $p < 0.05$ (two-tailed Student's t test).

^c Significantly different from cypermethrin treatment in same species with $p < 0.05$ (two-tailed Student's t test).

^d Significantly different from deltamethrin treatment in same species with $p < 0.05$ (two-tailed Student's t test).

mated depolarization produced by *Leiurus* venom in rat brain synaptosomes was 45 \pm 3 mV, with an estimated EC_{50} of 0.15 \pm 0.02 μM (assuming a molecular weight of 6700 for the α -toxin component of *Leiurus* venom [Tamkun and

Catterall, 1981]). In trout brain synaptosomes *Leiurus* venom produced a maximal estimated membrane depolarization of 20 \pm 3 mV with an EC_{50} of 0.30 \pm 0.04 μM .

Membrane Depolarization Produced by Pyrethroid Insecticides

Pyrethroid insecticides have been divided into two classes on the basis of their chemical structures, which is reflected in their neurophysiological and toxicological actions (Verschoyle and Aldridge, 1980; Ray, 1982; Casida *et al.*, 1983). Pyrethroids containing an α -cyano substituent on the alcohol moiety of the pyrethroid molecule have been classified as type II pyrethroids and include the potent insecticides, deltamethrin and cypermethrin. Type I pyrethroids, which lack the α -cyano moiety, include the descyano analog of cypermethrin, permethrin, and the descyano analog of deltamethrin, NRDC 157. Both type I and type II pyrethroids produced concentration-dependent increases in membrane depolarization in rat and trout brain synaptosomes (Fig. 5). The effects of pyrethroids on membrane potential were apparent from 100 nM to 100 μM . Pyrethroid-induced membrane depolarization achieved statistical significance at 1 μM for the type II pyrethroids and at 10 μM for the type I pyrethroids, in both species. Maximal depolarizing effects were observed in both species at pyrethroid concentrations between 10 and 100 μM . In rat brain synaptosomes, the type II pyrethroids, deltamethrin and cypermethrin, were significantly more effective and potent depolarizing agents than their descyano analogs, NRDC 157 and permethrin (Table 1). Deltamethrin and cypermethrin produced similar profiles of depolarization in

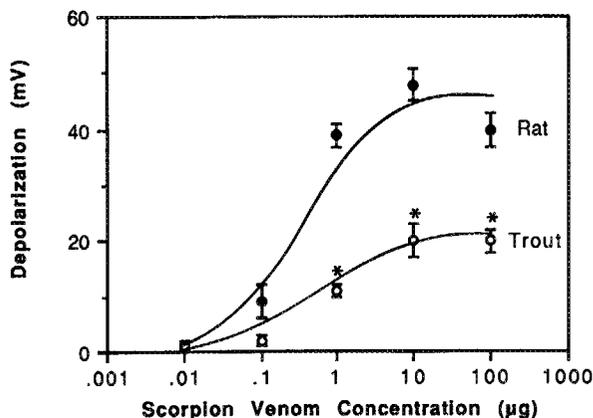


FIG. 4. Scorpion (*L. quinquestriatus*) venom-induced synaptosomal depolarization in rat and trout brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM [³H]TPP⁺ at 37°C (rat) or 15°C (trout) for 15 min to allow for TPP⁺ equilibration. Scorpion venom was added at the concentrations indicated, in the presence of ouabain (1 mM), and the mixture was incubated for 10 additional minutes. 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 neurotoxin-treated synaptosomes and their respective vehicle-treated (0.1% DMSO; 1 mM ouabain) controls. Shown are the mean values \pm SE from 5 experiments. An asterisk indicates $p < 0.05$ compared to depolarization measured in rat brain synaptosomes using Dunnett's modification of the t test.

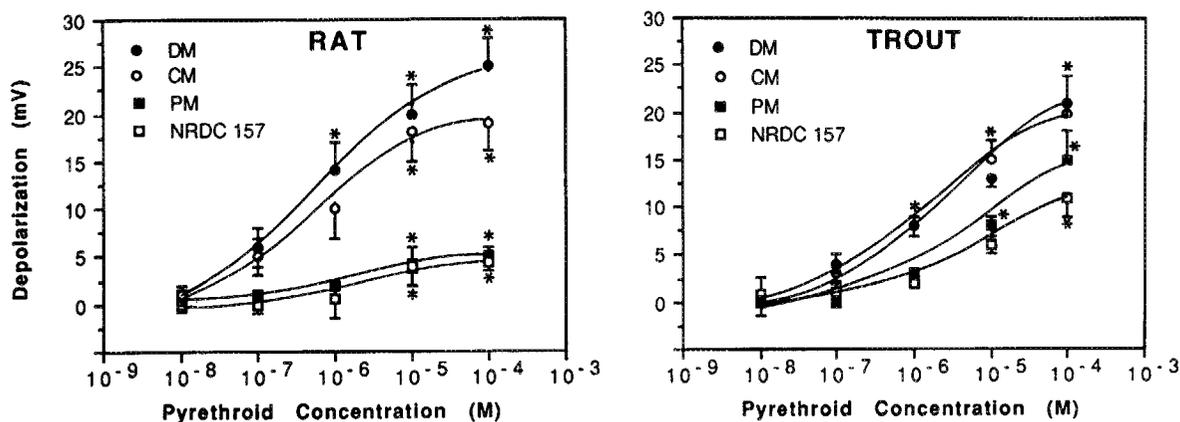


FIG. 5. Pyrethroid insecticide-induced synaptosomal membrane depolarization in rat and trout brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM [^3H]TPP $^+$ at 37°C (rat) or 15°C (trout) for 15 min to allow for TPP $^+$ equilibration. Deltamethrin (DM), cypermethrin (CM), permethrin (PM), or NRDC 157 was then added at the concentrations indicated, in the presence of 1 mM ouabain, and the mixture was incubated for 10 additional minutes. 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) controls. Shown are the mean \pm SE from 4 to 6 experiments. An asterisk indicates $p < 0.05$ compared to depolarization measured in the absence of pyrethroid using Dunnett's modification of the t test. Synaptosomal depolarization elicited by permethrin and NRDC 157 was significantly greater in trout brain synaptosomes than in rat brain synaptosomes (two-way ANOVA with repeated measures, $p < 0.01$).

rat brain synaptosomes, with maximal depolarization values of 25 ± 3 and 20 ± 3 mV and EC_{50} values of 0.4 ± 0.1 and 0.6 ± 0.3 μM , respectively. The type I pyrethroids, NRDC 157 and permethrin, produced maximal membrane depolarizations of 4 ± 1 and 5 ± 1 mV with EC_{50} values of 5.4 ± 1.0 and 3.5 ± 0.9 μM , respectively. In contrast, in trout brain synaptosomes, both the intrinsic activity and the potency of type I and type II pyrethroids were similar (Table 1). The type II pyrethroids, deltamethrin (E_{max} , 22 ± 3 mV; EC_{50} , 2.0 ± 0.3 μM) and cypermethrin (E_{max} , 20 ± 1 mV; EC_{50} , 2.0 ± 0.4 μM) exhibited nearly equivalent intrinsic activity and potency in trout brain synaptosomes, analogous to their actions in rat brain synaptosomes (Fig. 5). Maximal estimated membrane depolarization produced by the type II pyrethroids was also similar in the two species (Table 1). The descyano pyrethroids, NRDC 157 and permethrin, were significantly more effective depolarizing agents in trout brain synaptosomes than in rat brain synaptosomes, producing maximal depolarizations of 11 ± 2 and 15 ± 2 mV, with apparent EC_{50} values of 5.0 ± 0.7 and 3.8 ± 0.9 μM , respectively (Table 1 and Fig. 5).

Effect of Assay Temperature on Pyrethroid-Induced Membrane Depolarization

To determine if the observed differences in the depolarizing actions of the pyrethroid insecticides in rat and trout brain synaptosomes were due to the differences in assay temperatures, experiments were conducted on synaptosomal preparations from both species incubated at the same temperature. Figure 6 compares the membrane-depolarizing actions of the type I and type II pyrethroids in rat and

trout brain synaptosomes incubated at 25°C. Membrane depolarization produced by both type I and type II pyrethroids in rat brain synaptosomes was significantly decreased at 25°C relative to 37°C. In trout brain synaptosomes, no assay temperature-related differences were apparent in the depolarizing actions of the type I and type II pyrethroids. Maximal estimated depolarization (E_{max}) produced by the type II pyrethroids, deltamethrin and cypermethrin, in trout synaptosomes, was 16 ± 3 and 18 ± 1 mV, respectively, compared to E_{max} values of 13 ± 1 (deltamethrin) and 10 ± 2 mV (cypermethrin) in rat synaptosomes. For the type I pyrethroids, NRDC 157 and permethrin, trout E_{max} values were 9.5 ± 0.8 and 10 ± 1 mV, respectively, compared with 2.5 ± 1 (NRDC 157) and 3 ± 1 mV (permethrin) in the rat. Calculated EC_{50} values for the pyrethroids in rat and trout synaptosomes were similar at 25°C relative to EC_{50} values obtained at physiological assay temperatures (data not shown), indicating that pyrethroid potency was not affected by changing the assay temperature. Importantly, the relationship between the depolarizing actions of type I and type II pyrethroids in each species was not altered by changing the assay temperature. In rat brain synaptosomes, the type II pyrethroids were more potent and efficacious membrane-depolarizing agents than the type I pyrethroids, whereas, in trout brain synaptosomes both classes of pyrethroids exhibited similar potency and efficacy.

Species Differences in Pyrethroid Stereoselectivity

The neuroexcitatory and neurotoxic actions of the pyrethroid insecticides in the mammalian nervous system in

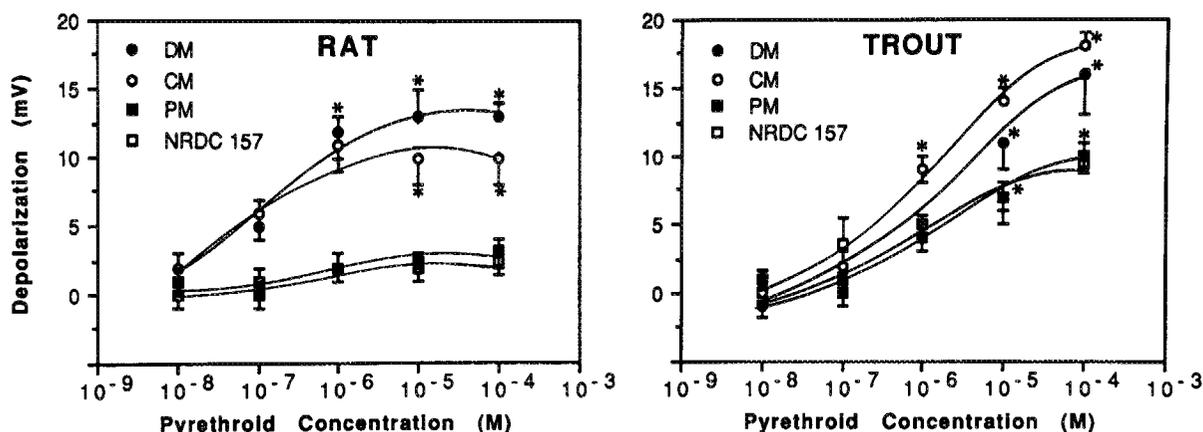


FIG. 6. Pyrethroid insecticide-induced synaptosomal membrane depolarization in rat and trout brain synaptosomes incubated at 25°C. Synaptosomes were incubated in buffer containing 2 nM [^3H]TPP $^+$ at 25°C for 15 min to allow for TPP $^+$ equilibration. Deltamethrin (DM), cypermethrin (CM), permethrin (PM), or NRDC 157 was then added at the concentrations indicated, in the presence of 1 mM ouabain, and the mixture was incubated for 10 additional minutes. 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) controls. Shown are the mean \pm SE from 4 to 6 experiments. An asterisk indicates $p < 0.05$ compared to depolarization measured in the absence of pyrethroid using Dunnett's modification of the t test. Synaptosomal depolarization elicited by each pyrethroid was significantly greater in trout brain synaptosomes than in rat brain synaptosomes (two-way ANOVA with repeated measures, $p < 0.001$). Depolarizing responses measured at 25°C in rat brain synaptosomes were significantly attenuated compared to corresponding responses measured at 37°C (two-way ANOVA with repeated measures, $p < 0.01$).

in vivo and in mammalian neuronal preparations *in vitro* have been shown to be stereospecific. For the phenoxybenzyl pyrethroids, including permethrin, cypermethrin, deltamethrin, and NRDC 157, isomers possessing an *S*-configuration at the cyclopropane C-1 and an *S*-configuration at the α -cyano moiety (for type II compounds) are neurotoxic and their corresponding *1S* and αR enantiomers are nontoxic (Lawrence and Casida, 1982; Gray, 1985; Eells *et al.*, 1992). As shown in Fig. 7, the depolariz-

ing actions of deltamethrin in rat brain synaptosomes were only observed with the *1R-cis- αS* isomer. The *1R-cis- αR* isomer had no effect on membrane potential as measured by TPP $^+$ accumulation. In trout, both the *1R-cis- αS* and the *1R-cis- αR* isomers of deltamethrin depolarized trout brain synaptosomes, producing estimated membrane depolarizations of 22 ± 3 and 13 ± 2 mV, respectively, at concentrations of 100 μM . We also compared the effects of the *1R-cis- αS* and the *1S-cis- αR* isomer of cypermethrin and

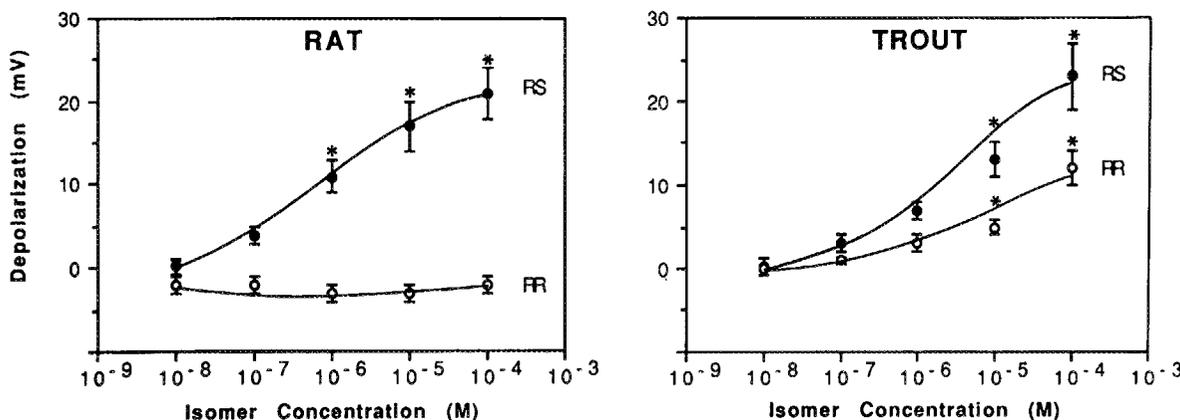


FIG. 7. Stereospecificity of deltamethrin-induced membrane depolarization in rat and trout brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM [^3H]TPP $^+$ at 37°C (rat) or 15°C (trout) for 15 min to allow for TPP $^+$ equilibration. Deltamethrin (RS) or its *1R-cis- αR* enantiomer (RR) was then added at the concentrations indicated, in the presence of 1 mM ouabain, and the mixture was incubated for 10 additional minutes. 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 neurotoxin-treated synaptosomes and their respective vehicle-treated (0.1% DMSO; 1 mM ouabain) controls. Shown are the mean \pm SE from 5 experiments. An asterisk indicates $p < 0.05$ compared to depolarization measured in the absence of pyrethroid using Dunnett's modification of the t test.

the *1R-cis* and the *1S-cis* isomer of permethrin in both rat and trout synaptosomes. In both species, reductions in TPP^+ accumulation were specific for the *1R-cis* isomer of permethrin and the *1R-cis-\alpha S* isomer of cypermethrin (data not shown), suggesting that the *1R* conformation is as essential for biological activity in trout as it is in rat. However, the conformation of the α -cyano group does not appear to be an important determinant of neuroactivity in trout brain *in vitro*.

Experiments were also conducted to determine if the *1R-cis-\alpha R* enantiomer of deltamethrin was neurotoxic to trout *in vivo*. The *1R-cis-\alpha R* isomer of deltamethrin was administered at a dose of 5 mg/kg (ip) to rainbow trout. Within 5 min of the injection, seizure activity was observed in all of the trout. Convulsions were manifested by wide opercular flaring, increased ventilation rate, and myoclonic head swaying. Similar symptoms were observed in trout treated with an equivalent dose of *1R-cis-\alpha S* deltamethrin. Rats given 100 mg/kg (ip) of the *1R-cis-\alpha R* isomer of deltamethrin exhibited no neurotoxic symptoms, whereas administration of the same dose of *1R-cis-\alpha S* deltamethrin produced the classic type II poisoning syndrome characterized by salivation and choreoathetosis. Lawrence and Casida (1983) have also reported no evidence of neurotoxicity in rats treated with the *1R-cis-\alpha R* isomer of deltamethrin.

Inhibition of Pyrethroid- and Neurotoxin-Induced Membrane Depolarization by Tetrodotoxin

Tetrodotoxin (TTX) binds to the voltage-sensitive sodium channel to block the influx of sodium through the channel (Narahashi *et al.*, 1964; Catterall, 1988). As shown in Table 1, TTX at a concentration of 1 μM fully antagonized the membrane-depolarizing actions of veratridine, aconitine, *Leiurus* venom, and the type I and type II pyrethroids in both rat and trout synaptosomes. To characterize the TTX sensitivity of rat and trout brain sodium channels, we determined the concentration-dependence of TTX inhibition of veratridine-induced membrane depolarization as shown in Fig. 8. In these experiments, we used 100 μM veratridine in trout brain synaptosomes and 10 μM veratridine in rat brain synaptosomes to achieve comparable initial levels of membrane depolarization. The threshold concentration for TTX antagonism of veratridine-induced depolarization was 10 nM in both species and depolarization was completely blocked at a TTX concentration of 1 μM . The concentration of TTX producing 50% inhibition of veratridine-induced depolarization (IC_{50}) was 20 ± 1 nM. The apparent IC_{50} for TTX inhibition of deltamethrin-induced (100 μM) depolarization was also 20 nM in both rat and trout synaptosomes (data not shown). TTX alone had no effect on membrane potential as determined by TPP^+ accumulation (data not shown).

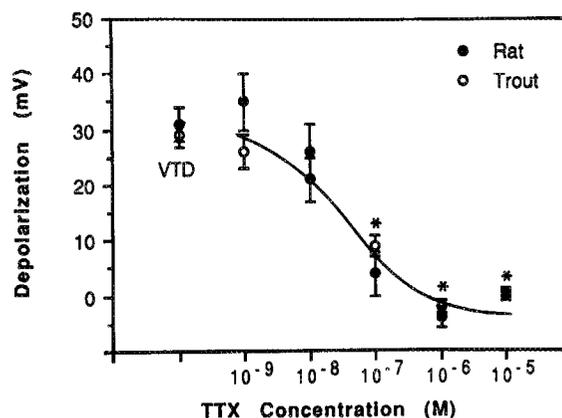


FIG. 8. Inhibition of veratridine-induced membrane depolarization by tetrodotoxin in rat and trout brain synaptosomes. Synaptosomes were incubated in buffer containing 2 nM $[\text{H}^3]\text{TPP}^+$ at 37°C (rat) or 15°C (trout) for 15 min to allow for TPP^+ equilibration. TTX was added at the concentrations indicated, in the presence of veratridine (10 μM in rat and 100 μM in trout) and ouabain (1 mM), and the mixture was incubated for 10 additional minutes. 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 neurotoxin-treated synaptosomes and their respective vehicle-treated (0.1% DMSO; 1 mM ouabain) controls. Shown are the mean values \pm SE from 4 experiments. An asterisk indicates $p < 0.05$ compared to depolarization measured in the absence of TTX (point labeled VTD) using Dunnett's modification of the t test.

DISCUSSION

The data presented in this paper show that the effects of pyrethroid insecticides and other sodium channel-specific neurotoxins on nerve membrane potential can be estimated in trout brain synaptosomal preparations using the biochemical probe $[\text{H}^3]$ tetraphenylphosphonium to measure membrane potential changes. These findings confirm and extend our previous work providing evidence that TPP^+ accumulation is a sensitive and direct measure of the neuroexcitatory actions of pyrethroid insecticides and other sodium channel-specific neurotoxins in nerve terminal preparations from two species with documented differences in pyrethroid sensitivity. Experiments were conducted in similarly prepared nerve terminal preparations from rat and trout brain and were incubated at temperatures which are physiological for each species (37°C for rat and 15°C for trout). Under these conditions, synaptosome preparations from both species exhibited similar time courses of TPP^+ accumulation and virtually identical profiles of potassium-dependent membrane depolarization. Moreover, the calculated resting potential of -78 ± 2 mV for trout brain synaptosomes is in good agreement with measured values in fish neuronal preparations (Frank and Campbell, 1992) and is also similar to the calculated resting potential in rat brain synaptosomes (-80 ± 3 mV).

In both species, type I and type II pyrethroid insecticides and the sodium channel-specific neurotoxins, veratridine, aconitine, and *Leiurus* venom, were potent synaptosomal depolarizing agents producing measurable changes in membrane potential at concentrations as low as 100 nM, with maximal responses observed between 10 and 100 μ M. Moreover, pyrethroid and neurotoxin-induced changes in membrane potential were tetrodotoxin-sensitive in both rat and trout brain synaptosomes, indicating that these agents depolarize the plasma membrane by interacting with the voltage-sensitive sodium channel. The potency of TTX (IC_{50} , 20 ± 1 nM) as a blocker of both deltamethrin and veratridine-induced membrane depolarization was identical in synaptosomes isolated from both species, suggesting that the TTX binding site is similar in both species. Similar IC_{50} values for TTX inhibition of veratridine-induced influx of radiolabeled sodium through voltage-sensitive sodium channels have been reported in neuroblastoma cells (Catterall, 1977) and in rat (Tamkun and Catterall, 1981), mouse (Ghiasudden and Soderlund, 1985), and trout (Stuart *et al.*, 1987) synaptosome preparations.

The neurotoxins, veratridine, aconitine, and *Leiurus* venom, which have been widely used to characterize the pharmacological properties of voltage-sensitive sodium channels in mammalian neuronal preparations, were used in our studies to compare sodium channel properties in rat and trout brain synaptosomes. Veratridine and aconitine bind to the sodium channel, inducing persistent channel activation, and the α -toxin in *Leiurus* venom delays sodium channel inactivation. The rank order of potency for these neurotoxins was the same in rat and trout synaptosome preparations [*Leiurus* venom (rat EC_{50} , 0.15 μ M; trout EC_{50} , 0.30 μ M) > veratridine (rat EC_{50} , 2.3 μ M; trout EC_{50} , 5 μ M) > aconitine (rat EC_{50} , 5 μ M; trout EC_{50} , 10 μ M)]. In addition, conversion of the EC_{50} values to ratio form (1:16:33) shows that this relationship is identical in both species, suggesting that the relative affinity of these neurotoxins for their respective binding sites on the voltage-sensitive sodium channel does not differ in rat and trout. Although the relative affinity for these neurotoxins was similar in both species, the potency of veratridine, aconitine, and *Leiurus* venom in trout brain synaptosomes was two-fold lower than that in rat brain synaptosomes. In fact, trout brain synaptosomes were one-half as responsive as rat brain synaptosomes to the depolarizing actions of these neurotoxins with respect to both potency and intrinsic activity. These differences in neurotoxin potency and efficacy were not due to differences in the number of sodium channels, since binding studies indicate that both species have approximately equal densities of sodium channels (Tamkun and Catterall, 1981; Rubin and Soderlund, personal communication). Moreover, a different pharmacological profile from that observed in rat was also apparent in terms of intrinsic neurotoxin activity in trout brain synaptosomes. In rat

brain synaptosomes, veratridine and aconitine exhibited nearly equivalent efficacy and both produced greater maximal depolarizations than *Leiurus* venom. In contrast, in trout brain synaptosomes veratridine was a significantly more effective depolarizing agent than either aconitine or *Leiurus* venom. These findings suggest that the specificity of the neurotoxin binding domains of the trout brain sodium channel for the site 2 toxins, aconitine and veratridine, and the site 3 toxin, *Leiurus* venom, differ from their mammalian counterparts. Radiolabeled sodium flux studies in synaptosome preparations also indicate that trout brain sodium channels (Stuart *et al.*, 1987) differ from those of rat (Tamkun and Catterall, 1981) and mouse (Ghiasuddin and Soderlund, 1984; Bloomquist and Soderlund, 1988) in their responses to aconitine and veratridine, providing additional pharmacological evidence for differences between neurotoxin binding domains of mammalian and piscine brain sodium channels.

Rat and trout brain synaptosome preparations also differed in their responses to the pyrethroid insecticides. The difference in pyrethroid sensitivity was most apparent in studies with the type I pyrethroids, NRDC 157 and permethrin, which produced maximal depolarization in trout brain synaptosomes of 11 and 15 mV compared to 4 and 5 mV in rat brain. In trout brain synaptosomes, permethrin produced a depolarizing response three times greater than the effect produced at the same concentration in rat brain synaptosomes. The three-fold difference in efficacy of permethrin (*IR-cis*) in our studies is analogous to the difference in brain concentrations of this isomer of permethrin associated with the onset of toxic symptoms in trout and mice (Glickman and Lech, 1982). It is interesting that the EC_{50} values for pyrethroid-induced synaptosomal depolarization were similar in both species for permethrin and NRDC 157, in contrast to the pronounced differences in their E_{max} values and the species difference in permethrin toxicity. These findings suggest that pyrethroid-induced neurotoxicity may be a function of efficacy rather than affinity. A similar conclusion was reached by Brown *et al.* (1988) in studies measuring pyrethroid enhancement of [3 H]batrachotoxin A 20- α -benzoate binding in rat brain synaptoneuroosomes.

The species-specific differences in pyrethroid-induced membrane depolarization in rat and trout brain synaptosomes were not due to differences in assay temperatures. Pyrethroid-induced membrane depolarization was similar at both assay temperatures in trout brain synaptosomes and was significantly attenuated at 25°C relative to 37°C in rat brain synaptosomes. However, the relationship between the intrinsic activities of type I and type II pyrethroids as membrane-depolarizing agents was not altered in either species by changing the assay temperature. In addition, the potency of these agents was also unaffected by assay temperature. In some biological systems, pyrethroid action has

been shown to be enhanced at lower temperatures (Vijverberg and De Weille, 1985; Vijverberg and van den Bercken, 1990). The opposite effect was observed in rat brain synaptosomes in the present studies. Other investigators have reported both negative and positive temperature effects on pyrethroid actions depending upon the temperature range studied and the physiological response measured (Adams and Miller, 1979, 1980; Vijverberg and van den Bercken, 1990). It is therefore likely that the processes involved in the excitation of neuronal membranes by pyrethroid insecticides are differentially modified by changes in temperature.

Although our findings with type I pyrethroids correlate with *in vivo* studies of pyrethroid toxicity in fish relative to mammals, the type II pyrethroids were more potent and equally efficacious depolarizing agents in rat brain synaptosomes compared with fish brain synaptosomes. These findings indicate that the differences in toxicity of type II pyrethroids in fish and rodents are not simply due to differences in synaptic membrane depolarization secondary to sodium channel activation. We have initiated studies to determine if there are species-specific differences in synaptic events secondary to sodium channel activation and synaptic membrane depolarization which may contribute to the observed hypersensitivity of fish to type II pyrethroid insecticides.

In rat brain synaptosomes there was a clear distinction between the depolarizing properties of type I and type II pyrethroids with respect to both potency and efficacy. In this species, type II pyrethroids were significantly more effective and potent depolarizing agents than their descyano analogs. This distinction was much less apparent in trout brain synaptosomes in which similar potencies and maximal depolarizing responses were observed with all of the pyrethroids studied. Similarity in the actions of type I and type II pyrethroids has also been described *in vivo* in trout and other species of fish (Bradbury and Coats, 1989). In contrast to rodents, in which there are clearly defined differences in the poisoning syndromes produced by type I and type II pyrethroids (Verschoyle and Aldridge, 1980; Ray, 1982), the symptoms associated with type I and type II pyrethroid toxicity in trout are indistinguishable from each other (Glickman and Lech, 1981, 1982; Holcombe *et al.*, 1982; Edwards *et al.*, 1986; Bradbury and Coats, 1989). Both classes of pyrethroids produce hyperactivity, erratic swimming, whole body convulsions, and myoclonic head swaying. In addition, similar LC₅₀ concentrations for type I and type II pyrethroids following aqueous exposure have been reported. LC₅₀ values for permethrin ranged from 0.4 to 7.0 µg/liter, and those for cypermethrin ranged from 0.5 to 1.0 µg/liter (Stephenson, 1982; Holcombe *et al.*, 1982; Kumaraguru and Beamish, 1981). Differences in the toxicities of type I and type II pyrethroids are more pronounced in rodents. In rodent toxicity studies type II pyrethroids have been reported to be at least 10 times more potent than

their noncyano analogs (Gray, 1985). Verschoyle and Aldridge (1980) have reported LD₅₀ values of 2 mg/kg for cypermethrin and 135 mg/kg for permethrin in rats following intravenous administration, and Ghiasuddin and Soderlund (1985) have reported LD₅₀ values of 0.019 mg/kg for deltamethrin and 0.19 mg/kg for NRDC 157 following intracerebral ventricular administration in mice.

Differences in susceptibility to the toxic actions of pyrethroids have been related to structural variations in the pyrethroid binding domain of the voltage-sensitive sodium channel. The neurotoxic actions of pyrethroid insecticides in mammals are highly dependent upon the absolute conformation of the pyrethroid molecule and exhibit well-defined stereochemical requirements (Elliott *et al.*, 1974; Lawrence and Casida, 1982, 1983; Gray, 1985). In contrast, fish show less specific stereoisomeric requirements (Glickman and Lech, 1982; Edwards *et al.*, 1986; Bradbury and Coats, 1989), indicative of species differences in binding site structure. In our studies, the *IR-cis-αR* isomer of deltamethrin, which had no effect in rat brain synaptosomes, depolarized trout synaptosomes. These findings provide a neurochemical correlate for the differences in pyrethroid stereochemistry requirements in fish and mammals (Glickman and Lech, 1982; Edwards *et al.*, 1986; Bradbury and Coats, 1989) and further support the concept of structural variations in the pyrethroid binding domain. Importantly, this enantiomer of deltamethrin, which has been shown to be nontoxic in mammalian studies, produced toxic symptoms when administered to trout, further substantiating the relevance of *in vitro* measurements of pyrethroid-induced membrane depolarization to whole-animal toxicity.

Neurochemical and electrophysiological evidence from other laboratories also support the concept that the hypersensitivity of fish to pyrethroid insecticides is determined, in part, by the intrinsic properties of fish brain sodium channels. Radiolabeled sodium flux studies have shown striking interspecies differences in the effects of deltamethrin on aconitine-dependent sodium channel activation in mammalian and piscine brain preparations (Rubin and Soderlund, 1992). However, sodium flux assays do not possess adequate sensitivity to assess pyrethroid potencies in fish brain preparations and are additionally complicated by the requirement for sodium channel-activating neurotoxins to assess their activity. Eshleman and Murray (1991) have provided indirect evidence that pyrethroid insecticides stimulate sodium conductance to a greater extent in trout than in mammalian brain by showing a profound increase in ³⁶Cl⁻ influx in trout brain synaptoneuroosomes secondary to pyrethroid-induced sodium channel activation. Similar effects were not observed in mammalian synaptoneurosome preparations. Preliminary studies reported by Frank and Campbell (1992) show that permethrin more effectively prolongs sodium channel closing in trout than in mouse sensory nerve preparations and that this effect is

temperature-sensitive and only apparent at temperatures which are physiological for each species.

In summary, we have presented pharmacological evidence for differences between mammalian and piscine sodium channel modulation by sodium channel-specific neurotoxins and pyrethroid insecticides. Differences in the functional properties of trout and mammalian brain sodium channels may be due to structural differences in pyrethroid and other neurotoxin binding domains or may reflect differences in their membrane lipid environment. Although pharmacological characterization of sodium channel function in trout and mammalian brain provides valuable and important information, this approach may be confounded by differences in membrane lipids and assay parameters. To gain further insight into the mechanisms of enhanced neuronal sensitivity it will be necessary to separate the effects of the membrane environment and differences in assay conditions from those involving structural changes in one or more binding domains of the sodium channel. Studies have been initiated in our laboratory to clone and sequence the sodium channel α subunit from trout brain, thus enabling exploration of these differences at the molecular level.

ACKNOWLEDGMENTS

This study was supported, in part, by National Institutes of Health Grants ES05006 and ES04184 from the National Institute of Environmental Health Sciences. P. A. Bandettini and J. M. Propp were recipients of Research Experience for Undergraduate Awards from the National Science Foundation (NSF-OCE-8713207). The excellent technical assistance of Ms. P. A. Holman is appreciated. The authors thank Dr. C. J. Hillard and Dr. S. G. Frackman for helpful discussions and critical comments on the manuscript and acknowledge the secretarial assistance of Ms. C. Knapp.

REFERENCES

- Adams, M. E., and Miller, T. A. (1979). Site of action of pyrethroids: Repetitive backfiring in flight motor units of housefly. *Pestic. Biochem. Physiol.* **11**, 218-231.
- Adams, M. E., and Miller, T. A. (1980). Neural and behavioral correlates of pyrethroid and DDT-type poisoning in the housefly, *Musca domestica* L. *Pestic. Biochem. Physiol.* **13**, 137-147.
- Aiuchi, T., Matsunaga, M., Nakaya, K., and Nakamura, Y. (1989). Calculation of membrane potential in synaptosomes with use of a lipophilic cation (tetraphenylphosphonium). *Chem. Pharm. Bull.* **37**, 3333-3337.
- Bloomquist, J. R., and Soderlund, D. M. (1988). Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. *Mol. Pharmacol.* **23**, 543-550.
- Bradbury, S. P., and Coats, J. R. (1989). Comparative toxicology of the pyrethroid insecticides. *Rev. Environ. Contam. Toxicol.* **108**, 133-177.
- Bradford, M. M. (1976). 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.
- Brown, G. B., Gaupp, J. E., and Olson, R. W. (1988). Pyrethroid insecticides: Stereospecific allosteric interaction with the batrachotoxin-A benzothiazine binding site of mammalian voltage-sensitive sodium channels. *Mol. Pharmacol.* **34**, 54-59.
- Casida, J. E., Gammon, D. W., Glickman, A. H., and Lawrence, L. J. (1983). Mechanism of selective action of pyrethroid insecticides. *Annu. Rev. Pharmacol. Toxicol.* **23**, 413-418.
- Catterall, W. A. (1977). Activation of the action potential Na^+ ionophore by neurotoxins, an allosteric model. *J. Biol. Chem.* **252**, 8669-8676.
- Catterall, W. A. (1988). Structure and function of voltage-sensitive ion channels. *Science* **242**, 50-61.
- Edwards, R., Millburn, P., and Hutson, D. H. (1986). Comparative toxicity of *cis*-cypermethrin in rainbow trout, frog, mouse and quail. *Toxicol. Appl. Pharmacol.* **84**, 512-522.
- Eells, J. T., Bandettini, P. A., Holman, P. A., and Propp, J. M. (1992). Pyrethroid insecticide-induced alterations in mammalian synaptic membrane potential. *J. Pharmacol. Exp. Ther.* **262**, 1173-1181.
- Elliott, M., Farnham, A. W., Janes, N. F., Needham, P. H., and Pulman, D. A. (1974). Insecticidally active conformations of pyrethroids. In *Mechanism of Pesticide Action* (G. K. Kohn, Ed.), pp. 80-91. American Chemistry Society, Washington, DC.
- Eshleman, A. J., and Murray, T. F. (1991). Pyrethroid insecticides indirectly inhibit GABA-dependent $^{36}\text{Cl}^-$ influx in synaptoneuroosomes from the trout brain. *Neuropharmacology* **30**, 1333-1341.
- Frank, T. M., and Campbell, D. T. (1992). Ionic channel basis for the differential toxicity of permethrin to fish and mammals. *Biophys. J.* **61**, A111.
- Ghiasuddin, S. M., and Soderlund, D. M. (1984). Mouse brain synaptosomal sodium channels: Activation by aconitine, batrachotoxin and veratridine, and inhibition by tetrodotoxin. *Comp. Biochem. Physiol. C* **77**, 267-271.
- Ghiasuddin, S. M., and Soderlund, D. M. (1985). Pyrethroid insecticides: Potent, stereospecific enhancers of mouse brain sodium channel activation. *Pestic. Biochem. Physiol.* **24**, 200-206.
- Glickman, A. H., and Lech, J. J. (1981). Hydrolysis of permethrin, a pyrethroid insecticide, by rainbow trout and mouse tissues *in vitro*: A comparative study. *Toxicol. Appl. Pharmacol.* **60**, 186-192.
- Glickman, A. H., and Lech, J. J. (1982). Differential toxicity of *trans*-permethrin in rainbow trout and mice. II. Role of target organ sensitivity. *Toxicol. Appl. Pharmacol.* **66**, 162-171.
- Glickman, A. H., Weitman, S. D., and Lech, J. J. (1982). Differential toxicity of *trans*-permethrin to rainbow trout and mice. I. Role of biotransformation. *Toxicol. Appl. Pharmacol.* **66**, 153-161.
- Gray, A. J. (1985). Pyrethroid structure-toxicity relationships in mammals. *Neurotoxicology* **6**, 127-138.
- Holcombe, G. W., Phipps, G. L., and Tanner, D. K. (1982). The acute toxicity of kelthane, dursban, disulfoton, pydrin and permethrin to flathead minnows, *Pimephales promelas* and rainbow trout *Salmo gairdneri*. *Environ. Pollut.* **29A**, 167-178.
- Jacques, Y., Romey, G., Cavey, M. T., Kartalovski, B., and Lazdunski, M. (1980). Interaction of pyrethroids with the Na^+ channel in mammalian neuronal cells in culture. *Biochem. Biophys. Acta* **600**, 882-897.
- Kumaraguru, A. K., and Beamish, F. W. H. (1981). Lethal toxicity of permethrin (NRDC 143) to rainbow trout, *Salmo gairdneri*. *J. Fish. Biol.* **20**, 87-91.
- Lawrence, L. J., and Casida, J. E. (1982). Pyrethroid toxicology: Mouse intracerebral structure-toxicity relationships. *Pestic. Biochem. Physiol.* **18**, 9-14.
- Lawrence, L. J., and Casida, J. E. (1983). Stereospecific action of pyrethroid insecticides on the λ -amino butyric acid receptor ionophore complex. *Science* **221**, 1399-1401.
- Lichtshstein, D., Kaback, H. R., and Blume, A. J. (1979). Use of a lipophilic

- cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. *Proc. Natl. Acad. Sci.* **76**, 650-654.
- Lombet, A., Mourre, C., and Lazdunski, M. (1988). Interaction of insecticides of the pyrethroid family with specific binding sites on the voltage-dependent sodium channel from mammalian brain. *Brain Res.* **459**, 44-53.
- Lund, A. E., and Narahashi, T. (1983). 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.
- Narahashi, T. (1985). Nerve membrane ionic channels as the primary target of pyrethroids. *Neurotoxicology* **6**, 3-22.
- Narahashi, T., Moore, J. W., and Scott, W. R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* **47**, 965-974.
- Pauwels, P. J., and Laduron, P. M. (1986). TPP⁺ accumulation in rat brain synaptosomes as a probe for Na⁺ channels. *Eur. J. Pharmacol.* **132**, 289-293.
- Ramos, S., Grollman, E. F., Lazo, P. S., Dyer, S. A., Habig, W. H., Harder-gee, M. C., Kaback, H. R., and Kohn, L. D. (1979). Effect of tetanus toxin on the accumulation of the permeant lipophilic cation tetraphenylphosphonium by guinea pig brain synaptosomes. *Proc. Natl. Acad. Sci.* **76**, 4783-4787.
- Ray, D. E. (1982). The contrasting actions of two pyrethroids (deltamethrin and cismethrin) in the rat. *Neurobehav. Toxicol. Teratol.* **4**, 801-804.
- Roche, M., Frelin, C., Bruneau, P., and Meinard, C. (1985). Interaction of tralomethrin, tralocyrin, and related pyrethroids in Na channels of insect and mammalian neuronal cells. *Pestic. Biochem. Physiol.* **24**, 306-316.
- Rubin, J. G., and Soderlund, D. M. (1992). Interaction of naturally occurring neurotoxins and the pyrethroid insecticide deltamethrin with rainbow trout (*Oncorhynchus mykiss*) brain sodium channels. *Environ. Toxicol. Chem.* **11**, 677-685.
- Scott, I. D., and Nicholls, D. G. (1980). Energy transduction in intact synaptosomes. *Biochem. J.* **186**, 21-33.
- Stephenson, R. R. (1982). Aquatic toxicology of cypermethrin. I. Acute toxicity to some freshwater fish and invertebrates in laboratory tests. *Aquat. Toxicol.* **2**, 175-185.
- Stuart, A. M., Bloomquist, J. R., and Soderlund, D. M. (1987). Pharmacological characterization of the voltage-dependent sodium channels of rainbow trout brain synaptosomes. *Brain Res.* **437**, 77-82.
- Tamkun, M. M., and Catterall, W. A. (1981). Ion flux studies of voltage-sensitive sodium channels in synaptic nerve ending particles. *Mol. Pharmacol.* **19**, 78-86.
- Verschöyle, R. D., and Aldridge, W. N. (1980). Structure-activity relationships of some pyrethroids in rats. *Arch. Toxicol.* **45**, 325-332.
- Vijverberg, H. P. M., and De Weille, J. R. (1985). The interaction of pyrethroids with voltage-dependent Na⁺ channels. *Neurotoxicology* **6**, 23-34.
- Vijverberg, H. P. M., De Weille, J. R., Rught, G. S. F., and van den Bercken, J. (1986). The effect of pyrethroid structure on the interaction with the sodium channel in the nerve membrane. In *Neuropharmacology and Pesticide Action* (M. G. Ford, G. G. Lunt, R. C. Reay, and P. N. R. Usherwood, Ed.), pp. 267-285. Ellis Horwood, England.
- Vijverberg, H. P. M., and van den Bercken, J. (1990). Neurotoxicological effects and the model of action of pyrethroid insecticides. *Crit. Rev. Toxicol.* **21**, 105-126.
- Winer, B. (1972). *Statistical Principles in Experimental Design*. McGraw-Hill, New York.