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ARTICLE

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Pyrethroid Mode(s) of Action in the Context of Food Quality Protection Act (FQPA) Regulation†

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ABSTRACT: A Scientific Advisory Panel (SAP) in June 2009 concluded that a common mode of action existed for pyrethroids, with two subgroups. The purpose of this SAP was to advise the U.S. Environmental Protection Agency on the validity of regulation of pyrethroids as a single class under the Food Quality Protection Act of 1996. Two types of pyrethroid action were first described for clinical signs in the rat and clinical signs/nerve effects in the cockroach. In insects, Type I clinical signs correlate with repetitive firing in nerve axons, especially fine sensory axons. The Na^+ inward current is via a TTX-sensitive voltage-gated sodium channel (VGSC). Type II (α-CN) effects on VGSCs do not include repetitive firing following stimulation in these axons. Instead, Type II effects on VGSCs include prolonged Na^+ tail currents along with depolarization of nerve membrane. Other Type II effects have been measured on VG Ca^{2+} and K^+ channels and VG and GABA-activated Cl^- channels. In conclusion, in vivo pyrethroid effects in mammals should be linked with specific channel effects, allowing the use of specific clinical signs or ion channel effects for pyrethroid risk assessment.

KEYWORDS: pyrethroid insecticides, mechanism, sodium, calcium chloride, voltage-gated channels, GABA receptors, FQPA, cumulative exposure

BACKGROUND

After passage of the Food Quality Protection Act (FQPA) in 1996, the U.S. Environmental Protection Agency (EPA) developed guidelines for evaluating exposure to pesticides as classes if they shared a common mechanism of action. Organophosphate and carbamate insecticides were each considered separately as common classes because they both inhibit acetylcholine esterase, but via different mechanisms. As such, each class was subjected to "cumulative" exposure assessment, meaning that exposure to individual organophosphates or carbamates was combined and evaluated relative to an index chemical, methamidophos or oxamyl, respectively. Furthermore, aggregate exposure assessment for each chemical in each class was intended to capture multiple routes of exposure and combine them, that is, dietary, drinking water, and home/garden uses of each chemical. Occupational exposure was not included in FQPA because it had already been addressed in other legislation. After assessing organophosphates and carbamates, and canceling home and garden uses to reduce aggregate exposure, EPA then considered pyrethroid insecticides from the perspective of a common mechanism of action under FQPA. Any EPA involvement in the enactment of a U.S. law requires EPA to present its plan before an independent Scientific Advisory Panel (SAP). Their recommendations need to be taken into account by EPA. Such an SAP was convened in June 2009 to answer a series of questions designed to establish whether or not pyrethroids, based on a set of data supplied by EPA, could be considered sufficiently homogeneous as a class to regulate them "cumulatively" under FQPA. The four questions posed by EPA and the SAP answers will be described. Then, the background to the SAP will be addressed in this paper, much of it stemming from research conducted in J. E. Casida's laboratory at the University of California, Berkeley, in the 1980s.

† Part of the Symposium on Pesticide Toxicology in Honor of Professor John Casida.

Question 1a: Do pyrethroids share a common pathway to neurotoxicity?

SAP Panel response: Yes, it was agreed that all pyrethroids act on the voltage-gated sodium channel (VGSC), the common molecular target, increasing the open time. There are two types of action, quantitatively and qualitatively, at high doses.[1,2] The interactions on VGSC vary among different structures. Type I and II pyrethroids cause different physiological effects on neurons (excitation vs block) and two syndromes (T, CS). It was considered that extrapolation of channel effects to those on whole animals was difficult. Two or more (of the 10 panel members) thought pyrethroids should not be considered to have a common mechanism.

Question 1b: EPA is aware that pyrethroids can bind to Ca^{2+} and Cl^- channels and GABA-gated Cl^- channels. However, data are not sufficiently robust for a Common Mechanism Group (CMG) under FQPA. Please comment.

SAP Panel response: The voltage-gated calcium channel (VGCC) is affected by Type II, but not Type I, pyrethroids (at <pM). Cl^- channels are less likely as primary targets of Type II pyrethroids. $GABA_A$—Cl^- channels indicate a very weak Type II effect. The effects of Type II pyrethroids on VGCC and Cl^- channels do not discount placing them into the same CMG (based on effects on VGSC).

Question 2: Is heterogeneity of VGSC a problem?

SAP Panel response: Although it is recognized that there are multiple forms of VGSC proteins in mammals, this does not present a problem in CMG analysis.

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Question 3: The subgrouping of Type I and II pyrethroids is based on the absence or presence of a cyano group in the alcohol part of the structure, effects on VGSC, and toxicity syndromes. Please comment.

SAP Panel response: The in vivo evidence is unclear because reduced motor activity is nonspecific; measurement of pyrethroid effects on the acoustic startle response (ASR) may help. The classification of other pyrethroids that have not been categorized needs more than just structure. Mixed-type could be in either subgroup, both, or a third subgroup.

Question 4: Dose-additivity evaluation, for example, refs 3 and 4. Please comment.

SAP Panel response: Motor activity is reduced by both Type I and II pyrethroids and does not allow separation. Functional observational battery (FOB) effects need more study. ASR separates Types I and II. EPA microelectrode array (MEA) studies consider only VGSC effects,[5] but may help in the classification process.

In conclusion, it was agreed that pyrethroids could probably be placed in a CMG, with two subgroups, Types I and II, as suggested by EPA. The ASR was considered a better end point for evaluating toxicity than reduced motor activity. Mixture studies should use smaller groups of Type I or II pyrethroids, using type-specific end points, such as ASR. Several end points should be evaluated for each compound. Pyrethroid mixture studies should consider potential coexposure (www.epa.gov/scipoly/sap/meetings/2009/061609meeting.html).

INTRODUCTION

The early studies to establish pyrethroid mechanism of action were conducted using allethrin, the first commercial pyrethroid or synthetic analogue of the natural pyrethrins, by Narahashi and colleagues from 1962 to 1972 (see, e.g., refs 6−10). Most of these studies used giant axons from the cockroach, squid, or crayfish, in vitro, and the VGSC was clearly identified as a target site. Prolongation of VGSC inactivation, without an effect on axon membrane resting potential, was associated with nerve conduction blockage at low temperatures and repetitive firing following stimulation at high temperatures. Because allethrin is more toxic to insects at low temperatures, it was considered that nerve blockage was especially important in causing pyrethroid toxicity to insects. However, it should be noted that these experiments were conducted on giant axons, in vitro, at relatively high concentrations, with little or no data from in vivo nerve studies.

During the 1970s, a technique was developed to examine the mechanism of action of insecticides on the nervous system of the intact, free-walking cockroach.[11] As outlined in Figure 1, six electrodes comprising insulated Na/NaCl or W/Cu wires, were implanted into the abdomen or a cercus of the insect and sealed into position using beeswax/resin. The implanted cockroach was allowed to move freely in a small, temperature-controlled chamber, and evoked potentials were recorded at three sites: a response in a cercal sensory nerve and at two sites in the abdominal nerve cord (or central nervous system, CNS), as shown (Figure 1). Electrical stimulation of a cercal nerve (at S1) resulted in a compound spike at R1 and R2 in the CNS (Figures 2 and 3). These recordings involve transmission across cholinergic synapses in the sixth abdominal ganglion (A6). Further compound spikes without synaptic involvement were recorded by applying a stimulus at R1 and recorded at R2 (S2 in Figures 2 and 3). In addition, primarily mechanosensory responses were recorded in a cercal nerve, as well as in the CNS, following air movements caused by an air puff or sound pulse (Figure 4). Such responses were stable for several days in undosed cockroaches. It should be

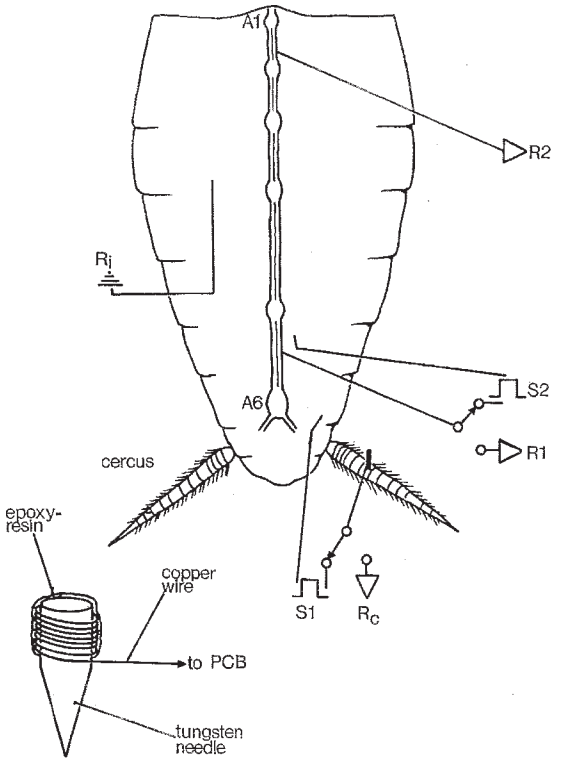

Figure 1. Placement of the six electrodes in the free-walking, electrode-implanted cockroach. Adapted from ref 11.

stressed that the insect VGSC in both cercal sensory nerves and CNS is very sensitive to tetrodotoxin (TTX) blockage.[12,13] TTX is also a potent blocker of some, but not all, isoforms of the mammalian VGSC. The $Na_v1.8$ isoform, from mammalian peripheral nerve, has been used extensively in pyrethroid mode of action studies, but is insensitive to TTX.[14]

TARGET SITES

Voltage-Gated Sodium Channel Effects. Following the administration of a topical LD_{95} dose of allethrin at 15 °C, there was little sign of repetitive firing in the CNS following electrical stimulation at either site (S1 or S2), as shown in Figure 2.[11,13,15] Following uncoordinated behavior, starting within minutes of dosing, leading to prostration at ca. 3 h, a gradual blockage of the CNS was observed. Occasionally, discharges in the CNS followed cercal nerve stimulation, and these were subsequently shown to likely be secondary effects of dosing related to physical stress, rather than having been a direct effect of allethrin. At 32 °C, dosing with a LD_{95} (ca. 10× greater than at 15 °C) resulted in repetitive firing in the CNS within 1−2 h of dosing (Figure 3). Blockage occurred many hours later at 32 °C. Although these results are consistent with those anticipated from the work of Narahashi, it should be noted that effects in peripheral nerves, such as cercal sensory nerves, at 15 °C, were not consistent with this earlier work. As shown in Figure 4, increased bursts of spikes were recorded within 20 min of dosing (Figure 4A), and repetitive firing followed mechanical stimulation at 40 min (Figure 4B) in a cercal sensory nerve.

Following these studies in the cockroach, a project was conducted to gain an understanding of insecticide resistance in the Egyptian cotton leaf worm (*Spodoptera littoralis*).[16,17] A strain of these insects had developed resistance to permethrin,

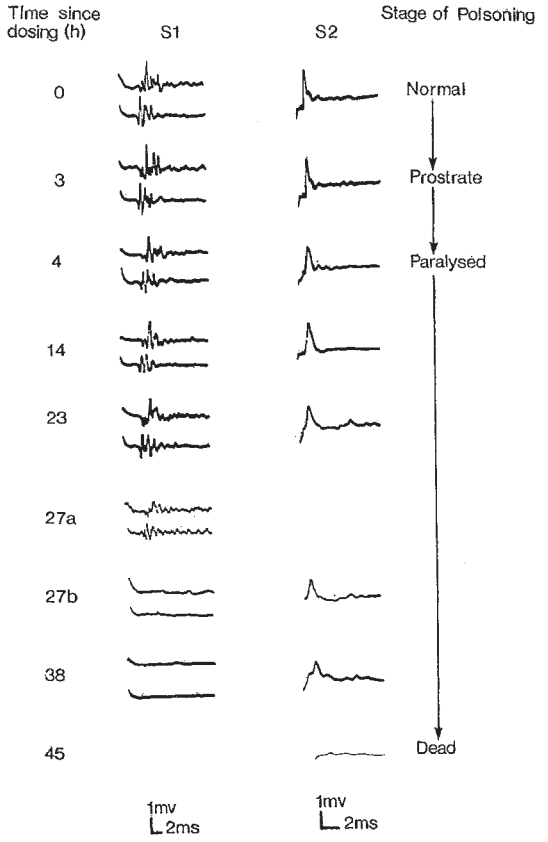

Figure 2. Nerve recordings in free-walking, electrode-implanted cockroach after dosing with allethrin (LD_{95} dose at 15 °C): cercal sensory nerve stimulation (S1) and nerve cord stimulation (S2) recorded in nerve cord (CNS). Adapted from refs 13 and 15.

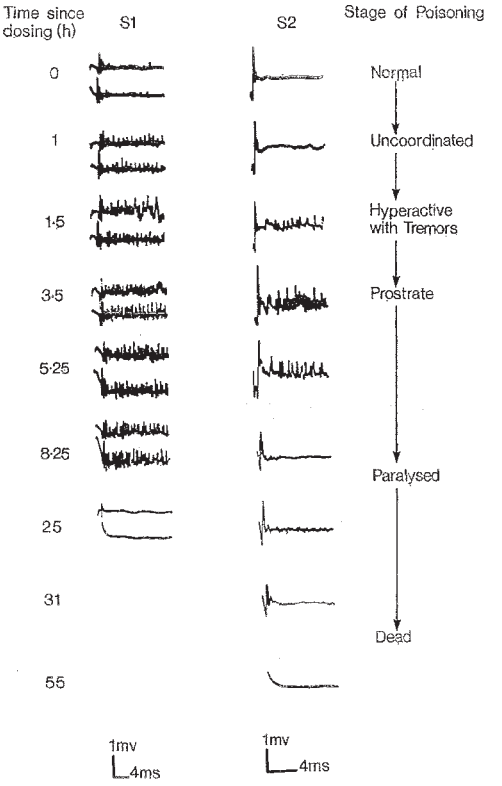

Figure 3. Nerve recordings in free-walking, electrode-implanted cockroach after dosing with allethrin (LD_{95} dose at 32 °C): cercal sensory nerve stimulation (S1) and nerve cord stimulation (S2) recorded in nerve cord (CNS). Adapted from refs 13 and 15.

although not to cypermethrin, which was shown to be not related to metabolic differences.[17] It was found that resistance was manifested only at high but not at low temperatures. Thus, on the basis of previous experiments with allethrin in the cockroach, it was considered that the CNS may be the most likely location to obtain an explanation for this resistance. An in vitro assay was therefore developed in which the isolated abdominal nerve cord was incubated in a chamber between pairs of suction electrodes. An evoked compound spike was recorded after electrical stimulation through the other pair of electrodes. At temperatures above 19 °C, permethrin at $\geq 10^{-7}$ M caused repetitive firing following electrical stimulation within a few minutes of dosing (Figure 5) followed by blockage. Abdominal nerve cords from resistant insects took significantly longer to develop repetitive firing, indicating that the CNS was less sensitive to permethrin in resistant than in susceptible larvae. For cypermethrin, however, there was a possible increase in background CNS activity at 15 and 20 min, but repetitive firing following stimulation was never observed (Figure 6). Increasing the cypermethrin concentration or changing the temperature of the nerve chamber failed to produce evidence of repetitive firing after stimulation for cypermethrin. Thus, the lack of resistance to cypermethrin of the permethrin-resistant strain was due to a qualitative rather than a quantitative difference between the pyrethroids; that is, cypermethrin had a different mechanism of action from permethrin, and so the latter's target site resistance mechanism was not relevant for cypermethrin.

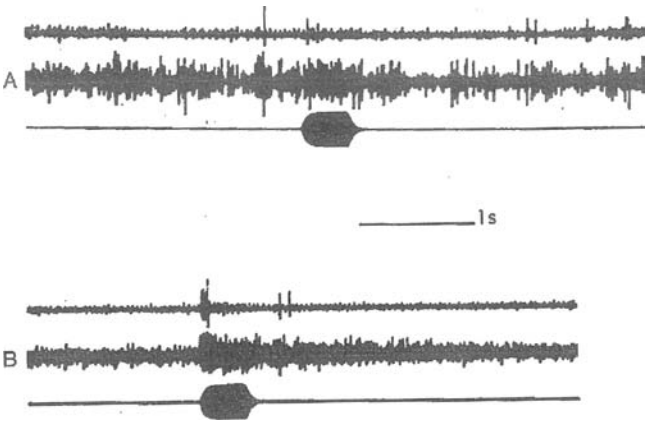

Figure 4. LD_{95} dose of allethrin effects at 15 °C: responses in cercal nerve (middle) and nerve cord (top) to a 200 Hz sound (bottom) at 20 min (A) and 40 min (B) from a restless cockroach. Adapted from refs 13 and 15.

An insect assay system was then developed, in Dr. Casida's laboratory, to quantify the excitatory effects of a range of pyrethroids. Because the cockroach cercal sensory nerves were susceptible to repetitive firing following stimulation over a broad range of temperatures when exposed to allethrin,[11,13,15] an in vitro assay was developed (Figure 7) in which evoked responses were recorded using a suction electrode following electrical stimulation of this nerve using a pair of insulated Ag/AgCl wire electrodes.[2] Repetitive firing after stimulation was recorded for all insecticidal non-cyano pyrethroids (Table 1). In addition, cockroaches were dosed in vivo ($2 \times LD_{50}$) and nerve recordings

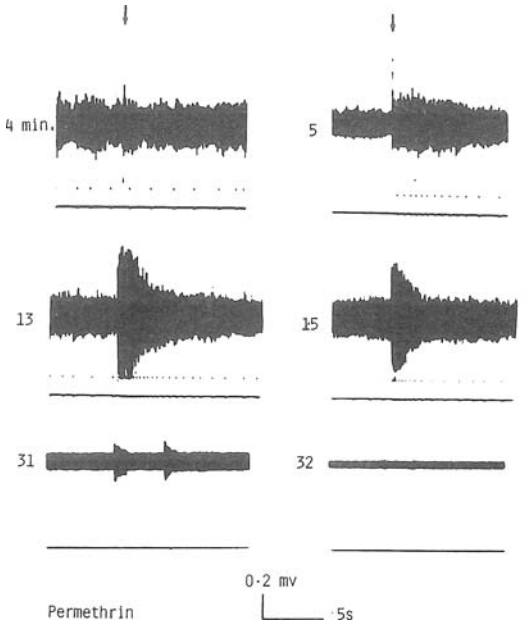

Figure 5. Permethrin (10^{-7} M) effects on CNS of *Spodoptera littoralis*, in vitro. Arrow indicates single electrical stimulation. The bottom trace is from a spike-counting device at a preset threshold. Adapted from ref 16.

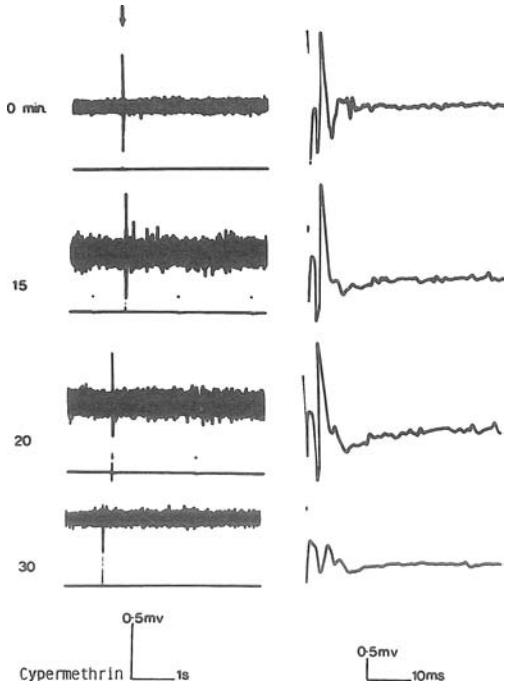

Figure 6. Cypermethrin (10^{-7} M) effects on CNS of *Spodoptera littoralis*, in vitro. Arrow indicates single electrical stimulation. Adapted from ref 16.

made in a cercus as well as in the CNS, using implanted electrodes. The results are summarized in Table 1. Non-cyano pyrethroids caused repetitive firing in cercal sensory nerves in vitro and in vivo, associated with a similar set of clinical signs for these pyrethroids. The main findings are summarized as follows:

1. Nontoxic stereoisomers had essentially no activity on this nerve, in vitro or in vivo, contradicting earlier neurophysiological studies.[18]

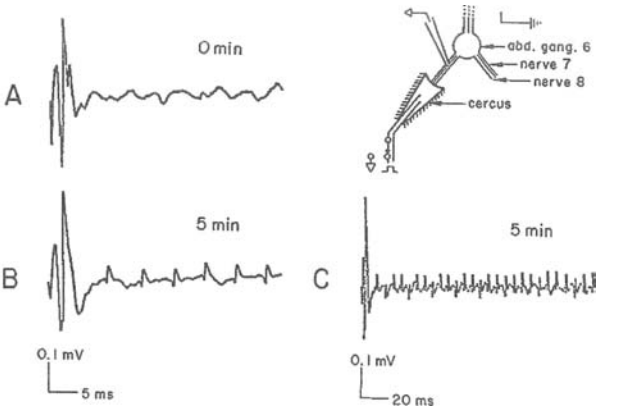

Figure 7. Type I effect: electrical stimulation of cercal sensory nerve caused repetitive firing at 5 min, shown with compound **19** of Table 1 at 10^{-7} M. Adapted from ref 2.

2. Geometric isomers of active pyrethroids (1*R*,*cis*, 1*R*,*trans*) had similar in vitro potencies, demonstrated for tetramethrin, resmethrin, phenothrin, and permethrin.

3. DDT had effects similar to these non-cyano pyrethroids, although with some differences in other nerves in the cockroach, as anticipated from a study of DDT in the free-walking cockroach.[19,20]

4. The most potent pyrethroid in vitro was tetramethrin, a pyrethroid associated with knock-down activity in insects.

5. The repetitive firing in the cercal sensory nerves, recorded in vitro and in vivo, along with characteristic clinical signs in the cockroach, was designated the Type I syndrome.

Pyrethroids containing an α-cyanophenoxybenzyl moiety, with the exception of fenpropathrin, did not cause repetitive firing in the cercal sensory nerves, in vitro or in vivo (Table 2). Other characteristics of these pyrethroids were as follows:

1. Clinical signs were different from those observed with non-cyano pyrethroids.

2. Only blockage in the cercal sensory nerves with no repetitive firing was recorded.

3. Long trains of efferent (motor) potentials were observed, in vivo, associated with cercal motor nerves/muscles at the base of each cercus.

4. The clinical signs in the cockroach were distinct from those caused by non-cyano pyrethroids and were referred to as the Type II syndrome.

5. Type I/II pyrethroid effects were considered analogous to the T (tremors) and CS (choreoathetosis/salivation) syndromes in the rat.[1]

6. However, whereas *cis*- and *trans*-cyphenothrin (**3** and **4** in Table 2) and *cis*- and *trans*-difluorocyphenothrin (**5** and **6** in Table 2) were all considered Type II, in ref 2, the *cis* isomers were designated CS type and the *trans* isomers T type.[1]

Structural derivatives of the isobutenyl group of phenothrin were made and assessed.[21] Phenothrin itself did not result in repetitive firing in a cercal sensory nerve at 10^{-9} M, but it caused inconsistent repetitive firing at 10^{-8} M and consistent repetitive firing at 10^{-7} M. Examples of nerve recordings are shown in Figure 8, along with examples of consistent repetitive firing caused by the epoxide (6×10^{-9} M), the cyclopropane analogue (10^{-9} M), and the thirane (10^{-10} M). Because the epoxide is a metabolite of phenothrin, this is the first example of a metabolite of a commercial pyrethroid having nerve activity. Furthermore,

Table 1. Pyrethroids Lacking an α-Cyano Substituent: Toxicity, Poisoning Signs, and in Vivo Qualitative and in Vitro Quantitative Ability To Cause Repetitive Firing in the Cockroach Cercal Sensory Nerves (Nerve 8, Figure 7; Adapted from Reference 2)

		ester		toxicity and symptomology		
no.	compound[a]	alcohol[b]	acid[c]	LD_{50} (or test dose)[d] (μg/g)	Type[e]	in vitro nerve response[f] (M)
1	pyrethrins[g]			1.2	I	4×10^{-9} (n = 10)
	allethrin					
2	(1*R*,*trans*)	Al	CA	0.5	I	2×10^{-9} (6)
	tetramethrin					
3	1*R*,*cis*	Tet	CA	~500	I	3×10^{-13} (14)
4	1*R*,*trans*			57	I	3×10^{-13} (7)
	resmethrin					
5	1*R*,*cis*	BFA	CA	0.3	I	8×10^{-10} (10)
6	1*RS*,*trans*			1.0	I	$\leq 10^{-9}$(1)
7	kadethrin	BFA	TCA	1.2	I[h]	6×10^{-10} (9)
	phenothrin	PB	CA			
8	1*R*,*cis*			0.7	I	9×10^{-9} (4)
9	1*R*,*trans*			0.7	I	8 X 10^{-9} (10)
10	1*S*,*cis*			>500	—[i]	5×10^{-7} (5)
11	1*S*,*trans*			260	I	7×10^{-7} (7)
	permethrin	PB	$C1_2CA$			
12	1*R*,*cis*[j]			0.09	I	7×10^{-9} (9)
13	1*R*,*trans*			0.6	I	8×10^{-9} (8)
14	1*S*,*cis*			>500	—[i]	$>10^{-6}$ (1)
15	1*S*,*trans*			>500	—[i]	$>10^{-6}$ (5)
16	descyanodeltamethrin (1*R*,*trans*)	PB	Br_2CA	(0.3)	I	
17	descyanofenpropathrin	PB	TMCA	(0.5)	I	
18	descyanofenvalerate (*RS*)	PB	CPMB	(0.2)	I	
19	oxime *O*-ether	PB	CCKO	1.1	I	5×10^{-8} (7)[k]
	comparison compound					
20	*p*,*p*′-DDT			(6−33)	I[l]	2×10^{-8} (5)

[a] Sources: (**1**, **6**) McLaughlin Gormley King Co. (Minneapolis, MN); (**2**, **5**, **7**−**15**) Roussel-Uclaf (Paris, France); (**3**, **4**, **18**) Sumitomo Chemical Co. (Takarazuka, Japan); (**16**, **17**) Luis O. Ruzo; and (**19**) Mark A. Brown (Pesticide Chemistry and Toxicology Laboratory, University of California, Berkeley). [b] Abbreviations used: Al, *S*-allethrolone; PB, 3-phenoxybenzyl alcohol; BFA, 5-benzyl-3-furylmethyl alcohol; Tet, *N*-(hydroxymethyl)-3,4,5,6-tetrahydrophthalimide. [c] Abbreviations used: CA, chrysanthemic acid; Br_2CA, 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid; $C1_2CA$, 3-(2,2-dichIorovinyl)-2,2-dimethylcyclopropylpropanecarboxylic acid; F_2CA, 3-(2,2-difluorovinyl)-2,2-dimethylcyclopropanecarboxylic acid; CCKO, (*EZ*)-(4-chlorophenyl)cyclopropyl ketoxime; CPMB, 2-(4-chlorophenyl)-3-methylbutyric acid; TCA, (1*R*,*cis*,*E*)-2,2-dimethyl-3-(2′-oxo-3′-thiocyclopentylidenemethyl)-1-cyclopropanecarboxylic acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid. [d] Parentheses designate arbitrary test dose where LD_{50} value was not determined. [e] Classification based on both in vivo repetitive firing in a cereal sensory nerve following an air-puff stimulus (recorded using a 50 μm insulated Ag wire electrode inserted through the cut end of a cercus) and symptoms at twice the LD_{50} value or at the designated test dose. [f] The minimum concentration effective within 2 min in inducing repetitive firing following a single electrical stimulus. [g] Pyrethrum extract consisting of 40% pyrethrins I and 46% pyrethrins II with dose calculated as average molecular weight. [h] Did not reliably cause repetitive firing in nerve 8, in vivo. [i] Slight incoordination but no mortality. [j] Minimum concentration of 1*RS*,*cis* isomer for repetitive firing is 1×10^{-8} M based on 12 determinations. [k] 8.9×10^{-8} M when DMSO used as cosolvent [l] Caused repetitive firing in nerve 8, in vivo, but poisoning signs peculiar to DDT

the potency of these analogues in vitro appears to be similar or greater than that of the parent, phenothrin.

In the case of the cyclopropyl analogue of tetramethrin, the most potent Type I pyrethroid,[2] consistent repetitive firing was recorded 11 min after dosing in vitro at 10^{-18} M (Figure 9). Such nerve potency is greater than any previously described chemical for any nerve assay. Insecticidal activity of the tetramethrin and phenothrin analogues, however, was not greater than that of the parent pyrethroids.[21,22]

In another study, to define the relevance of the ester link in the mechanism of action of non-cyano pyrethroids, four non-ester pyrethroids were studied.[23,24] These were the ether pyrethroids ethofenprox (MTI 500) and its analogue, with Si replacing the tetrahedral C atom, and an alkyl pyrethroid, MTI 800, along with its Si-containing analogue, silanophane. All of these resulted in Type I pyrethroid effects in the cockroach, in vitro and in vivo, and representative discharges are shown in Figures 10−12.

In other structure−activity studies, the commercial pyrethroid tefluthrin also caused Type I nerve discharges in the cockroach. Insects dosed at 2 or 4 times the LD_{50} (0.5 μg/g) displayed prolonged repetitive firing in the cercal sensory nerves within an hour of topical dosing. These discharges continued for several days after dosing, in vivo. However, another commercial pyrethroid bifenthrin (FMC 54800) failed to cause Type I discharges in the cockroach after dosing at 2−5 times the LD_{50} (0.2 μg/g).[25] There was some evidence for the inhibition of [^{3}H]-Ro5-4864 (the 4Cl-phenyl analogue of diazepam, see the following section) binding in rat brain membranes, but the published

Table 2. Pyrethroids with an α-Cyanophenoxybenzyl Substituent: Toxicity, Poisoning Signs, and in Vivo Qualitative and in Vitro Quantitative Ability To Cause Repetitive Firing in the Cockroach Cercal Sensory Nerves (Nerve 8, Figure 7; Adapted from Reference 2)

		ester		toxicity and symptomology		
no.	compound[a]	alcohol[b]	acid[c]	LD_{50} (or test dose)[d] (μg/g)	Type[e]	in vitro nerve response[f] (M)
1	oxime *O*-α-CN-ether (*RS*)	CN-PB	CCKO	1.0	I/II[g]	3×10^{-6} ($n = 7$)[h]
2	fenpropathrin (*RS*)	CN-PB	TMCA	0.04	I/II[g]	2×10^{-9} (5)
	cyphenothrin	CN-PB	CA			
3	1*R*,*cis*,α*S*			(0.6)	II	
4	1*R*,*trans*,α*S*			(0.3)	II	
	difluorocyphenothrin	CN-PB	F_2CA			
5	1*R*,*cis*,α*S*			(0.3)	II	
6	1*R*,*trans*,α*S*			(0.2)	II	
	cypermethrin	CN-PB	Cl_2CA			
7	1*R*,*cis*,α*S*			0.02	II	$>10^{-6}$(2)
8	1*R*,*trans*,α*S*			0.01	II	$>10^{-6}$(1)
9	deltamethrin (1*R*,*cis*,α*S*)	CN-PB	Br_2CA	0.01	II	$>10^{-6}$(1)
10	fenvalerate (*S*,*S*)	CN-PB	CPMB	0.1	II	$>10^{-6}$(1)

[a] Sources: (**1**) prepared by M. A. Brown (Pesticide Chemistry and Toxicology Laboratory, University of California, Berkeley); (**2** and **10**) Shell Development Co. (Modesto, CA); (**3**−**9**) Roussel-Uclaf (Paris, France). [b] Abbreviations used: CN-PB, α-cyano-3-phenoxybenzyl alcohol. [c] CA, chrysanthemic acid; Br_2CA, 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid: $C1_2$CA, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid; F_2CA, 3-(2,2-difluorovinyl)-2,2-dimethylcyclopropanecarboxylic acid; CCKO, (*EZ*)-(4-chlorophenyl)cyclopropyl ketoxime; CPMB, 2-(4-chlorophenyl)-3-methylbutyric acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid. [d] Parentheses designate arbitrary test dose where LD_{50} value was not determined. [e] Classification based on both in vivo repetitive firing in a cercal sensory nerve and symptoms at twice the LD_{50} value or at the designated test dose. Although the Type II syndrome did not involve repetitive firing in this nerve, long efferent discharges were recorded in the cerci. [f] The minimum concentration effective within 2 min in inducing repetitive firing following a single electrical stimulus, [g] Symptoms resembled Type II but consistent repetitive firing was observed in nerve 8, in vivo, i.e., Type I. [h] Lower concentrations caused repetitive firing but with a latency >2 min.

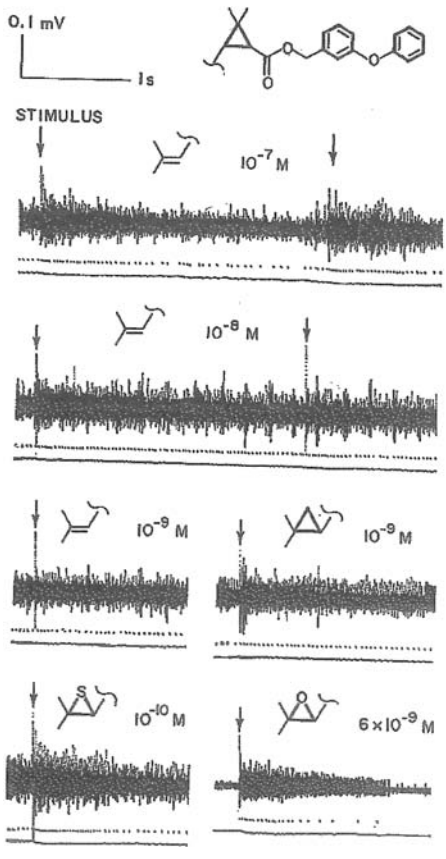

Figure 8. Type I repetitive firing in cercal sensory nerve after stimulation caused by phenothrin and cyclopropane, thiirane, and epoxide. Adapted from ref 21.

record is incomplete with regard to the precise mechanism of action of bifenthrin. For example, the interesting observation was made recently[26] that whereas ζ-cypermethrin, λ-cyhalothrin (Type II), and fenpropathrin (Type I/II) had a negative temperature coefficient of toxicity to a Hemipteran bug over the range of 27−37 °C, bifenthrin had a positive temperature coefficient of toxicity. Therefore, although bifenthrin lacks an αCN group and has thus been classed as Type I, its effects on the insect VGSC appear to show differences from Type I and II pyrethroids. Similarly, the effects of bifenthrin on the rat $Na_v1.8$ channel, expressed in *Xenopus* oocytes, were intermediate between those of Type I and II pyrethroids.[14]

GABA-Activated Chloride Channel Effects. In a previous study,[27] it had been suggested that a Type II pyrethroid had effects on the $GABA_A$ receptor complex because deltamethrin inhibited the binding of a tritiated picrotoxinin analogue to rat brain membranes. Moreover, because insects possess $GABA_A$ receptors in both the peripheral and central nervous systems, the cockroach was used to investigate this further. Attempts were made to quantify the trains of efferent/motor potentials caused by deltamethrin (Type II) in the cockroach, in vivo.[28] Electrodes were implanted just inside the cut end of a cercus to record sensory nerve activity as well as into the base of a cercus to also record efferent/motor activity. Long trains of motor spikes, unrelated to a stimulus, were observed within 20 min of dosing with 2 times the LD_{50} with no apparent change in sensory nerve activity (Figure 13). Prior injection of cockroaches with the benzodiazepine diazepam delayed the onset of these cercal motor discharges from 20 to 84 min, in this (typical) experiment. It was also found that prior dosing with diazepam delayed the onset of clinical signs in both the cockroach and the mouse.[28] This was the first demonstration of benzodiazepine receptors in invertebrates, including insects. In mammalian CNS, activation of these receptors amplifies the effects of GABA at the $GABA_A$ receptor complex, increasing the Cl^- current. The Cl^- channel is blocked by picrotoxinin and convulsant bicyclophosphates, acting at a separate receptor in the channel.

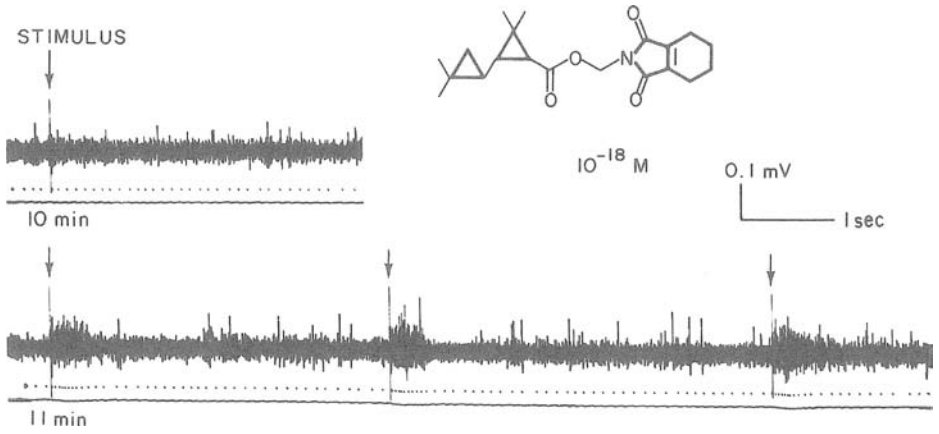

Figure 9. Type I repetitive firing in a cercal sensory nerve after electrical stimulation in vitro caused by tetramethrin analogue [1*RS*,1′*RS*,*cis*]-methanotetramethrin. Normal response was at 10 min and consistent repetitive firing at 11 min. Adapted from ref 22.

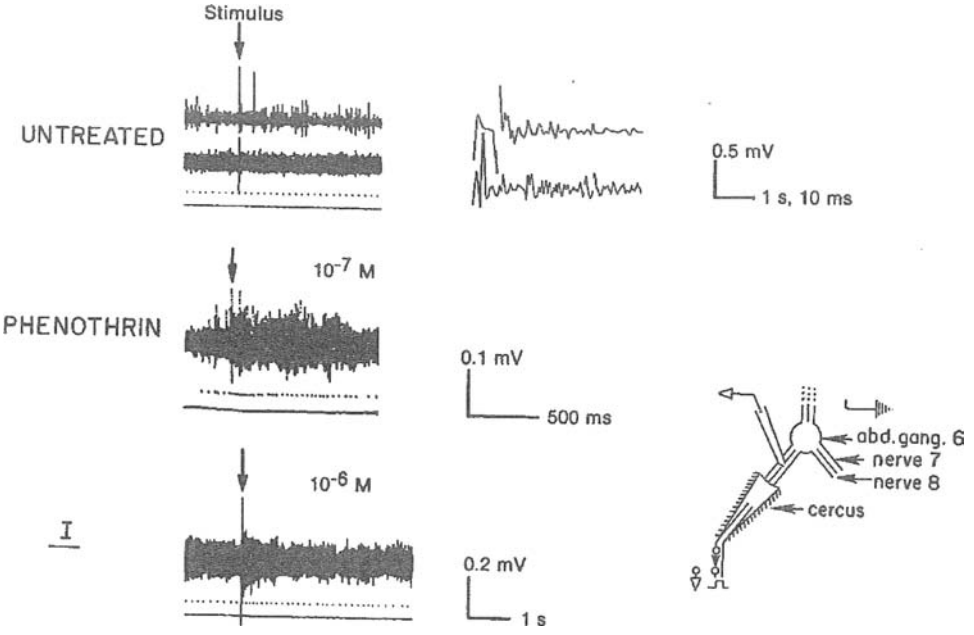

Figure 10. Type I repetitive firing in cercal sensory nerve after electrical stimulation in vitro caused by phenothrin and MTI-500 (I). Adapted from ref 23.

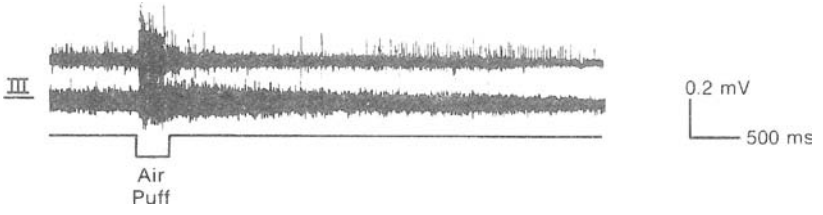

Figure 11. Type I repetitive firing in cercal sensory nerve after mechanical stimulation in vivo caused by MTI-800 (III). Adapted from ref 23.

The possible involvement of the benzodiazepine receptor in the mechanism of action of deltamethrin in the cockroach provided the impetus to evaluate the Type II syndrome on the $GABA_A$ receptor complex in the crayfish claw opener muscle, in vitro.[29] Deltamethrin at 5−40 μM, along with other Type II pyrethroids, acted as GABA antagonists, by increasing the input resistance of the muscle fiber membrane exposed to GABA (Figure 14). This effect was similar to the effect of picrotoxinin (PTX) at 5−10 μM (Figure 14). Inactive stereoisomers of Type II pyrethroids and Type I pyrethroids had no effect on input resistance. It was also found that benzodiazepines antagonized the Type II pyrethroid effect, shifting the dose/response curve to

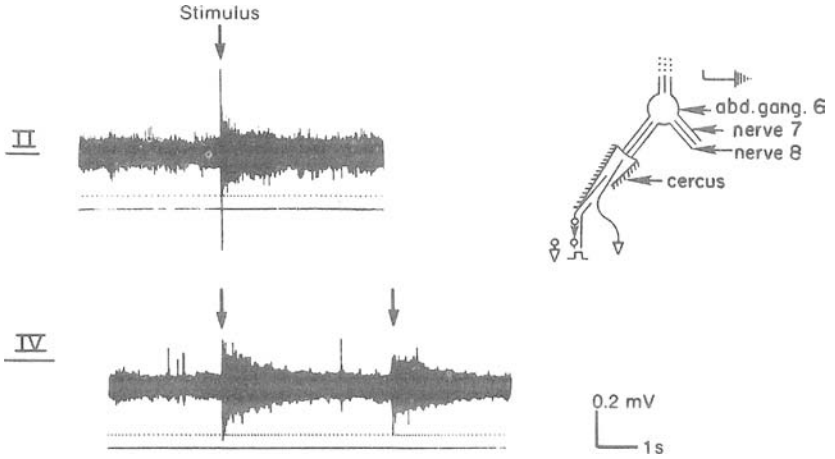

Figure 12. Type I repetitive firing in cercal sensory nerve after electrical stimulation in vivo caused by Si analogues of MTI-500 (II) and MTI-800 (IV). Adapted from ref 23.

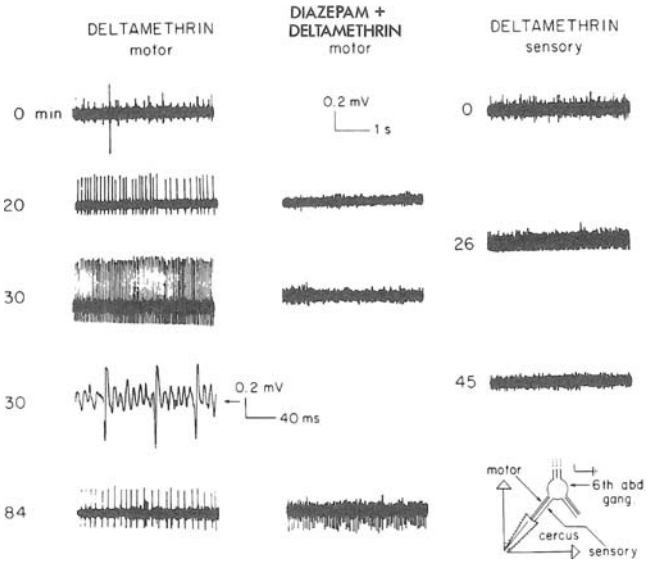

Figure 13. GABA-activated Cl^- channel. Deltamethrin caused trains of cercal efferent potentials, which were delayed by diazepam, in vivo, in cockroach. Adapted from ref 28.

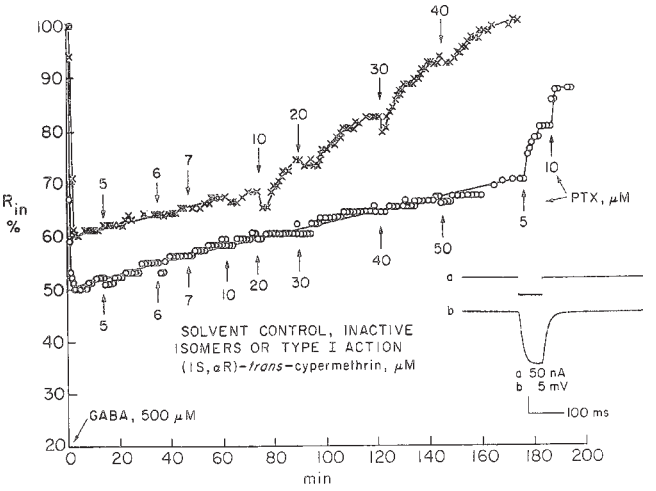

Figure 14. Crayfish claw opener muscle exposed to GABA plus deltamethrin (5−40 μM) in vitro gave increased R_{in} showing antagonism of GABA response. Adapted from ref 29.

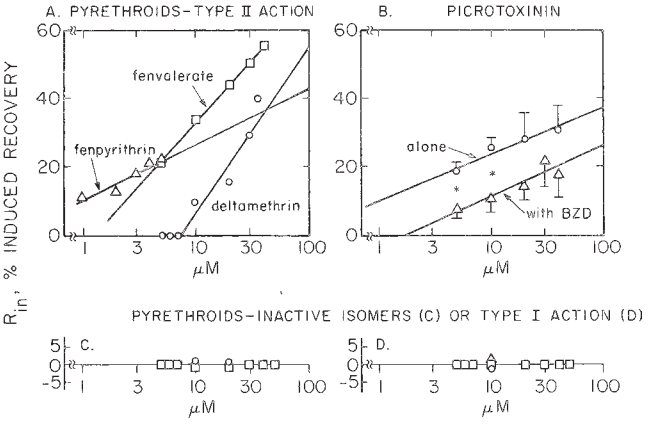

Figure 15. Effects of α-CN pyrethroids on R_{in} in GABA-exposed crayfish muscle fibers (A), inhibition of the effect of picrotoxinin by benzodiazepines (B), and lack of effect of nontoxic α-CN pyrethroids (C) or non-α-CN pyrethroids (D). Adapted from ref 29.

the right, that is, to higher concentrations (Figure 15). Simultaneously, Type II pyrethroids but not inactive isomers or Type I pyrethroids were found to inhibit the specific binding of [^{35}S]-TBPS (*tert*-butyl bicyclophosphorothionate) to mouse brain receptors.[30] This radioligand acts as an antagonist at the $GABA_A$-activated Cl^- channel, in the same manner as PTX. This site of action for Type II pyrethroids was confirmed in rat brain membranes.[31,32]

Further studies established that [^{3}H]-Ro5-4864 binding was inhibited at lower concentrations (than for [^{35}S]-TBPS binding) specifically by Type II pyrethroids.[25,33] This binding site is the so-called peripheral benzodiazepine receptor in mammals. Ro5-4864 lacks the ability to inhibit the binding of [^{3}H]-flunitrazepam to the central benzodiazepine receptor, although central benzodiazepine receptor ligands such as diazepam inhibit the binding of [^{3}H]-Ro5-4864 to peripheral or CNS receptors with high potency. Interestingly, Ro5-4864 was potent at inhibiting [^{3}H]-flunitrazepam at the insect benzodiazepine receptor.[34] Furthermore, the early clinical signs in the cockroach following injection of Ro5-4864 or diazepam were identical to those caused by Type II pyrethroids. One

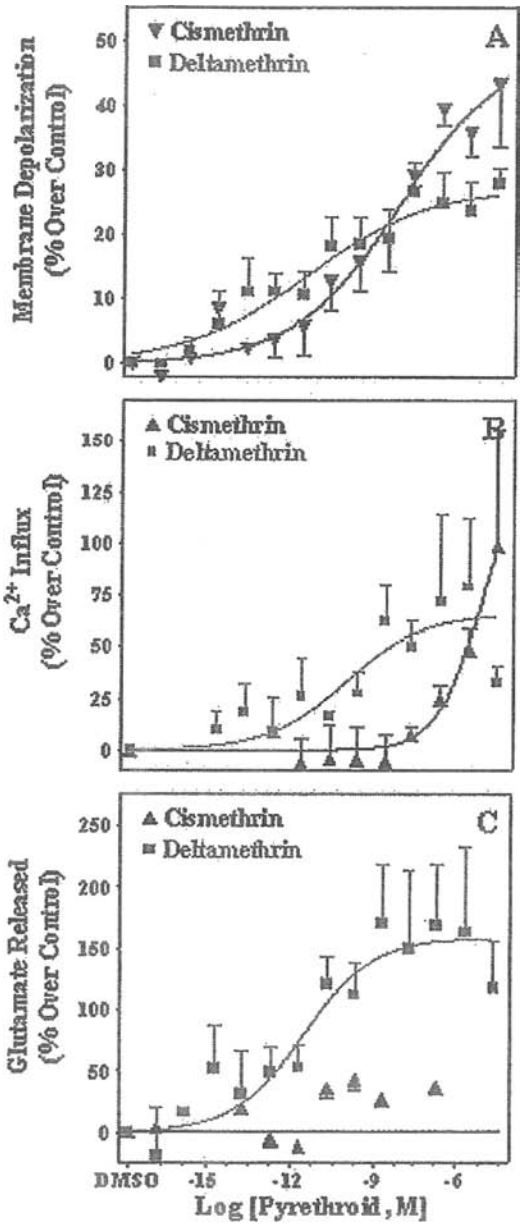

Figure 16. Pyrethroid effects on rat brain synaptosomes: (A) depolarization; (B) Ca^{2+} influx; (C) glutamate release. Adapted from refs 37 and 38.

difference was that insects recovered within an hour of dosing from the former but not the latter.[25] It remains plausible that the early clinical signs could result from an antagonist effect of Type II pyrethroids at the insect benzodiazepine receptor or its closely associated GABA Cl^- channel. It has also been noted that Ro5-4864 interacts with VGCCs, and Type II pyrethroid effects on the Ro5-4864 receptor may reflect such a site of action.[25,35]

Voltage-Gated Calcium Channel Effects. Other studies to define the mechanism(s) of action of pyrethroids have considered the voltage-gated calcium channel (VGCC). Initial studies established that the protozoan *Paramecium*, which lacks VGSCs and instead uses VGCCs, is extremely sensitive to pyrethroids such as deltamethrin.[36] Subsequently, pyrethroid effects on rat brain synaptosomes and *Xenopus* oocytes expressing specific types of VGCCs have been studied.[37] Distinct effects of Type II pyrethroids have been described on VGCCs, as well as on downstream events, such as glutamate release, following VGCC activation. These effects have not been found, in general, with Type I pyrethroids.

For example, both cismethrin and deltamethrin effects were studied on $Ca_v2.2$ channels in rat brain synaptosomes.[37,38] Both were potent inducers of depolarization (Figure 16A), but only deltamethrin potently also caused increased Ca^{2+} influx through VGCC (Figure 16B) as well as glutamate release (Figure 16C) at nanomolar concentrations. The deltamethrin effects on Ca^{2+} influx and glutamate release were blocked by ω-conotoxin, indicating that the N-type $Ca_v2.2$ VGCC was involved. In *Xenopus* oocytes engineered to express $Ca_v2.2$ channels, deltamethrin reduced Ca^{2+} influx in wild-type channels but increased this current in mutant channels (T422E). It was concluded that the mutant channel acts as though phosphorylated.[37,38]

A structure−activity analysis of six α-cyanophenoxybenzyl pyrethroids was conducted, using rat brain synaptosomes.[39] Cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, and esfenvalerate were potent stimulators of both Ca^{2+} influx and glutamate release (Figure 17). Only fenpropathrin, the Type I/II hybrid, was different from the others, resulting in glutamate release but without a significant increase in Ca^{2+} influx (Figure 17F). In the same study, five non-cyano pyrethroids were also examined (Figure 18). Four of them (bifenthrin, bioallethrin, permethrin, and tefluthrin) caused significantly increased glutamate release, whereas cismethrin did not (Figure 18C). Ca^{2+} influx, however, was variable in responses to these pyrethroids: bioallethrin and tefluthrin did not cause a significant increase, bifenthrin and cismethrin caused a significant increase only at high concentrations, and permethrin significantly increased Ca^{2+} influx and glutamate release in parallel (Figure 18D), similar to the majority of α-cyano pyrethroids (Figure 17).

The effects of 11 pyrethroids on Ca influx and glutamate release in rat brain synaptosomes are summarized in Figure 19.[39] Three groupings emerged when the dose−response relationships of these two parameters were compared. Five α-cyanophenoxybenzyl pyrethroids plus permethrin were potent stimulators of Ca influx and glutamate release. One (fenpropathrin) plus two non-CN pyrethroids (tefluthrin, bioallethrin) were inactive on Ca influx but were potent stimulators of glutamate release. The third group (cismethrin, bifenthrin) were low potency stimulators of Ca influx and low efficacy stimulators of glutamate release (Figure 19).

Other Systems. Recently, it has also been found that fenvalerate, an α-cyanophenoxybenzyl pyrethroid, induced Ca^{2+} transients via both intracellular and extracellular pathways in mouse GC-2spd (ts) cells.[40] Intracellular Ca^{2+} transient currents were activated via inositol triphosphate and ryanodine receptors, whereas extracellular Ca influx was found to be via a store-operated channel.

The voltage-gated Cl^- channel has also been found to be a target site specifically for pyrethroids causing the Type II syndrome.[41] Patch clamp analysis of mouse brain neurons, in vitro, has shown that α-cyanophenoxybenzyl pyrethroids block the so-called maxi voltage-gated Cl^- channel. This could be related, in terms of the binding site interaction, to the antagonism caused by such pyrethroids at the GABA-activated Cl^- channel, described previously.

Effects of pyrethroids have also recently been described on delayed rectifier K^+ currents in rat hippocampal neurons, in vitro, using patch clamp analysis.[42] α- and θ-cypermethrin (Type II), at

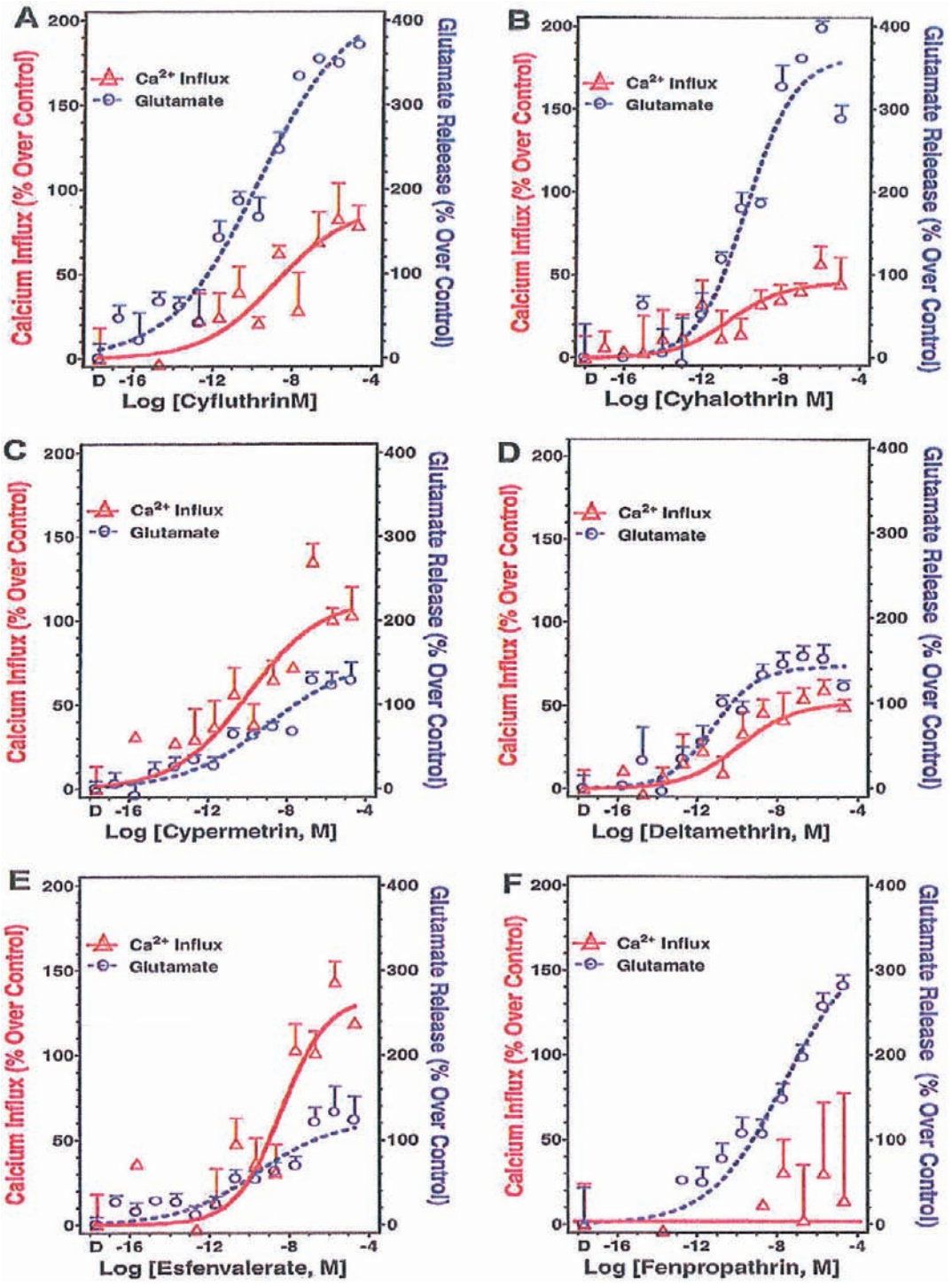

Figure 17. Effects of α-CN pyrethroids on Ca^{2+} influx and glutamate release in rat brain synaptosomes: cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, esfenvalerate, fenpropathrin (red, Ca influx; blue, glutamate release). Adapted from ref 39.

10^{-9}—10^{-7} M, reduced the steady-state (outward) current in these neurons in a concentration-dependent manner.

■ DISCUSSION

There is substantial evidence that pyrethroid-induced nerve effects are compound-specific. However, broad groupings can be made for Type I (non-CN pyrethroids), equivalent to T syndrome pyrethroids in the rat, and Type II (αCN pyrethroids), equivalent to CS syndrome pyrethroids in the rat. The groupings appear to be similar in insects and mammals. A limited number of pyrethroids, for example, fenpropathrin, appear to result in effects that are a hybrid of Types I and II. Type I effects appear to be associated with the VGSC, which in insects is TTX-sensitive. In mammals, broad sensitivity to pyrethroids is found for the $Na_v1.8$, which is TTX-insensitive. This apparent difference between insects and mammals should be further investigated. Other VGSC isoforms should also be studied in relation to

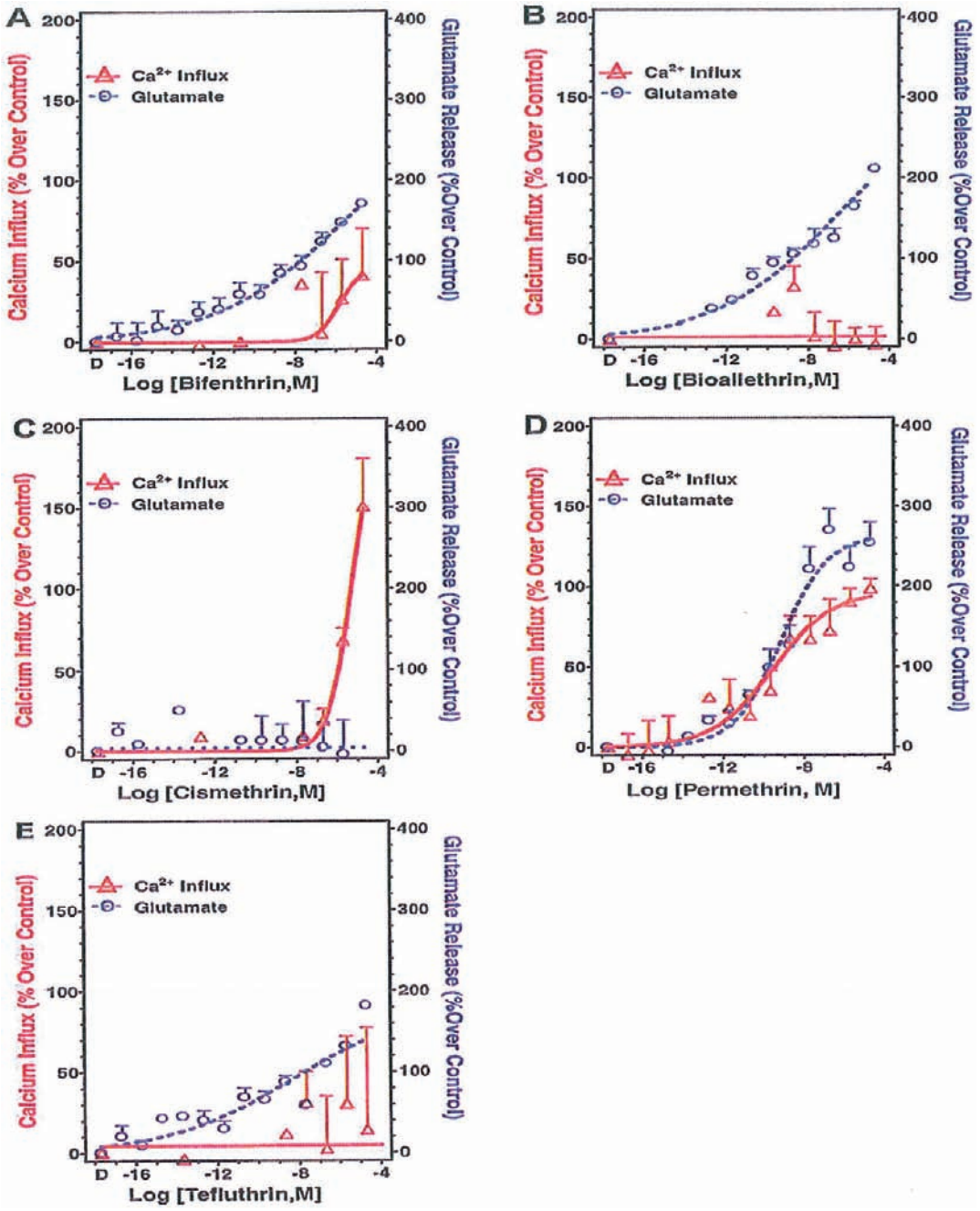

Figure 18. Effects of non-CN pyrethroids on Ca^{2+} influx and glutamate release in rat brain synaptosomes: bifenthrin, bioallethrin, cismethrin, permethrin, tefluthrin (red, Ca influx; blue, glutamate release). Adapted from ref 39.

selectivity and sensitivity to Type I and II pyrethroid effects. Studies currently being conducted in Soderland's laboratory should help to resolve this issue. Type II pyrethroid effects on the VGSC are qualitatively different from those associated with the Type I syndrome. Moreover, there is considerable evidence that the Type II syndrome, specifically, is also associated with effects on other ion channels, including VGCCs, voltage- and GABA-gated Cl^- channels, and voltage-gated K^+ channels. Attempts have recently been made to relate physiological effects on ion channels ($Na_v1.8$, $Ca_v2.2$, voltage-gated Cl- channel) with clinical signs in the rat, and two pyrethroid mechanisms of action have been reported.[14] The relative significance of each target site for the various clinical signs in insects and mammals is a subject worthy of further study. In particular, it is hoped that further studies will be conducted on pyrethroid effects on the ASR in the rat because these have been recommended by this and previous SAPs. It has been found that Type I pyrethroids increase the ASR amplitude, whereas Type II pyrethroids decrease the amplitude.[31,32] Differences are also apparent between Type I and II pyrethroids in the effects on latency of the ASR. The use of such an assay should enable (small) combinations of pyrethroids to be assessed, as also recommended by the SAP. This should allow a decision about whether all pyrethroids should be grouped together cumulatively or whether it is more appropriate to consider two separate groups, with perhaps a limited number of hybrid pyrethroids. It was agreed that, in general, ASR effects

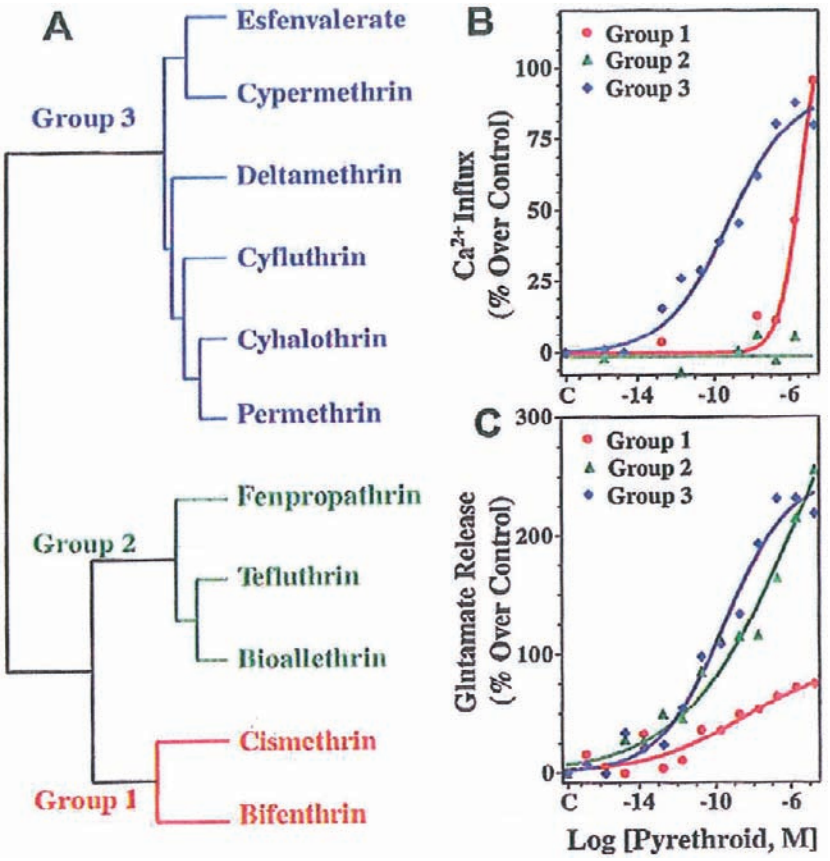

Figure 19. Separation of pyrethroids into three groups (A) based on effects on the stimulation of Ca^{2+} influx (B) and glutamate release (C). Adapted from refs 38 and 39.

were more likely to be pyrethroid-specific than effects such as reductions in motor activity, which are nonspecific, apical end points. Clearly, the work conducted in Dr Casida's laboratory in the 1980s has played a central role in our current understanding of pyrethroid toxicology.

Present Addresses

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