AN ABSTRACT OF THE THESIS OF

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Title: Pyrethroid Insecticide Interaction with the $GABA_A$ Receptor and the Peripheral-Type Benzodiazepine Receptor of Rainbow Trout Brain

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Thomas F. Murray

The peripheral-type benzodiazepine receptor (PTBR) of trout brain was pharmacologically characterized and pyrethroid interaction with this site investigated. High-affinity binding sites for [^{3}H]PK 11195 were detected in brain membranes of rainbow trout; these shared some of the characteristics of the PTBR of rodent brain (i.e., high affinity for PK 11195 and an endogenous ligand protoporphyrin IX) but were unique in the low affinity for Ro5-4864. Permethrin displaced [^{3}H]PK 11195 binding with micromolar affinity while deltamethrin had less than 50% efficacy at displacement. Thus the PTBR appeared not to be relevant to pyrethroid toxicity in rainbow trout.

Pyrethroid interaction with the $GABA_A$ receptor was investigated using [^{35}S]TBPS as a radioligand probe and by measurement of GABA-stimulated $^{36}Cl^-$ influx in vesicle preparations. At micromolar concentrations, deltamethrin,

cypermethrin isomers and other pyrethroids inhibited [^{35}S]TBPS binding by 55-95% with limited stereoselectivity. Pyrethroids were found to effect a GABA-dependent inhibition of [^{35}S]TBPS binding. Ro5-4864, which showed micromolar affinity for the trout PTBR, produced a GABA-modulated interaction with [^{35}S]TBPS binding. These results delineate the reciprocal allosteric interactions between a pyrethroid binding site, a Ro5-4864 binding site, the GABA recognition moiety and the TBPS binding site in trout brain. However, pyrethroids exhibited a modest affinity for this binding site on the $GABA_A$ receptor.

Pyrethroids indirectly inhibited the GABA-dependent influx of $^{36}Cl^-$ into trout brain synaptoneurosomes by increasing the basal uptake of chloride, thereby compromising the ability of the vesicles to respond to applications of GABA. This pyrethroid effect was of nanomolar potency, stereospecific, tetrodotoxin-sensitive and mimicked by veratridine. These results suggest that the primary effect of pyrethroids in trout brain, as measured by this assay, was due to an interaction with voltage-dependent sodium channels, increasing sodium conductance and thereby increasing the basal uptake of $^{36}Cl^-$ through a voltage-sensitive channel.

The convulsant activity of deltamethrin was tested in rainbow trout. The EC_{50} for convulsant severity was 32 μg/kg body weight. By comparison, pyrethroids at these concentrations in rodents produce no overt toxicity but act as potent proconvulsants.

Pyrethroid Insecticide Interaction with the $GABA_A$ Receptor
and the Peripheral-Type Benzodiazepine Receptor
of Rainbow Trout Brain

by

Amy J. Eshleman

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APPROVED:

Redacted for Privacy

Dr. Thomas F. Murray, Associate Professor of Pharmacy

Redacted for Privacy

Dr. Donald Buhler, Head of Toxicology Program

Redacted for Privacy

Dean of Graduate School

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TABLE OF CONTENTS

Chapter		Page
1.	Introduction	1
	Pyrethroids and the $GABA_A$ receptor	6
	Peripheral-type benzodiazepine receptor	17
2.	Differential Binding Properties of the Peripheral-Type Benzodiazepine Ligands, [^{3}H]PK 11195 and [^{3}H]Ro5-4864, in Trout and Mouse Brain Membranes	24
	Abstract	24
	Introduction	25
	Methods	28
	Results	32
	Discussion	37
3.	GABA Dependence of Pyrethroid and 4'-Chlorodiazepam Modulation of t-[^{35}S]Butylbicyclophosphorothionate Binding in Piscine Brain	59
	Abstract	59
	Introduction	60
	Methods	62
	Results	65
	Discussion	68
4.	Pyrethroid Insecticides Indirectly Inhibit GABA-Dependent $^{36}Cl^-$ Influx in Trout Brain Synaptoneurosomes	95
	Abstract	95
	Introduction	96
	Methods	98
	Results	100
	Discussion	104
5.	Convulsant Activity of Deltamethrin in Rainbow Trout	134
	Abstract	134
	Introduction	134
	Methods	135
	Results	137
	Discussion	138

6. Conclusions 145

7. References 152

LIST OF FIGURES

Figure		Page
1-1.	Chemical structure of Type I and II pyrethroids.	3
1-2.	Chemical structures of ligands for sites on the $GABA_A$ receptor.	8
1-3.	A model of the $GABA_A$ receptor.	12
1-4.	Chemical structures of CBR and PTBR ligands.	16
2-1.	Inhibition of [^{3}H]PK 11195 binding by Ro5-4864, PK 11195, and protoporphyrin IX in mouse forebrain and trout brain membranes.	43
2-2.	Saturation equilibrium binding of [^{3}H]PK 11195.	45
2-3.	Time course of [^{3}H]PK 11195 binding to trout brain membranes.	48
2-4.	Regional distribution of density of binding sites of [^{3}H]PK 11195 in trout brain membranes.	51
2-5.	Saturation equilibrium binding of [^{3}H]Ro5-4864 in mouse forebrain membranes.	53
3-1.	Equilibrium binding of [^{35}S]TBPS to trout brain membranes: uncompetitive inhibition by GABA.	74
3-2.	GABA modulation of deltamethrin concentration-response curves of inhibition of [^{35}S]TBPS binding.	77

3-3. Inhibition of [^{35}S]TBPS binding by three cypermethrin isomers. 79

3-4. The influence of GABA on Ro5-4864 modulation of specific [^{35}S]TBPS binding. 81

3-5. PK 11195 inhibition of specific [^{35}S]TBPS binding. 83

3-6. Lack of effect of PK 11195 on Ro5-4864-induced enhancement of [^{35}S]TBPS binding in the presence of 10 μM GABA. 85

3-7. Ro5-4864-induced shift of GABA concentration-response curves for inhibition of [^{35}S]TBPS binding. 87

4-1. Time course of GABA-dependent $^{36}Cl^-$ influx. 112

4-2. GABA, muscimol, and pentobarbital concentration-response-curves. 114

4-3. Concentration-response curve for deltmethrin. 117

4-4. Noncompetitive inhibition by deltamethrin of GABA concentration-response curve. 119

4-5. Concentration-response curves for (1RαS)-*cis* and (1SαR)-*cis*-cypermethrin and 1R-*cis*-permethrin. 121

4-6. Tetrodotoxin (TTX) inhibition of the deltamethrin-induced increase in basal uptake. 124

4-7. Veratridine-induced increase in basal uptake is inhibited by TTX. 126

4-8. Lack of additivity of basal uptake of deltamethrin and veratridine. 128

4-9. Lack of effect of deltamethrin on control, avermectin

(AVA), or pentobarbital-induced chloride efflux. 130

5-1. Deltamethrin dose-response curve for seizure activity score in rainbow trout. 141

5-2. PK 11195 modulation of deltamethrin-induced seizure activity following simultaneous administration. 143

6-1. A model of the $GABA_A$ receptor-chloride ion channel complex. 150.

LIST OF TABLES

Table		Page
2-1.	Potency of PTBR, CBR, and calcium channel ligands and pyrethroids as inhibitors of the specific binding of [^{3}H]PK 11195 to piscine and murine brain membranes.	55
2-2.	Inhibition of [^{3}H]PK 11195 binding by pretreatment with DEPC in rat cerebral cortex and trout brain.	56
2-3.	Parameter estimates of Ro5-4864 inhibition of [^{3}H]PK 11195 binding to mouse forebrain membranes.	57
2-4.	[^{3}H]PK 11195 and [^{3}H]Ro5-4864 binding in mouse forebrain membranes are nonadditive.	58
3-1.	Influence of GABA on the deltamethrin inhbition of [^{35}S]TBPS binding to trout brain membranes.	89
3-2.	Inhibition of [^{35}S]TBPS binding to trout brain membranes by 3 *cis*-cypermethrin isomers.	90
3-3.	Deltamethrin modulation of GABA inhibition of [^{35}S]TBPS binding.	91
3-4.	Pyrethroid inhibition of [^{35}S]TBPS binding in the presence of 5 μM GABA.	92
3-5.	Influence of GABA on PK 11195 inhibition of [^{35}S]TBPS binding.	93
3-6.	Ro5-4864 modulation of GABA inhibition of [^{35}S]TBPS binding.	94

4-1. Pyrethroid inhibition of GABA-dependent $^{36}Cl^-$ influx. 132

4-2. $^{36}Cl^-$ efflux from trout brain synaptoneurosomes. 133

Pyrethroid Insecticide Interaction with the $GABA_A$ Receptor
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Chapter 1

Introduction

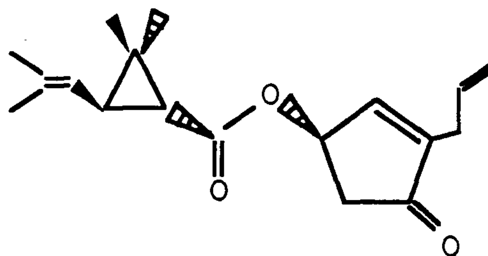
Synthetic pyrethroids are broad-spectrum, non-systemic insecticides, which have insecticidal activity as nerve poisons (Hill, 1985). These compounds are replacing the chlorinated hydrocarbons, such as DDT, which have unacceptable persistence and bioaccumulation, and insecticides which have a low margin of safety between insecticidal activity and lethality to mammals as exemplified by the organophosphates (Casida et al., 1983). The pyrethroids have a relatively short environmental half-life and a favorable selectivity between insecticidal potency and mammalian toxicity as measured by lethality. Worldwide use of these insecticides is increasing, as evidenced by the facts that the value of the world market consumption of pyrethroids increased from ten million to 630 million U.S. dollars and the pyrethroid percentage of the world foliar insecticide market increased from less than 5% to 20-25% from 1976 to 1983 (Herve, 1985). This widespread use will result in more non-target species being exposed to these insecticides. An effective antidote remains to be established (Aldridge, 1982).

Synthetic pyrethroids are derivatives of natural pyrethrins from Chrysanthemum species. Manufacture began in 1949 with allethrin; in 1976 the first α-cyano-pyrethroid, fenvalerate, was synthesized (Davies, 1985). The newer

compounds are more photostable and potent in insecticidal toxicity than pyrethrins. Structurally, most pyrethroids are esters of carboxylic acid and alkyl or aralkyl alcohol. The most potent pyrethroids are esters of cyclopropanecarboxylic acid and phenoxybenzyl alcohol (Davies, 1985; Fig. 1-1). Toxicity is highly dependent on stereochemistry: 1R but not 1S cyclopropanecarboxylates are active, while the presence of a cyano substituent at the α-position of the phenoxybenzyl group increases toxicity (Soderlund and Bloomquist, 1989).

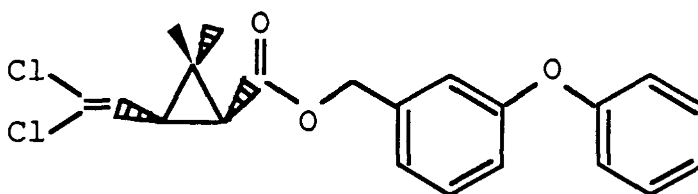
Pyrethroids have been classified as either type I or type II compounds, depending on structural characteristics and signs of poisoning in insects and mammals (Gammon et al., 1981; Gray, 1985; Lawrence et al., 1985), although a continuum of effects between these two syndromes may be more accurate (Narahashi, 1985). Type I compounds include allethrin, tetramethrin, phenothrin and permethrin, all of which lack a cyano substituent in the alcohol moiety (Fig. 1-1). The type I action in cockroach cercal sensory nerve assay is associated with repetitive firing following stimulation of the nerve. Electrophysiological experiments using intracellular microelectrodes with crayfish giant axons showed that exposure to nanomolar concentrations of tetramethrin increased the depolarizing after-potential until the after-potential reached the threshold membrane potential for repetitive after-discharges (Lund and Narahashi, 1981). Voltage-clamp analysis revealed that the mechanism of this depolarization involved a slowing of sodium channel inactivation (Narahashi and Lund, 1980). Patch clamp experiments revealed that tetramethrin modified sodium channels in an all-or-none manner, in which poisoned channels stayed open for a much

Figure 1-1. Chemical structure of Type I and II pyrethroids.

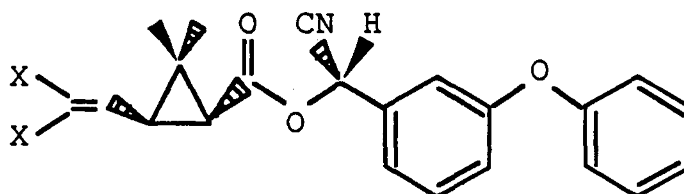


Type I Pyrethroids

Allethrin



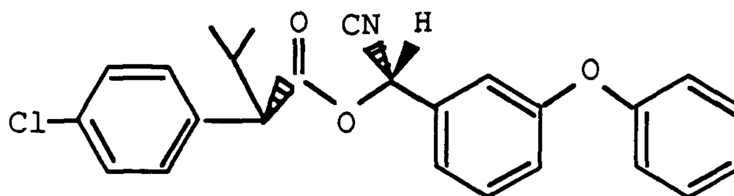
Permethrin



Type II Pyrethroids

Deltamethrin: X = Br

Cypermethrin: X = Cl



Fenvalerate

longer period of time (Narahashi, 1985).

Toxicity of Type I pyrethroids in insects is manifested as restlessness, incoordination, prostration and paralysis (Gammon et al., 1981). Mammalian signs of toxicity (i.e., the T syndrome) include aggressive sparring, sensitivity to external stimuli, intense hyperactivity, an increase in body temperature, whole body tremors which progress from fine to gross, prostration, and clonic convulsions (Gray, 1985).

The Type II pyrethroids include deltamethrin, cypermethrin and fenvalerate, which have a cyano group on the phenoxybenzyl portion of the molecule (Figure 1-1). Poisoning manifestations of these compounds are distinguishable from the Type I syndrome. The signs of poisoning by these pyrethroids in the cockroach include lack of coordination, convulsions and intense hyperactivity (Gammon et al., 1981). The progression of the signs of toxicity in mammals (i.e., the CS syndrome) is profuse salivation, coarse whole body tremor, sinuous writhing (choreoathetosis) and clonic/tonic seizures. If the latter occurs, death usually results (Lawrence et al., 1985). Electrophysiologically, these pyrethroids cause a block of conduction without an induction of repetitive firing in nerve axons.

Fish are exquisitely sensitive to both types of insecticides. Toxicity in rainbow trout exposed via the water column has been measured to be 76 $\mu g/L$ for a 24 hr LC_{50} for fenvalerate (Coats and O'Donnell-Jeffery, 1979), 14 $\mu g/L$ for a 24 hr LC_{50} for trans-permethrin (Glickman et al., 1982), and 0.5 $\mu g/L$ for a 96 hr LC_{50} for cypermethrin (Khan, 1982). Comparing toxicity in rainbow trout versus mouse in terms of LD_{50} values, permethrin, by intraperitoneal or

intravenous administration, was 37 fold more potent while technical grade fenvalerate was more than 300 fold more potent in fish (Bradbury and Coats, 1989). In general, fish exhibited 1-3 orders of magnitude greater sensitivity for pyrethroids as opposed to mammals. Brain concentrations at time of death underscore the sensitivity of this species: trout had 0.15 ppm (Bradbury et al., 1987) while quail, an insensitive species, had 1.26 ppm of fenvalerate (Bradbury and Coats, 1982). Signs of toxicity due to fenvalerate exposure in fish include initial hyperexcitability, significantly elevated cough rate, fine tremors which culminate in tonic seizures in which the head is held at a 30° angle from the body, opercular flaring, and a state of tetany (Bradbury et al., 1987).

Three possible explanations have been proposed for piscine sensitivity: efficient gill uptake of the compounds, low levels of enzymatic detoxification, or increased sensitivity at the site of action (Bradbury et al, 1986). Bradbury et. al. (1986) measured a relatively inefficient gill uptake of 28%. This uptake level followed the passive diffusion model of xenobiotic absorption which predicted that uptake efficiency would drop from 60% at log P values of 3-6 to an efficiency of 20-30% at log P values of 6-7, the pyrethroid octanol/water coefficients (McKim et al., 1985). Thus, uptake efficiency does not explain the extreme toxicity of these compounds to fish.

Glickman et. al. (1981) investigated the role of detoxification rates in differential toxicity of mouse and trout. The major routes of metabolism of pyrethroids are hydroxylation and ester hydrolysis followed by conjugation, the latter predominating in mammals. Trout have lower levels of esterases than

mammals (Glickman et al., 1982); however, following inactivation of both cytochrome-P_{450} with piperonyl butoxide and esterase inhibition by tri-o-tolyl phosphate in both species, trans-permethrin was still 65 times more toxic to trout than to mice (Glickman and Lech, 1982). Therefore, while slower metabolism rates play a role in the piscine susceptibility to pyrethroids, target organ sensitivity must also be involved.

Thus, the third proposed explanation, that of increased sensitivity of trout at the site of action, remains. Consistent with this proposal, the brain levels in trout required to produce signs of toxicity have been shown to be 3, 8, and 18 fold lower for *cis* permethrin, 1RαS-*cis*-cypermethrin, and *trans* permethrin, respectively, than in the mouse (Glickman and Lech, 1982; Edwards et al., 1986). Based on lethal brain concentrations, the sensitivity of rainbow trout and frog to pyrethroids is approximately one order of magnitude greater than the sensitivity of mouse or quail (Bradbury and Coats, 1989). The investigation of several of the possible sites of action in trout may lead to an increased understanding of the mechanism of pyrethroid toxicity.

Pyrethroids and the $GABA_A$ receptor

The $GABA_A$ receptor is a possible target of pyrethroid insecticides. GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates (Costa, 1988). There are at least two types of receptors for GABA in the CNS of mammals: the $GABA_A$ and $GABA_B$ receptors. While the latter is thought to be linked by a pertussis toxin-sensitive G protein to K+ channels and adenylate cyclase (Bowery, 1989), the former is

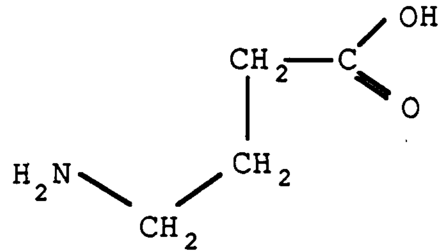
a possible target for epileptogenic compounds such as pyrethroids. The $GABA_A$ recognition site is part of a transmembrane protein composed of several subunits which form an anion channel that is ligand-gated. Following binding of GABA, the anion channel opens and chloride ions flow down their concentration gradient, producing a hyperpolarization. Other agonists at this site include muscimol and THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) while bicuculline acts as a competitive antagonist (Fig. 1-2). Bicuculline-sensitive GABA binding has been detected in the brains of vertebrates ranging from the primitive hagfish to mammals but is not present in invertebrate nervous tissue (Mann and Enna, 1980). Molecular biological analysis has shown that binding sites for GABA are present on both α (Mr 53K) and β (Mr 58K) subunits of the $GABA_A$ receptor. The stoichiometry of the subunits of the $GABA_A$ receptor remains to be defined.

The pharmacology of the $GABA_A$ receptor is rich, as evidenced by the numerous binding sites present on this protein which interact allosterically, influencing binding to other sites and the functioning of the chloride channel. The central benzodiazepine receptor (CBR) is the site of action for positive allosteric modulators such as diazepam, clonazepam and flunitrazepam (Braestrup and Squires, 1977; Mohler and Okada, 1977). These compounds increase the affinity of GABA for the receptor (Braestrup et al., 1982) and increase the frequency of channel opening (Barker et al, 1984). This enhancement is reciprocal; GABA enhances the affinity of positive benzodiazepine modulators for their binding site (Skerritt et al., 1983). Negative modulators such as β-carbolines decrease the affinity of GABA for its receptor;

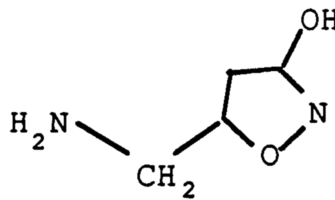
Figure 1-2. Chemical structures of ligands for sites on the $GABA_A$ receptor.

Agonists:

GABA



Muscimol



Competitive antagonist:

Bicuculline

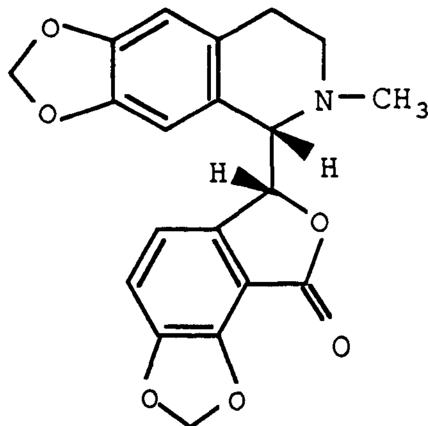
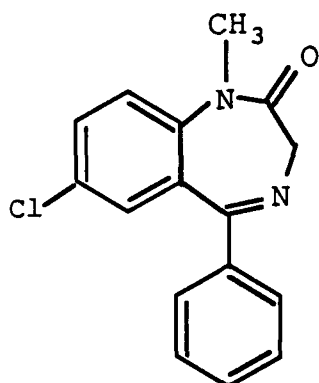
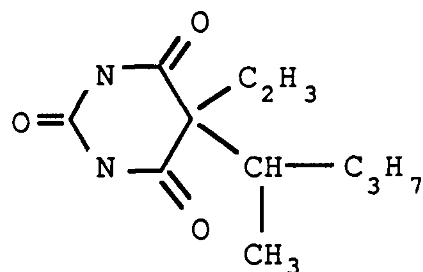


Figure 1-2 continued.

Allosteric Enhancers:

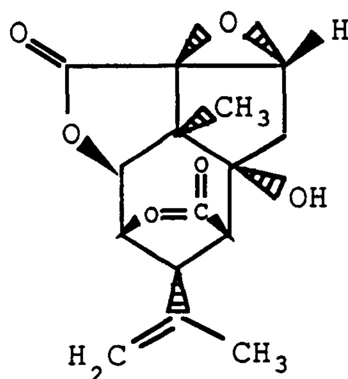


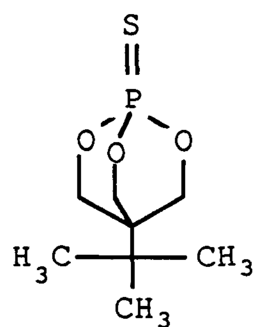


Diazepam

Pentobarbital

Uncompetitive Inhibitors:





Picrotoxinin

(PTX)

t-Butylbicyclophosphorothionate

(TBPS)

antagonists such as flumazenil (Ro15-1788) block the activity of both positive and negative modulators. These compounds are anxiolytic and anticonvulsant, anxiogenic, and without effect, respectively, when administered *in vivo*. Functional expression of benzodiazepine (BZ) ligands has been shown to require the presence of the γ-subunit in addition to the α and β subunits of the $GABA_A$ receptor (Puia et al., 1989). BZ receptors have been detected in rainbow trout brain with similar density, affinity for BZ positive modulators, and modulation by GABA as in rodent brain (Wilkinson et al., 1983).

A distinct site, associated with the anion channel, binds picrotoxinin (PTX) and t-butylbicyclophosphorothionate (TBPS). PTX is a polycyclic compound that is the toxic component of picrotoxin, which is found in the seeds of several plants. This toxin blocks anion flux and is a convulsant. TBPS, a cage convulsant, blocks anion movement and has higher affinity for the channel site than PTX (Squires et al., 1983). The negative allosteric interactions between PTX or TBPS and GABA agonists are complex. TBPS equilibrium binding is inhibited in the presence of GABA agonists. This is in contrast to noncompetitive inhibitors at other neurotransmitter-regulated ion channels, in which binding increases upon application of agonist and opening of the channels. Examples of this latter relationship include increases of phencyclidine binding at the nicotinic acetylcholine receptor in response to applications of carbamylcholine (Oswald et al., 1984) or MK 801 binding at the glutamate NMDA receptor in response to applications of NMDA receptor agonists (Ransom and Stec, 1988).

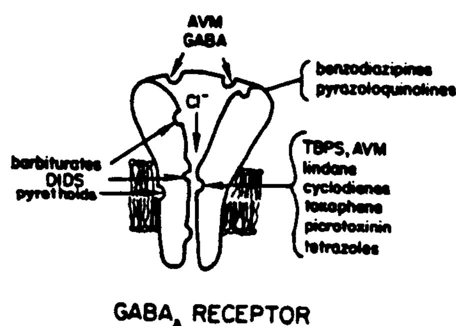
Early studies with the lobster leg opener muscle indicated that picrotoxin inhibited GABA-dependent responses in a "mixed-antagonism" manner- i.e. decreasing the potency of GABA as well as the maximum effect elicited. The authors suggested that this effect was due to a reciprocal effect of GABA in changing the affinity of PTX (Constanti, 1978). Later analysis with the same biological system suggested that PTX may stabilize the closed form of the activated channel (Smart and Constanti, 1986). Kinetic analysis of GABA modulation of TBPS binding revealed that GABA accelerated association and dissociation rates of TBPS binding with a greater effect on dissociation (Maksay and Simonyi, 1986). The authors suggested that this effect was caused by a shift to a more easily accessible open form of the channel in the presence of GABA agonists, while in the absence of GABA agonists the closed form of the channel resulted in slower binding of TBPS but a higher amount at equilibrium.

Following injection of chick brain mRNA into Xenopus oocytes and subsequent translation of $GABA_A$ receptors, TBPS effects on GABA-evoked current were studied using the voltage-clamp technique. Onset and recovery of TBPS block of current were stimulated in the presence of GABA while the degree of antagonism by TBPS decreased with increasing concentrations of GABA, supporting the contention that TBPS stabilized a closed form of the liganded receptor-channel complex. TBPS, like PTX, produced a mixed inhibition of GABA-dependent current; the maximal current evoked by GABA and the potency of GABA were reduced in the presence of TBPS (Van Renterghen et al., 1987; Barnard et al., 1987a).

Both PTX and TBPS act as convulsants due to the blockade of the chloride channel. Insecticides which interact with this site competitively, as measured by inhibition of [^{35}S]TBPS binding, include polychlorocycloalkanes such as endrin, dieldrin, heptaclor epoxide, and lindane (Lawrence and Casida, 1984).

A barbiturate binding site appears to be associated with the channel and interacts allosterically with, but is distinct from, the TBPS site. The existence of separate sites was established by the ability of low concentrations of phenobarbital to increase TBPS binding (Honore and Drejer, 1985). Agonists for this site cause a prolongation of the GABA channel open time and up to a 6-fold increase in potency and binding of GABA agonists (reviewed in Simmonds and Turner, 1987). A possible model for the $GABA_A$ receptor, including the various binding sites, is shown in Figure 1-3.

Figure 1-3. A model of the $GABA_A$ receptor.



(from Eldefrawi and Eldefrawi, 1987)

Research results over the last ten years have indicated that the $GABA_A$ receptor may be a target in pyrethroid toxicity and that pyrethroids may affect the allosteric interactions of the other binding sites. The primary site of action for pyrethroids has been shown to be in the central nervous systems of mammals; direct injection of cypermethrin, deltamethrin, permethrin and other pyrethroids into the ventricles of the brain produced many of the signs of toxicity, displayed increased potency as compared to i.v. administration, and retained the high stereospecificity of toxicity (Lawrence and Casida, 1982; Staatz et al., 1982). Consistent with this finding, signs of toxicity for type II pyrethroids were delayed by diazepam, a central benzodiazepine agonist (Gammon et al., 1982). The poisoning symptoms elicited by type II pyrethroids resemble those caused by GABA antagonists such as picrotoxin and other cage convulsants (Lawrence et al., 1985).

These facts have led to investigations in recent years concerning the interaction of type II pyrethroids with the GABA-receptor/Cl^- channel complex. Interaction of pyrethroids with this complex could alter the ability of the major inhibitory neurotransmitter system to control the excitability of brain neurons and therefore could underlie the neurotoxic effects of these insecticides.

In 1983, Lawrence and Casida reported the ability of Type II pyrethroids, such as cypermethrin and deltamethrin, to displace [35S]TBPS, a radioligand specific for the chloride ionophore of the $GABA_A$ receptor complex (Squires et al., 1983). The displacement occurred in a stereospecific manner, and studies with 37 pyrethroids revealed an absolute correlation with no false negatives or

positives between *in vivo* toxicity and displacement: only toxic cyano compounds were able to displace [^{35}S]TBPS, not their nontoxic stereoisomers, and noncyano pyrethroids were much less potent. Maximum inhibition of the specific binding was 37%. Seifert and Casida (1985a) reported that GABA facilitated and increased the ability of cypermethrin to displace [35S]TBPS from water-dialyzed membranes. Maximal inhibition without GABA was 10-15%; in the presence of 5 μM GABA cypermethrin inhibited 83% of radioligand binding in rat brain membranes.

In contrast to the results obtained at the TBPS site, equivocal results of pyrethroid interaction with two other sites within the GABA-receptor chloride ionophore complex, the central benzodiazepine receptor and the GABA recognition site of this complex, have been reported. Crofton et. al. (1987) detected no displacement of ligands from these receptors by pyrethroids while Lummis et. al. (1987) reported a decrease in [3H]-diazepam binding and an increase in [3H]-muscimol ($GABA_A$ ligand) binding in the presence of deltamethrin.

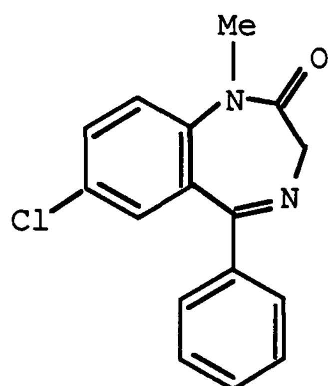
Binding studies alone are unable to define the effect of a compound on a physiological function. Binding of a compound to the $GABA_A$ receptor could increase or decrease the agonist-evoked anion movement. Measurement of the modulation of chloride flux assesses the functional coupling of $GABA_A$ receptors to their associated chloride channel (Bloomquist et al., 1986). This functional response can be measured by several techniques with differing time domains.

Chloride flux due to GABA-receptor opening of a chloride channel has been measured in various brain preparations using $^{36}Cl^-$ as a measure of chloride

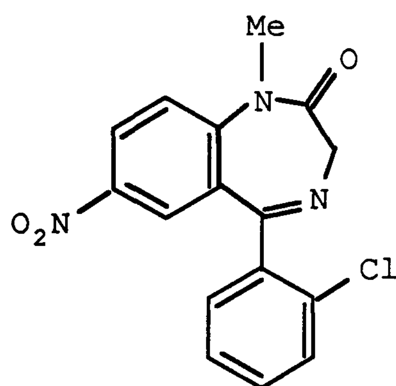
movement: in crayfish muscle strips, rat brain slices, embryonic chick neuronal cultures, and rat and mouse brain homogenates (reviewed by Allan and Harris, 1986). A brain homogenate preparation, referred to as synaptoneurosomes, has been widely used to study the effect of ligands of the various sites on the functional expression of the system. As a partial listing of the utility of this methodology, the biochemical assay has been used to measure the influence of a variety of compounds including alcohols (Allan and Harris, 1987), barbiturates (Schwartz et al., 1985), cage convulsants (Obata et al., 1988), and steroids (Morrow et al., 1987) in rodent brain membranes. Electron microscopic characterization of this system revealed a cell-free population of intact vesicles with pre-and postsynaptic profiles which were often attached (Allan and Harris, 1986). Guinea pig brain vesicles have been shown to establish a transmembrane potential of -58 to -78 mV at physiological K^+ concentrations (Creveling et al., 1980) which indicates that synaptoneurosomes establish normal chloride and sodium gradients.

Using $^{36}Cl^-$ uptake methodology, several groups have investigated the ability of pyrethroids to perturb this system in rodent membranes. Stereoselective inhibition of chloride flux by cypermethrin isomers and a greater potency of type II than type I pyrethroids has been measured in rodent synaptoneurosomes (Abalis et al, 1986; Ramadan et al, 1988). Bloomquist et al. (1986) measured a 50% inhibition of GABA dependent uptake by high concentrations of deltamethrin, but also observed less-potent inhibition by a non-toxic enantiomer. Deltamethrin and (1RαS)-*cis*-cypermethrin decreased GABA

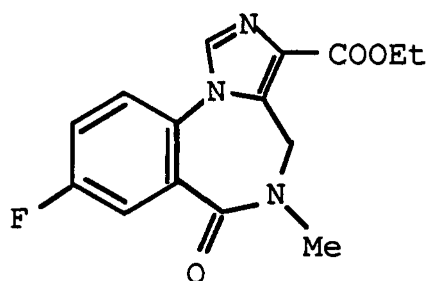
Figure 1-4. Chemical structures of CBR and PTBR ligands.



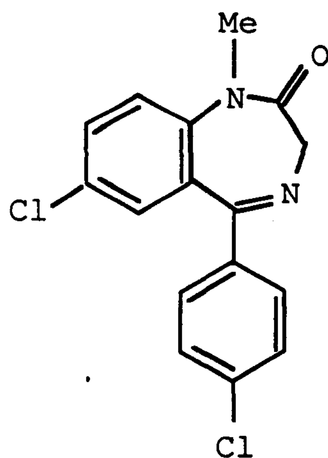
Diazepam



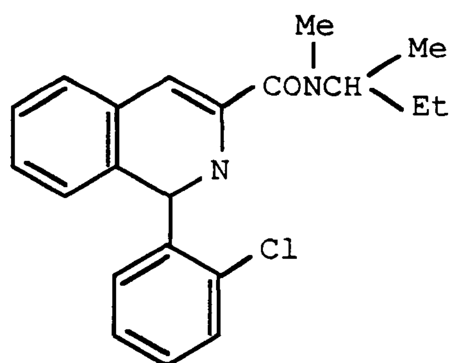
Clonazepam



Ro15-1788



Ro5-4864



PK 11195

dependent influx by more than 90% with potency in the nanomolar range (Devaud, 1988). These reports give support to an allosteric interaction of pyrethroids with the GABA site, although with conflicting assessments of stereospecificity.

Peripheral-type benzodiazepine receptor

Another site which may be relevant in understanding the mechanism underlying pyrethroid toxicity is the peripheral-type benzodiazepine receptor (PTBR). This site is distinct from the central benzodiazepine receptor (CBR) of the $GABA_A$ receptor, both in terms of biochemical properties and pharmacological profile. In rodents, the PTBR can be distinguished from the CBR by unique pharmacological profiles by use of Ro5-4864 (4'-chlorodiazepam) and PK 11195 (an isoquinoline carboxamide) which have high affinity for the PTBR in rodents and little activity at the CBR, and clonazepam, which has high affinity for the CBR and low affinity for the PTBR (Figure 1-3). Diazepam binds with relatively high affinity to both receptors. PTBR have been found in many tissues of mammals including brain, heart, lung, kidney, adrenal gland, and testis (Marangos et al., 1982). Ro5-4864 has been termed an agonist for this site and PK 11195, an isoquinoline carboxamide, an antagonist, originally based on thermodynamic studies of their binding kinetics (Le Fur et al., 1983) and more recently on physiological studies such as the ability of PK 11195 to reverse the proconflict action of Ro5-4864 (Mizoule et al., 1985), although this classification has recently been questioned (vide infra).

The physiological function and biochemical effects of ligands binding at the neuronal PTBR are being investigated. Clues to function can come from the elucidation of endogenous compounds which interact with a given receptor. Inhibitory activity from both high and low molecular weight components of blood and urine (Beaumont et al., 1983) indicated that an endogenous ligand exists for these sites. Recent work by Verma et. al. (1987) revealed that hemin and dicarboxylic porphyrins such as protoporphyrin IX are endogenous inhibitors of [3H]PK 11195 binding in both guinea pig brain membranes and rat kidney mitochondria. Protoporphyrin IX and PK 11195 have relatively constant affinities for PTBR in different organs and species, while Ro5-4864 has potencies which vary over several orders of magnitude (Verma and Snyder, 1988).

Numerous studies implicate an interaction of PTBR ligands with voltage-gated calcium channels. PK 11195 reversed the effects of three classes of calcium channel antagonists (phenylalkylamines, dihydropyridines, and benzothiazepines) on the transmembrane action potential of isolated guinea pig papillary muscle (Mestre, 1985). Both Ro5-4864 and PK 11195 inhibited the fast phase of $^{45}Ca^{2+}$ uptake in guinea pig cortical synaptosomes. Ro5-4864 blocked dihydropyridine induced release of β-endorphin from a pituitary tumor cell line which appeared to be due to a blockade of the calcium channel (Bisserbe et al., 1986). These results suggest an interaction of PTBR ligands with a cardiac and neuronal voltage dependent Ca^{2+} channel, although the enigma of this link is that all of these effects require micromolar concentrations, while DHP compounds and PTBR ligands have subnanomolar and nanomolar affinities for their receptors, respectively.

In vitro effects of Ro5-4864 include regulation of gene expression such as the induction of melanogenesis in B16/C3 melanoma cells (Matthew et al., 1981), inhibition of proliferation of mouse thymoma cells (Wang et al., 1984a), and induction of synthesis of hemoglobin in Friend erythroleukemia cells (Wang et al., 1984b), and physiological effects such as the promotion of human monocyte chemotaxis, which is inhibited by PK 11195 (Ruff et al., 1985). These receptors have also been shown to be regulated by various physiological changes. Downregulation of Ro5-4864 binding sites occurred *in vitro* after exposure of Friend erythroleukemia cells to PTBR ligands (Johnson et al., 1986) and *in vivo* in rat adrenal gland and testis after hypophysectomy (Anholt et al, 1985a) and in the pineal gland following either surgical cervical ganglionectomy or exposure to constant light to decrease neural input to the pineal gland (Weissman et al., 1984). An increase in the number of sites has been measured in the kidney following chronic progesterone treatment (Gavish et al., 1987), in the kidney and the olfactory bulb after acute swimming stress (Novas et al., 1987), in platelet membranes following chronic diazepam treatment of anxious patients (Weizman et al., 1987), in the brains of mice chronically exposed to ethanol (Syapin and Alkana, 1988) and in the hypothalamus and striatum after chemical sympathectomy (Basile and Skolnick, 1988). These multiple effects and the dynamic regulation of the number of sites strengthen the argument for a physiological function for these receptors that is common to many cell types.

The subcellular localization of the PTBR has been investigated. Anholt et. al. (1985b) studied the binding of [3H]Ro5-4864 to whole body sections of

neonatal rats and determined that high levels of binding occurred in tissues that derived their metabolic energy from oxidative phosphorylation while low levels were detected in tissues that derived energy from glycogenolysis. Histochemically visualized distribution of cytochrome oxidase activity overlapped the autoradiographic pattern of [3H]Ro5-4864 binding sites. In subcellular fractions, the highest levels of binding were found in the crude P_1 fraction of rat brain (Basile and Skolnick, 1986) and were present in the mitochondrial and synaptosomal fractions. [^{3}H]PK 11195 binding sites in the adrenal gland were distributed in a similar pattern to that of the mitochondrial marker enzymes, cytochrome oxidase (inner membrane) and monoamine oxidase (outer membrane)(Anholt et al., 1986a), which indicated an association with the mitochondrial compartment. Titration of isolated mitochondria with increasing concentrations of digitonin revealed that PTBR were co-released with monoamine oxidase activity, demonstrating that these receptors are associated with the mitochondrial outer membrane. PK 11195 binding sites subfractionated in a nearly identical pattern as the mitochondrial enzyme succinate dehydrogenase, but the density of binding was tissue dependent and regulated independently from other mitochondrial markers (Antkiewicz-Michaluk et al., 1988a).

Ligands for the PTBR affected mitochondrial respiration in well-coupled rat kidney mitochondria, including an increase in state IV (succinate added) and a decrease in state III (ADP added) respiration rates, which resulted in a decrease in the respiratory control ratio (the degree of coupling between

mitochondrial respiration and oxidative phosphorylation) (Hirsch et al., 1988). PTBR binding affinities and potencies at inhibiting respiratory control were correlated for these ligands, including Ro5-4864, PK 11195, and two porphyrins. PK 11195 and Ro5-4864 modulated mitochondrial activity in the same manner, indicating that the antagonist/agonist labelling of these compounds may not be accurate. The mechanism of uncoupling was not that of a classical uncoupler such as 2,4-dinitrophenyl because there was no dissipation of the inner mitochondrial membrane potential. Modulation of mitochondrial function would account for the effects of PTBR ligands on cell growth and differentiation listed above. Another possible interaction of PTBR ligands is with pyruvate dehydrogenase (Daval et al., 1989). This enzyme, located on the inner mitochondrial membrane, inhibited [^{3}H]Ro5-4864 binding. These results suggest that the endogenous role of PTBRs may involve actions of porphyrins linked with the outer mitochondrial membranes.

Solubilization and purification as well as cloning of the PTBR has been accomplished. The receptor's behavior during solubilization and reassembly suggested that it was an integral protein of the outer mitochondrial membrane (Anholt et al. 1986b). Photoaffinity labelling of the PTBR with PK 14105 resulted in this isoquinoline carboxamide covalently binding to an 18K Da membrane protein of cardiac membrane (Doble et al., 1987). Using the same photoaffinity probe and solubilizing with digitonin, the receptor was purified by ion exchange chromatography and reversed-phase HPLC and shown to have a M_r of 17K (Antkiewicz-Michaluk et al., 1988b).

Interactions of pyrethroids and PTBR ligands have been established. Pyrethroids have been shown to have potent proconvulsant activity in the rodent pentylenetetrazol (PTZ) seizure-threshold paradigm; that is, they significantly reduce the amount of PTZ required to elicit a seizure (Devaud et al, 1986; Devaud and Murray, 1988). PK 11195 inhibited this proconvulsant activity of pyrethroids. Pyrethroids also displaced [^{3}H]Ro5-4864 from rat brain membranes in a stereospecific manner with higher potency than they displaced [^{35}S]TBPS binding (Gammon and Sander, 1985; Lawrence et al., 1985; Devaud and Murray, 1988). In addition the IC_{50} values for pyrethroid inhibition of [^{3}H]Ro5-4864 binding were significantly correlated with the ED_{50} values for proconvulsant activity of both Type I and Type II pyrethroids. These results suggest an interaction of pyrethroids with a binding site of PTBR ligands.

Recent binding studies have indicated that PK 11195 and Ro5-4864 may interact with a subpopulation of $GABA_{A}$ receptors, which are termed the $GABA_{A3}$ receptors, as well as with the PTBR (Costa, 1988). This interaction has been measured by [^{35}S]TBPS binding modulation in rodent membranes; the potency of PTBR ligands in this assay is several orders of magnitude lower than their interaction with the rodent PTBR but in the micromolar range (Gee et al, 1988). Thus pyrethroid interactions with these ligands may be at the PTBR or at the $GABA_{A3}$ receptor.

Bolger et.al.(1985) reported that there was no detectable high-affinity binding of [^{3}H]Ro5-4864 in the membranes of non-mammalian vertebrate brains,

including pigeon, lizard and fish. However, this did not preclude the possibility of high affinity binding of PK 11195 in these animals.

The research presented in this thesis was directed toward a greater understanding of the sites and mechanisms of action of pyrethroid insecticides. Rainbow trout were chosen as a model system both because of their sensitivity to the neurotoxic activity of these insecticides as well as providing a basis for phylogenetic comparisons to be drawn with the greater body of mammalian data available in the literature. The target proteins investigated, the PTBR and the $GABA_A$ receptor, were chosen based on published interactions of pyrethroids in mammalian systems.

Acknowledgement: The author acknowledges Thomas Jacobsen's contribution of the chemical structures presented in this chapter.

Chapter 2

Differential Binding Properties of the Peripheral-Type Benzodiazepine Ligands, [^{3}H]PK 11195 and [^{3}H]Ro5-4864, in Trout and Mouse Brain Membranes

Amy J. Eshleman and Thomas F. Murray

Abstract

High affinity binding sites for [^{3}H]PK 11195 have been detected in brain membranes of rainbow trout (*Oncorhynchus mykiss*) and mouse forebrain, where the densities of receptors were 1030 and 445 fmol/mg protein, respectively. Ro5-4864 (4'-chlorodiazepam) was 2200 fold less potent as a competitor of [^{3}H]PK 11195 binding in the piscine than the murine membranes. Investigation of regional distribution of these sites in trout yielded a rank order of density of spinal cord > olfactory bulb = optic tectum = rhombencephalon > cerebellum > telencephalon. This site in trout shared some of the characteristics of the peripheral-type benzodiazepine receptor (PTBR) (also known as the mitochondrial benzodiazepine receptor) in rodents, i.e. high affinity for PK 11195 and the endogenous ligand protoporphyrin IX, but was unique in the low affinity of Ro5-4864 (41 μM) and diazepam and the relatively high affinity of the calcium channel ligand diltiazem and two central benzodiazepine ligands CGS 8216 and CGS 9896. The differential affinity for the two prototypic PTBR ligands in trout is similar to that previously observed in calf and human brain

membranes. Structural differences for the trout sites are indicated by the relative inability of diethyl pyrocarbonate to modify histidine residues of the binding site in trout as compared with mouse membranes. Heterogeneity of binding of the two prototypic PTBR ligands in mouse brain membranes was indicated by additivity studies, equilibrium competition experiments and saturation isotherms, which together support the hypothesis that Ro5-4864 discriminates between two [^{3}H]PK 11195 binding sites having high (nanomolar) and low (micromolar) affinity, respectively.

Introduction

Benzodiazepines and related compounds interact with high affinity with two distinct receptors in mammalian CNS. The more-well defined receptor is the central benzodiazepine receptor (CBR) (Braestrup and Squires, 1977; Mohler and Okada, 1977) which is a component of the supramolecular complex of the γ-aminobutyric acid (GABA) receptor/chloride ionophore (Barnard et al., 1987a). It is through this receptor that the anxiolytic and anticonvulsant properties of CBR agonists have been shown to be mediated by enhancement of GABA-gated chloride conductance (Tallman and Gallager, 1985; Morrow and Paul, 1988). The second receptor has been termed the peripheral-type benzodiazepine receptor (PTBR), which has been detected in the membranes of several tissues of mammals including heart, lung, kidney and testis as well as brain (Marangos et al., 1982), in which it has a different regional distribution than CBR (Schoemaker et al., 1983; Benavides et.al., 1983). The two receptors in rodent brain can be distinguished by pharmacological profiles using Ro5-4864

(4'-chlorodiazepam), which has high affinity for only the PTBR, and clonazepam, with high affinity for the CBR and low affinity for the PTBR; diazepam binds with relatively high affinity to both sites (Marangos et al., 1982). Ro5-4864 has been termed an agonist for PTBR and PK 11195, an isoquinoline carboxamide, an antagonist, originally based on thermodynamic studies of their binding kinetics (Le Fur et al., 1983) and more recently on physiological studies such as the ability of PK 11195 to reverse the proconflict action of Ro5-4864 (Mizoule et al., 1985) or to inhibit the promotion of human monocyte chemotaxis by Ro5-4864 (Ruff et al., 1985), or to block the diuresis and increased renal PTBR density produced by Ro5-4864 (Basile et al., 1988).

The physiological functions and biochemical effects of ligands binding at the PTBR are being investigated. Numerous studies implicate an interaction of PTBR ligands with voltage-gated calcium channels (Cantor et al., 1984; Bender and Hertz, 1985; Mestre et al., 1986; Rampe and Triggle, 1987) and with anion transport (Lueddens and Skolnick, 1987; Basile et al., 1988). The cellular locus of a receptor protein and endogenous ligands also provide insights into function. In brain homogenates from rats, PTBRs are most abundant in the mitochondrial and synaptosomal fractions (Basile and Skolnick, 1986). In adrenal gland tissue, PTBRs fractionate with markers of the mitochondrial outer membranes (Anholt et al., 1986a) and have been solubilized from these membranes (Anholt et al., 1986b). This localization has resulted in another nomenclature for this receptor: the mitochondrial benzodiazepine receptor (Anholt et al., 1986b; Verma et al., 1987). PTBRs, as defined by [^{3}H]PK 11195 binding, were also enriched in the

mitochondrial fraction of human, cat, and rat brain membranes (Doble et al., 1987). Endogenous ligands for this site include hemin and dicarboxylic porphyrins such as protoporphyrin IX, which inhibit [^{3}H]PK 11195 binding in both guinea pig brain membranes and rat kidney mitochondria with nanomolar affinities (Verma et al., 1987).

Recent reports have indicated that the 2 prototypic PTBR ligands, Ro5-4864 and PK 11195, may not label identical populations of binding sites in all mammals: [^{3}H]PK 11195 labels 40 times the number of sites that [^{3}H]Ro5-4864 labels in bovine brain, and unlabelled PK 11195 is 50-200 times more potent than Ro5-4864 in inhibiting [^{3}H]PK 11195 binding in cat, calf and human brain (Awad and Gavish, 1987; Doble et al, 1987) and 7800 times more potent in bovine pineal gland (Basile et al., 1986). Sites labelled by these two ligands have been shown to be differentially influenced by several agents, including arachidonate (Skowronski et al., 1987; Beaumont et al., 1988), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Lueddens and Skolnick, 1987), and detergents such as Triton X-100, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and Tween 20 (Awad and Gavish, 1988), and by chemical modification by diethyl pyrocarbonate (DEPC) (Benavides et al., 1984; Skowronski et al., 1987), and phospholipase A_2 (Havoundjian et al., 1986). Densities of [^{3}H]Ro5-4864 binding sites also differ greatly across mammalian species. The cat and guinea pig brain have high densities, whereas mice, rats, dogs, and monkeys have low concentrations in brain (Cymerman et al., 1986); however, the two rodent species have abundant binding sites in their olfactory

bulbs. Human brains contain a low density of PTBRs when labelled by [^{3}H]Ro5-4864, which are distributed relatively homogeneously (Pazos et al., 1986). In contrast [^{3}H]PK 11195 binding sites in human brain are heterogeneously distributed and restricted to gray matter (Doble et al., 1987).

Bolger et al.(1985) have reported that there is no detectable high-affinity binding of [^{3}H]Ro5-4864 in the membranes of non-mammalian vertebrate brains, including pigeon, lizard or fish. However, this does not preclude the possibility of high affinity binding of [^{3}H]PK 11195 in these animals. The aim of the present study was to characterize the binding sites for [^{3}H]PK 11195 in rainbow trout brain membranes, to contrast these with the PTBR in murine brain, and to explore the heterogeneity of binding of these two prototypic PTBR ligands in the two species.

Methods

Rainbow trout (*Oncorhynchus mykiss*), weighing 200-300 g, were obtained from the Food Toxicology and Nutrition Laboratory of Oregon State University (Corvallis, OR, U.S.A.), where they were maintained on a 12-hour light/dark cycle and fed Oregon Test Diet. Male Swiss Webster mice were obtained from Simonsen Laboratories (Gilroy, CA, U.S.A.). The trout were killed by decapitation and the mice were killed by cervical dislocation. Whole brains from trout and forebrains from mice were removed, cleaned, rapidly frozen on solid dry ice and stored at -70° C until used. The ligand binding characteristics of mouse brains stored at -70°C were similar to those used immediately after

dissection.

For competition studies, either a P_2 membrane preparation or a total particulate fraction was used; these preparations gave equivalent results. The P_2 preparation was prepared by homogenizing the brains in 0.27M sucrose solution (100:1 vol/wt) with a Brinkman Polytron at a setting of 7 for 20 s. The homogenate was centrifuged at 1000 g for 10 min; the supernatant was then centrifuged at 16,000 g for 20 min. The pellet was resuspended in the assay buffer (50 mM Tris buffer, pH 7.8) with a Polytron as described above. The total particulate fraction was prepared by homogenizing the brains in 100:1 (vol/wt) assay buffer, centrifuging at 30,000 g for 20 min and resuspending in assay buffer.

All radioligand binding assays were conducted at 4°C. The assay volume was 2 ml, including 250 μl membrane preparation (trout, 100 μg protein/assay; mouse 200 μg protein/assay), displacer or solvent carrier, and [^{3}H]PK 11195. For competition studies, the [^{3}H]PK 11195 concentration was 0.8-1.2 nM. In the equilibrium saturation binding assay, 12 concentrations of [^{3}H]PK 11195 ranging from 0.5 to 11 nM were used. Non-specific binding was defined as that remaining after incubation with 10 μM PK 11195 and was typically $<10\%$ of total binding. All competitors except protoporphyrin IX and DIDS were dissolved in dimethyl sulfoxide and added in 5 μl aliquots; this concentration (0.25%) of dimethyl sulfoxide did not affect radioligand binding. DIDS was dissolved in 0.1 M NaOH or distilled water. Protoporphyrin IX was dissolved in 0.1 M Tris base, and serial dilutions were made from this stock with assay buffer such that the pH of the final assay was not affected. Incubations with this light-

sensitive compound were performed in a dark room under a safe light, which transmitted light only in the red portion of the spectrum.

Equilibrium binding reactions were incubated for 60-90 minutes at 4°C. Incubation was terminated by vacuum filtration with a Brandel Cell Harvester (Brandel, Gaithersburg, MD, U.S.A.) through polyethylenimine (0.5%)-soaked GF/B filters, which were washed four times with 4 ml each of ice-cold assay buffer. Filters were placed in vials, and Biocount scintillant (RPI, Mount Prospect, IL, U.S.A.) was added; samples were counted for radioactivity 6 h later in a Beckman LS 6800 counter at an efficiency of 53%.

Experiments using [^{3}H]Ro5-4864 as the radioligand followed essentially the same procedure as the protocol for [^{3}H]PK 11195, with specific binding defined as the difference of binding in the absence and presence of 10 μM PK 11195 and using concentrations of [^{3}H]Ro5-4864 ranging from 0.3 to 30 nM. For the additivity experiments, concentrations of radioligands were used to reach 93% saturation, based on their respective K_D values determined by saturation experiments and the equation

$$Y = [d^*]/([d^*] + K_D)$$

where Y is the fractional occupancy and [d*] is the concentration of radiolabelled drug in the assay.

Histidine residue modification of the PTBR was performed as described by Skowronski et al. (1987) with minor modifications. In brief, a washed P_2 fraction was prepared from rat cerebral cortex or trout brain membranes. These P_2 fractions were resuspended in 45:1 (vol/wt) 50 mM Tris-HCl, pH 6.0. DEPC was added in 10 μl of ethanol/ml membrane suspension, followed by a 5 min

incubation, except where noted otherwise. Following treatment, 10 volumes of 50mM Tris (pH 7.5) was added to stop the reaction, and the suspension was centrifuged at 30,000 g for 20 minutes. The resultant pellet was resuspended in assay buffer and used directly in binding assays.

Protein content was determined by the method of Lowry et al. (1951) following solubilization with 0.5 M NaOH, using bovine serum albumin as the standard.

Non-linear least squares curve fitting programs were used to analyze both competition and saturation data. For the former LIGAND (Munson and Rodbard, 1980) was used, with background counts always defined as that not displaceable by unlabelled 10 μM PK 11195; whereas for the latter Lundon I was used. To obtain estimates of parameters for two-site fits with the Ro5-4864 displacement data, Newfitsites 2 on the PROPHET computer system was used. Kinetic parameters were determined by linear regression. For comparison of means, Student's t test was used.

Materials: [^{3}H]PK 11195 (specific activity=37.6 Ci/mmol) was purchased from DuPont/NEN (Boston, MA, U.S.A.). Ro5-4864, Ro15-1788, and clonazepam was generously supplied by Dr. Peter Sorter (Hoffman-LaRoche, Inc., Nutley, NJ, U.S.A.). Unlabelled PK 11195 was a generous gift of Dr. G. Le Fur (Pharmuka Laboratories, Gennevilliers, France). The technical grade permethrin, nifedipine and deltamethrin were gifts from FMC Corporation (Princeton, NJ, U.S.A.), Pfizer (New Groton, CT, U.S.A.) and Dr. P. Foulhoux (Roussel Uclaf, Romainville, France), respectively. CGS 8216 and CGS 9896 were gifts from Ciba Geigy (Summit, NJ, U.S.A.). SITS (4-acetamido-4'-

isothiocyanostilbene-2,2'-disulfonic acid) was purchased from US Biochemical (Cleveland, OH, U.S.A.), DIDS from Calbiochem (La Jolla, CA, U.S.A.), verapamil and diltiazem from Research Biochemicals, Inc. (Natick, MA, U.S.A.), and the balance of the reagents from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Preliminary experiments with trout brain membranes using [3H]Ro5-4864 detected no high affinity binding, whether nonspecific binding was defined by 10 μM Ro5-4864 or 10 μM PK 11195. However, when [3H]PK 11195 was used as the radioligand probe and nonspecific binding was defined in the presence of 10 μM PK 11195, high affinity binding sites were detected. The IC_{50} for unlabelled PK 11195 in trout brain was 1.64 ± 0.44 nM (mean ± SEM) and in mouse brain membranes was 1.87 ± 1.26 nM. In contrast, when Ro5-4864 was used as the competitor, the IC_{50} in mouse membranes was 18.9 ± 5.8 nM while for trout the IC_{50} was 41 ± 5 μM. These species differences in the potency of Ro5-4864 as an inhibitor of [^{3}H]PK 11195 binding are illustrated in the displacement curves shown in Fig. 1. Thus, Ro5-4864 was 2200 times more potent in the mouse than in the trout as a displacer of [^{3}H]PK 11195 binding. Similarly, diazepam and clonazepam were 100 and 10 times more potent at the site in the murine brain, as shown in Table I.

Equilibrium saturation experiments were performed in brain membranes from both species. In the mouse, the density of [^{3}H]PK 11195 binding sites was measured to be 445 ± 14 fmol/mg of protein and the dissociation constant was

calculated to be 0.58 ± 0.07 nM (Fig. 2A), whereas in the trout, these parameters were, respectively, 1030 ± 24 fmol/mg of protein and 1.03 ± 0.08 nM (Fig. 2B). The linear Scatchard replots indicated one population of binding sites, which was confirmed by nonlinear regression analysis. The density of receptors in trout brain has been calculated to be approximately twice that in the mouse forebrain. These sites, while differing by 3.5 orders of magnitude in affinity for Ro5-4864, have similar affinity for PK 11195.

Kinetic experiments showed that the association of [^{3}H]PK 11195 with trout brain membranes was rapid, reaching equilibrium in 10-15 minutes with a k_{obs} of 0.29 min^{-1} (Fig. 3A). Dissociation was also relatively rapid, with k_{-11} of 0.121 min^{-1} and a $t_{1/2}$ for dissociation of 2.4 min (Fig. 3B). The K_D for [^{3}H]PK 11195 in trout computed from kinetic data is 0.68 nM, which is in excellent agreement with that derived from equilibrium saturation studies.

The regional distribution of these recognition sites in trout brain is shown in Fig. 4. The density was highest in the spinal cord, lowest in the telencephalon and cerebellum, and approximately equal in the olfactory bulb, optic tectum and rhombencephalon. Thus, it appears that these binding sites are heterogeneously distributed in the piscine brain.

To characterize further the pharmacological profiles of [^{3}H]PK 11195 binding sites in the two species, we compared the potencies of an array of compounds as inhibitors of [^{3}H]PK 11195 binding. The CBR antagonist Ro15-1788 had low potency in trout and no effect in the mouse. The pyrazoloquinolines, CGS 9896 and CGS 8216, with agonist and antagonist

activity, respectively, at the CBR in the rat brain (Petrack et al., 1983), showed efficacy only in the trout, with respective IC_{50} values of 1.1 and 4.0 μM. Thus, the rank order of potency for CBR and PTBR ligands in mouse brain membranes was PK 11195 > Ro5-4864 > diazepam > clonazepam > Ro15-1788 = CGS 8216 = CGS 9896, which is consonant with previously reported pharmacological profiles for the PTBR in rodents. The profile for these ligands was strikingly different in the trout: PK 11195 > CGS 9896 > CGS 8216 > Ro5-4864 > Ro15-1788 > diazepam > clonazepam (see Table 1).

The benzothiazepine calcium channel antagonist diltiazem had low micromolar affinity in the trout only and was able to displace 93% of the specific binding. Two other structurally dissimilar calcium channel antagonists- nifedipine, a dihydropyridine, and verapamil, a phenylalkylamine- were both ineffective at displacing the radioligand. The glycine receptor antagonist strychnine was ineffective as a competitor in either species, as were the chloride channel blockers SITS and DIDS.

The ability of a representative type I and type II pyrethroid insecticide to interact with this site was investigated. The type I pyrethroid permethrin displayed similar potency in both species, with IC_{50} values of 10.3 and 13 μM in the trout and mouse, respectively, whereas the type II pyrethroid deltamethrin had no efficacy in the trout and an IC_{50} of 440 nM in the mouse. The limited aqueous solubility of these compounds precluded using concentrations of >30 μM.

The most potent inhibitor of [^{3}H]PK 11195 binding in trout is protoporphyrin IX (Fig. 1). This compound had an IC_{50} of 5 nM in the trout

and 149 nM in the mouse. Thus, this compound was 30-60 times more potent in the trout brain under these conditions; however, it is possible that differences in the content of endogenous heme-containing compounds decreased the potency of the exogenously added protoporphyrin IX in the mouse membranes.

Modification of histidine residues by DEPC was performed in both the rat and the trout. In rat cerebral cortex membranes, DEPC decreased binding of 1 nM [^{3}H]PK 11195 in a dose-dependent manner with 72% inhibition at 2 mM, as shown in Table 2. In contrast to these results obtained with rat brain membranes, in the trout the maximal decrement in binding was 19.5% at 2 mM DEPC, which was not increased by prolonging the preincubation with DEPC from 5 to 30 minutes. This low level of susceptibility precluded the possibility of protection experiments in trout brain membranes using unlabelled Ro5-4864 co-incubated with DEPC.

Given the extensive evidence for differences in the binding properties of PK 11195 and Ro5-4864 in trout brain membranes, it was of interest to investigate more thoroughly the sites labelled by these compounds in rodent brain, which have been reported to be similar and/or overlapping. Simultaneous analysis of pooled data from three competition experiments for Ro5-4864 displacement of [3H]PK 11195 in the mouse brain indicated that a two-site model best described these data (Table 3). This is in agreement with results previously reported in the rat and guinea pig brain (Awad and Gavish, 1987). These curves were composed of a high affinity site (IC_{50} = 11.5 nM) making up 87% of the binding, and a lower affinity site (46 μM), making up the remaining 13%. This lower affinity site was similar to the potency of Ro5-4864 in the trout

brain.

Additional support for the contention that these ligands do not label an identical population of binding sites was provided by the following additivity experiments performed in parallel using identical incubation conditions and tissue preparations. [^{3}H]PK 11195 and [^{3}H]Ro5-4864 were diluted with unlabelled PK 11195 and Ro5-4864, respectively, to a final specific activity of 35.7 Ci/mmol. Concentrations of [^{3}H]PK 11195 (8.3 nM) and [^{3}H]Ro5-4864 (32.9 nM) were chosen to produce identical fractional occupancies (93%) (see Methods for the calculation). In this series of experiments, the respective K_D values were determined to be 0.6 and 2.6 nM. At the concentrations used, both [^{3}H]PK 11195 and [^{3}H]Ro5-4864 were ~13-14-fold above their respective K_D values. [^{3}H]PK 11195 and [^{3}H]Ro5-4864, either alone or in combination, were then used to label sites in mouse forebrain. As summarized in Table 4, [^{3}H]Ro5-4864 labelled only a fraction (61%) of the total population of sites labelled by [^{3}H]PK 11195 ($P<0.005$). The binding of [^{3}H]PK 11195 and [^{3}H]Ro5-4864 in combination was not additive, and the number of sites labelled did not differ significantly from the number of sites labelled by [^{3}H]PK 11195 alone (Table 4).

These results were corroborated by equilibrium saturation experiments with each ligand. For [^{3}H]PK 11195, the density in mouse brain was computed to be 445 ± 14 fmol/mg of protein (Fig. 2A), whereas for [^{3}H]Ro5-4864 the density was computed to be 308 ± 27 fmol/mg of protein ($n=3$) (Fig. 5), a result confirming that the number of high- affinity sites for [^{3}H]PK 11195 is greater in this species. The calculated densities are also in excellent agreement with the

amount of ligand bound in the experiment demonstrating the nonadditivity of binding: 487 and 306 fmol/mg of protein for [^{3}H]PK 11195 and [^{3}H]Ro5-4864, respectively.

Discussion

The definition of a PTBR is critical to the ensuing discussion. If one accepts that these sites can be characterized by high affinity binding of <u>either</u> [^{3}H]Ro5-4864 or [^{3}H]PK 11195 (Le Fur et al., 1983), then the trout has abundant PTBRs distributed heterogeneously throughout its brain, in contrast to a previous report claiming a late evolutionary appearance of these receptors when defined only by the binding of [^{3}H]Ro5-4864 (Bolger et al., 1985).

This site/receptor in trout is characterized by low nanomolar displacement of [^{3}H]PK 11195 by unlabelled PK 11195. However, it does not have the same rank order potency for CBR and PTBR ligands as that of rodents. The rodent site is characterized by a rank order of potency of Ro5-4864 > diazepam > clonazepam > Ro15-1788 = CGS 8216 = CGS 9896 with the affinity for the first two compounds in the low and high nanomolar range, respectively. The trout profile is CGS 9896 > CGS 8216 > Ro5-4864 > Ro15-1788 > diazepam > clonazepam. The low affinity of Ro5-4864 indicates that this site may be more similar to the bovine or human brain [^{3}H]PK 11195 binding sites (Awad and Gavish, 1987; Doble et al., 1987) than to the rodent site, because the affinity of Ro5-4864 is ~200 nM and 10 μM for the PTBR in human and bovine tissue, respectively. Another dissimilarity from rodent is the inability of DEPC to modify histidine residues which are critical for binding in

the trout brain.

Interactions of rodent PTBR ligands with the function of voltage-gated calcium channels have been reported. PK 11195 reversed the effects of the calcium channel antagonists verapamil, diltiazem, and nitrendipine as well as the agonist BAY K 8644 on the transmembrane action potential of isolated guinea pig papillary muscle (Mestre et al.,1985) and reduced by half the maximum contraction induced by the dihydropyridine agonist in rabbit aorta (Mestre et al., 1986). PK 11195 and Ro5-4864 both inhibited the fast phase of $^{45}Ca^{2+}$ uptake in guinea pig cortical synaptosomes (Rampe and Triggle, 1987). Nitrendipine displaced [^{3}H]Ro5-4864 from rat brain membranes with low micromolar potency (Cantor et al., 1984). These results suggest that the PTBR ligands interact with a cardiac and a neuronal voltage-dependent calcium channel.

We therefore investigated the ability of three structurally dissimilar calcium channel antagonists to interact with the PTBR in murine and piscine brain. Our results indicated that neither a dihydropyridine nor a phenylalkylamine calcium channel ligand was able to displace [^{3}H]PK 11195 in either species. In contrast, diltiazem had low micromolar affinity for the site in trout. Previous studies have shown that PK 11195 and diltiazem were able to increase the affinity of nitrendipine for its binding site in rodent synaptosomes in a similar manner (Boles et al., 1984; Rampe and Triggle, 1987), although the effects were not identical, as the diltiazem effect was temperature dependent, whereas the enhancement by PK 11195 was not. It has been suggested (Rampe and Triggle, 1987) that structural similarity exists between the two compounds

and that PK 11195 may interact with the diltiazem site associated with the calcium channel. In our assay, diltiazem may interact with the PK 11195 site also because of similarity of structure. Dihydropyridine-sensitive binding sites do exist in piscine brain (Bolger et al., 1986), hence, on the basis of our results, one cannot definitively state the locus of interaction of these two compounds.

Because the regional distribution studies indicated that the highest density of binding sites was in the spinal cord, we attempted displacement with the glycine receptor antagonist strychnine, as this receptor has been shown to be abundant in mammalian spinal cord (reviewed by Betz, 1987). We also used the chloride transport blockers SITS and DIDS, which interact with both receptor-gated and anion-exchange channels (Cabantchik et al., 1978; Schwartz et al., 1986). Previous work has shown that DIDS was able to inhibit binding of [^{3}H]Ro5-4864 but not [^{3}H]PK 11195 in rat kidney membranes (Lueddens and Skolnick, 1987). None of these compounds was able to displace [^{3}H]PK 11195 in trout brain.

Pyrethroid insecticides have been shown to have proconvulsant actions in rats in the pentylenetetrazol seizure-threshold paradigm that are reversed by PK 11195 (Devaud et al., 1986; Devaud and Murray, 1988). The type I pyrethroid permethrin (technical grade) had low micromolar potency, whereas the type II pyrethroid deltamethrin was ineffective at displacing [^{3}H]PK 11195 in the trout. Thus, this binding site appears not to be involved in the exquisite sensitivity of rainbow trout to these insecticides (Glickman et al., 1982).

The compound which appears to have the highest affinity for this site in trout besides PK 11195 is protoporphyrin IX. This precursor to heme has an IC_{50} of 4.9 nM in trout, 200-300 fold more potent than any other compound tested. Thus, because this endogenous compound exhibits high affinity for this site, it may be more properly termed a high-affinity porphyrin receptor. This is similar to results reported by Verma et al. (1987) in guinea pig brain and rat adrenal cortex. These authors suggest that these rodent sites are outer mitochondrial membrane proteins that may be involved in the movement of porphyrins from the cytosol to the mitochondria. Whether these high-affinity sites are the locus for interaction with the voltage- gated calcium channels discussed earlier has still to be resolved, but this proposal seems unlikely due to the difference in cellular compartments of these two proteins and the difference in affinity and potency in the two systems.

Heterogeneity of the binding sites of the two prototypic PTBR ligands has been demonstrated through differential chemical modification of binding. DEPC modification of histidine residues of the PTBR in kidney membranes caused a decrease in the number of binding sites of [^{3}H]PK 11195 with no change in affinity but had no effect on [^{3}H]Ro5-4864 binding; however, Ro5-4864 was able to protect the PK 11195 sites from modification (Benavides et al., 1984; Skowronski et al., 1987). Phospholipase A_2 pretreatment of membranes from rat cerebral cortex produced a dose-dependent decrease in [^{3}H]Ro5-4864 affinity with no change in density of binding sites but caused an increase in the number of binding sites of [^{3}H]PK 11195 (Havoundjian et al., 1986). Arachidonate

decreased the affinity of the PTBR of rat kidney mitochondrial membranes for Ro5-4864 without any changes in the affinity for PK 11195, whereas preincubation with dithiothreitol, a thiol reagent, resulted in inhibition of binding of both compounds. DIDS inhibited [^{3}H]Ro5-4864 binding up to 80% in rat kidney membranes but had no effect on [^{3}H]PK 11195 binding (Lueddens and Skolnick, 1987).

Our results support the proposal that Ro5-4864 and PK 11195 bind non-identical populations of sites in rodents. Using an investigative strategy that has been previously used to substantiate the contention that pirenzepine recognized a subset of muscarinic receptors labelled by [^{3}H]quinuclidinyl benzilate (Luthin and Wolfe, 1984), we have shown that at equal fractional occupancy (93%), significantly more sites are labelled by [^{3}H]PK 11195 than by [^{3}H]Ro5-4864. Moreover, the lack of additivity in binding observed when [^{3}H]Ro5-4846 and [^{3}H]PK 11195 were combined suggests that [^{3}H]Ro5-4864 labels a subset of [^{3}H]PK 11195 recognition sites in this species. The competition studies showed that Ro5-4864 has high affinity for most of the sites labelled by [^{3}H]PK 11195, but a population of sites exists with which it can interact only at micromolar concentrations. Comparison of the density of sites for the two radiolabelled ligands as determined by equilibrium saturation experiments indicated a higher density of sites for [^{3}H]PK 11195: 445 vs. 320 fmol/mg of protein.

The results in the trout indicate that it may be a good model species to study recognition sites for which Ro5-4864 exhibits only micromolar affinity, as we have found that Ro5-4864 stimulates t-[^{35}S]butylbicyclophosphorothionate

binding in micromolar concentrations (authors' unpublished data). Use of the trout for *in vivo* studies with Ro5-4864 would facilitate determination of whether high- or low-affinity sites are important in the proconvulsant and convulsant activities of this compound, because only low-affinity sites exist in this species. The similarities in affinity of the trout and human PTBR for the prototypic ligands indicate that the trout may be useful in probing the pharmacological and physiological significance of this receptor protein.

Acknowledgements

The excellent word processing assistance of Ms. Elaine Luttrull is gratefully acknowledged. This research was supported in part through NIEHS Center grant ESO3850 and by grant ESO4892. Permission to include this paper in the thesis was given by Raven Press.

Figure 2-1. Inhibition of [^{3}H]PK 11195 binding by Ro5-4864, PK 11195, and protoporphyrin IX in mouse forebrain and trout brain membranes. (Open symbols and solid symbols represent mouse forebrain and trout brain membrane results, respectively). Details of the binding assay are described in Materials and Methods. Data are from a representative experiment, repeated three to five times with similar results.

Figure 2-1.

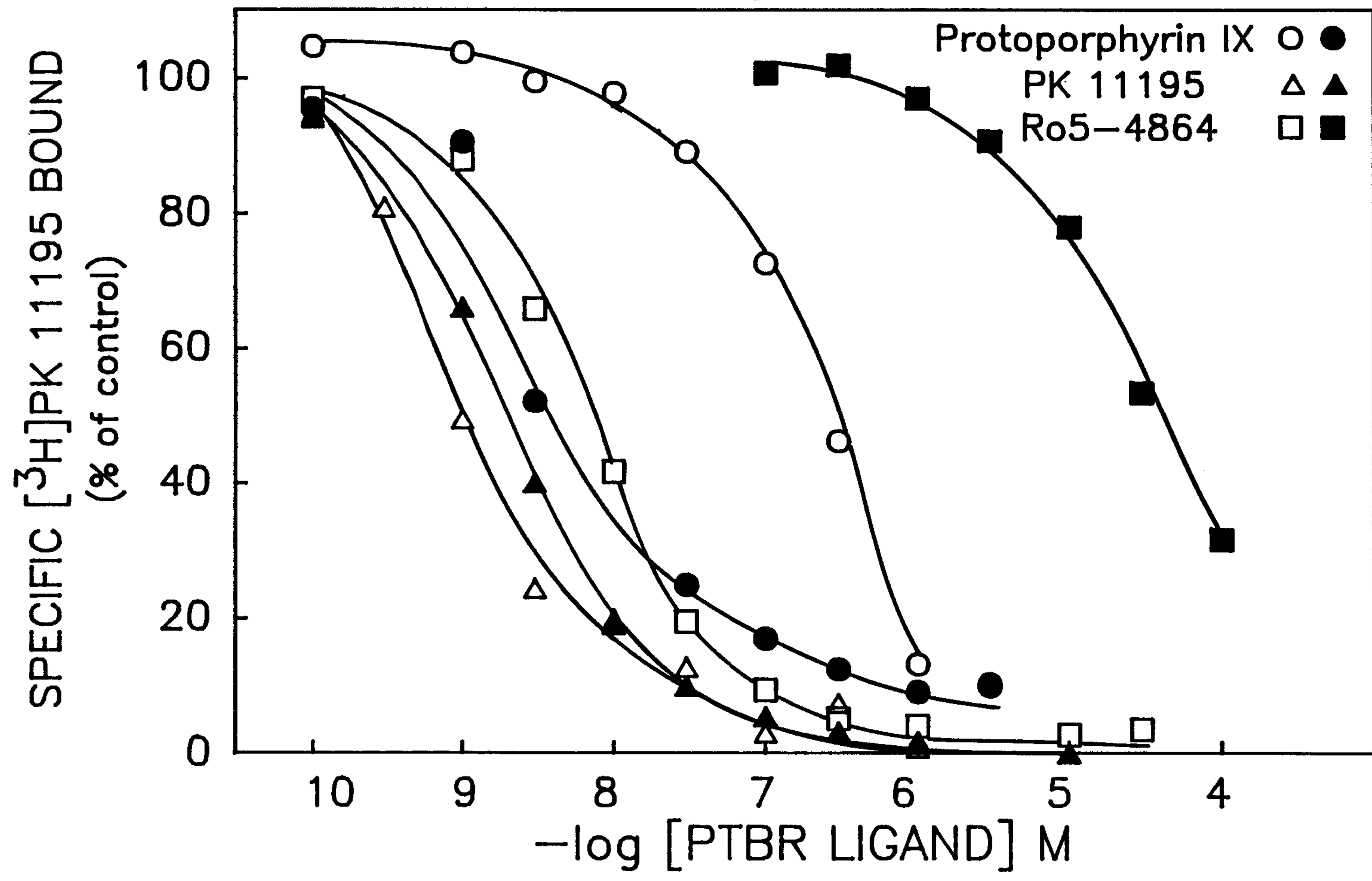


Figure 2-2. Saturation equilibrium binding of [^{3}H]PK 11195. A: Mouse forebrain. B: Trout brain. A total particulate membrane preparation was used. Data are the results of a representative experiment consisting of duplicate measurements, which was replicated with similar results. **Insets**: Scatchard plots of the same data.

Figure 2-2A.

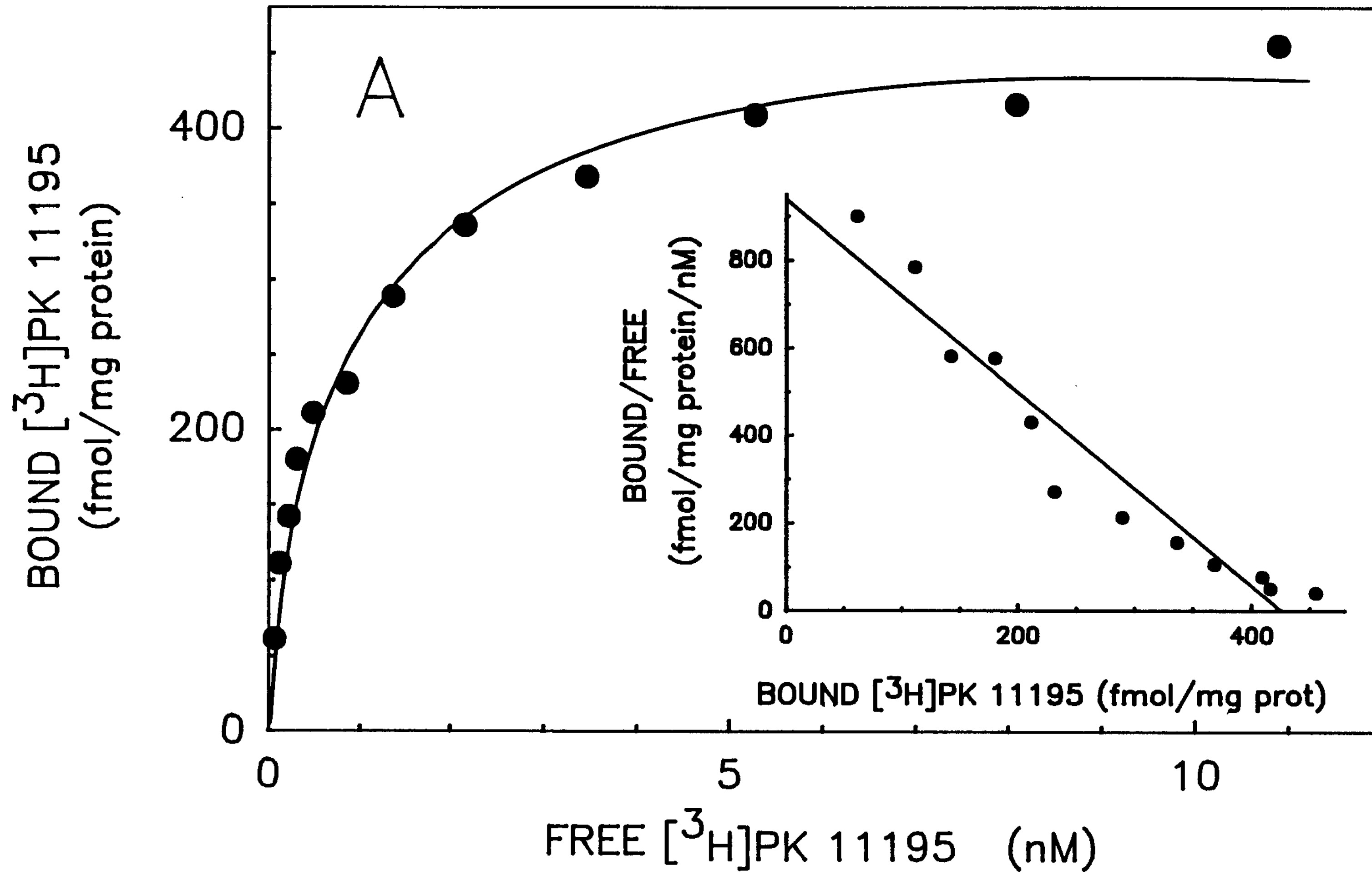


Figure 2-2B.

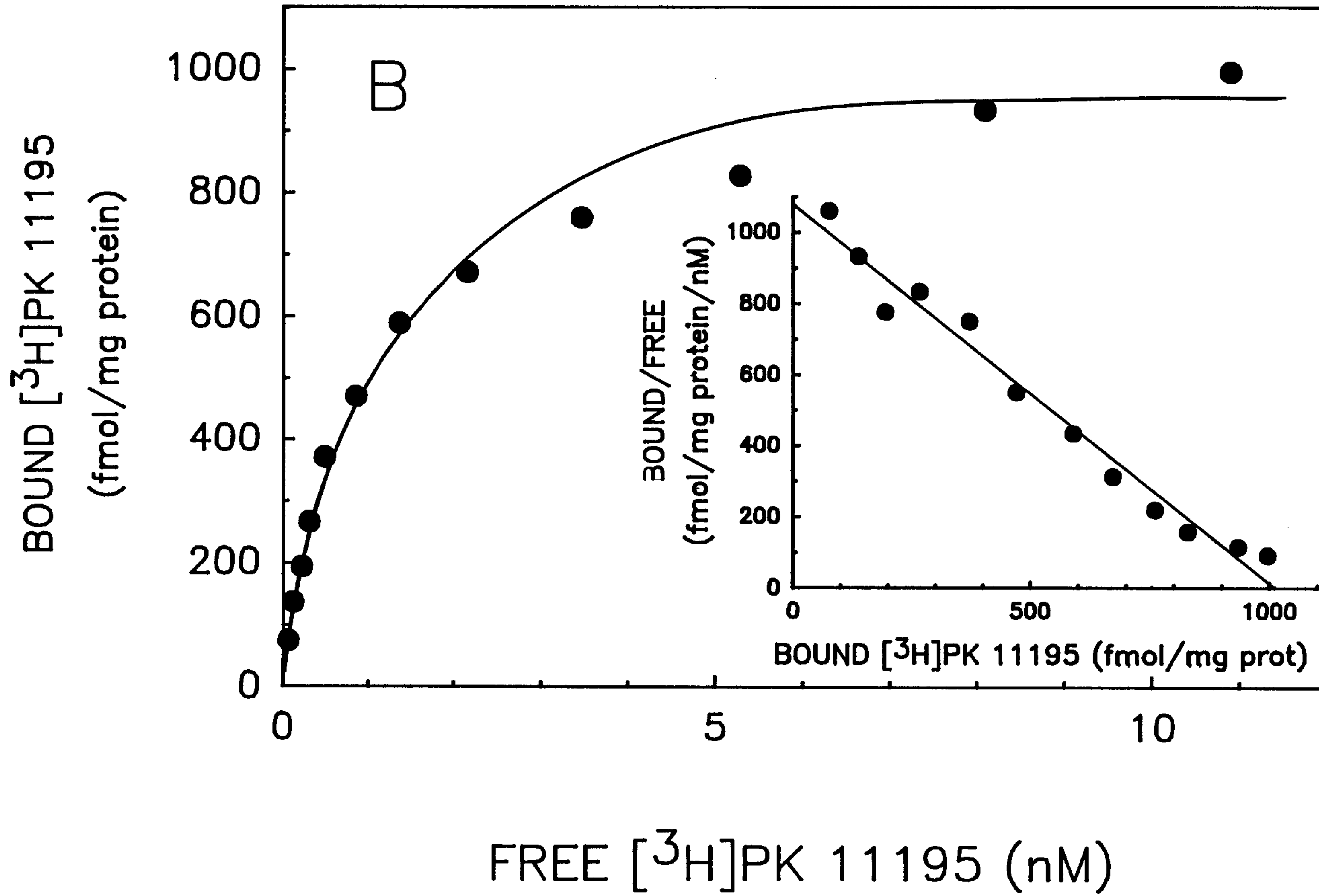


Figure 2-3. Time course of [^{3}H]PK 11195 binding to trout brain membranes. A: Association was measured by addition of 1 nM [^{3}H]PK 11195 to the incubation mixture at various time points. LR is the amount of [^{3}H]PK 11195 bound at a given time point, and LR_e is the amount bound at equilibrium. B: Dissociation. All membranes were preincubated with [^{3}H]PK 11195 for 90 min. before initiation of dissociation by addition of 10 μM PK 11195 at the indicated times. LR_0 is the amount of [^{3}H]PK 11195 bound at time 0 before addition of 10 μM PK 11195.

Figure 2-3A.

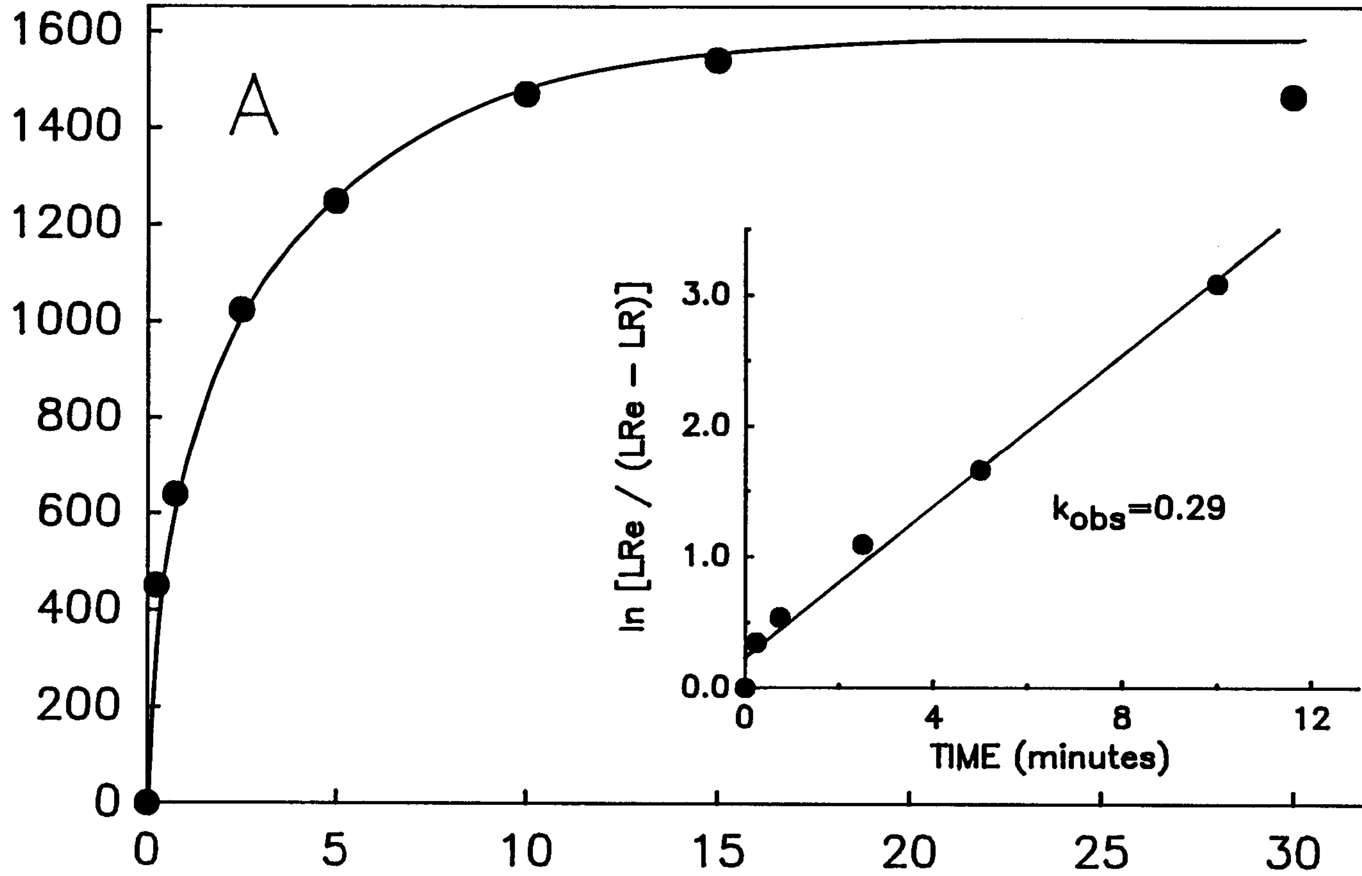


Figure 2-3B.

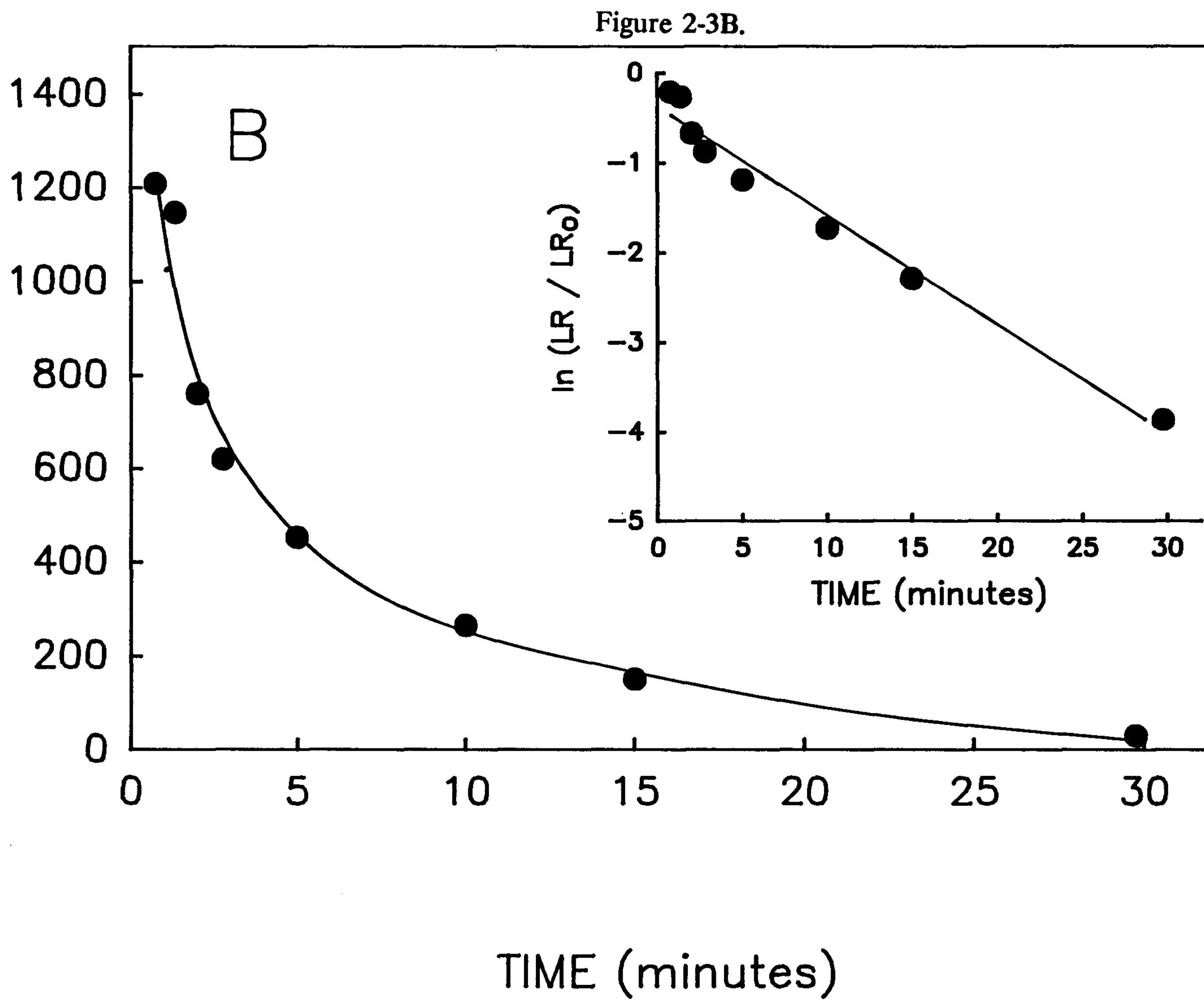


Figure 2-4. Regional distribution of density of binding sites of [^{3}H]PK 11195 in trout brain membranes. Total particulate membrane preparations were prepared from the indicated regions. Data are mean $\pm$ SEM (bars) values from three separate experiments, each of which consisted of quadruplicate measurements.

Figure 2-4.

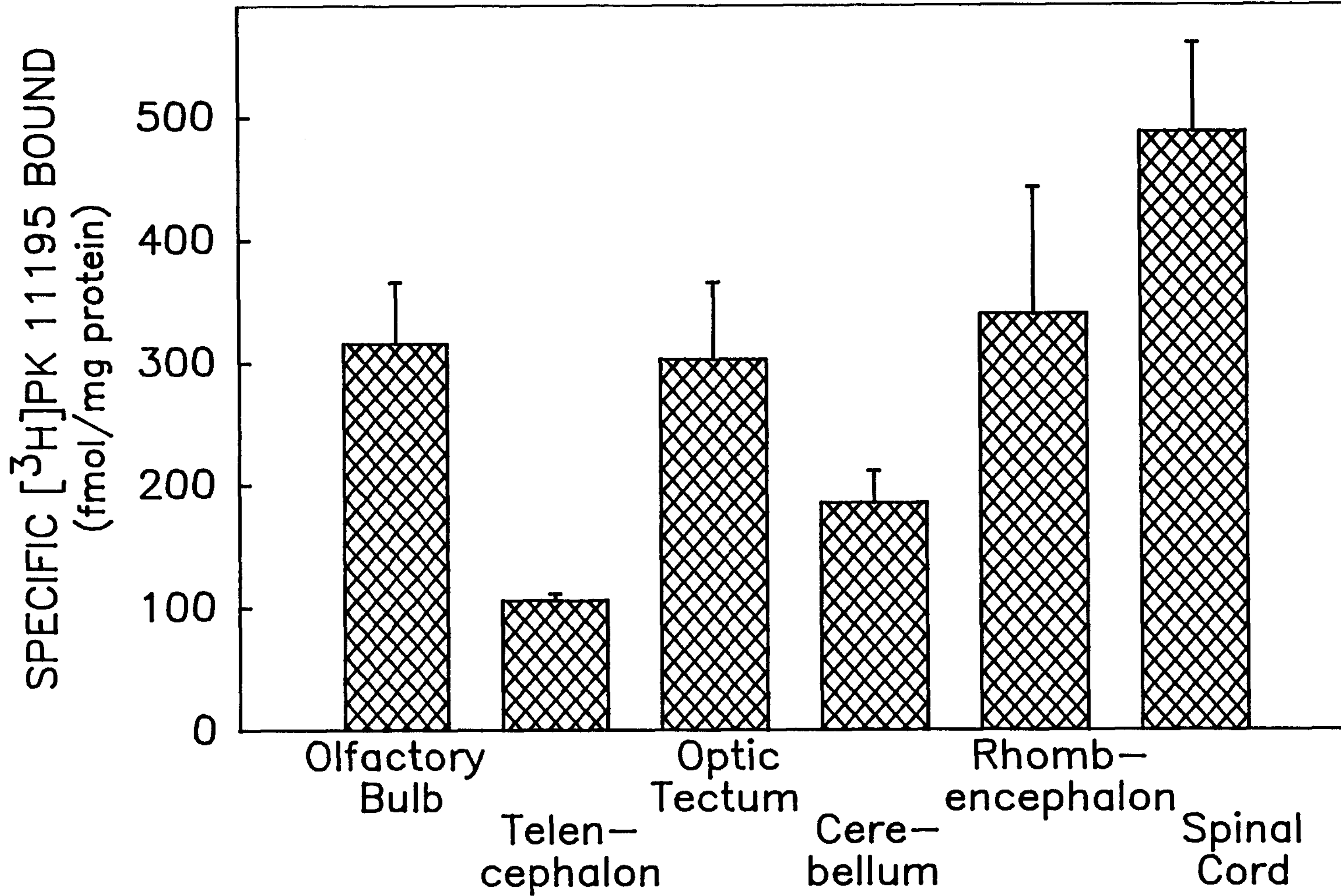


Figure 2-5. Saturation equilibrium binding of [^{3}H]Ro5-4864 in mouse forebrain membranes. Twelve concentrations of radioligand were used, ranging from 0.5 to 21.0 nM. Data are the results of duplicate measurements using a total particulate membrane preparation. The mean $\pm$ SEM values for the density of binding sites and the dissociation constant were determined to be 308 $\pm$ 27 fmol/mg of protein and 5.8 $\pm$ 1.6 nM. respectively (n=3). **Inset:** Scatchard plot of the same data.

Figure 2-5.

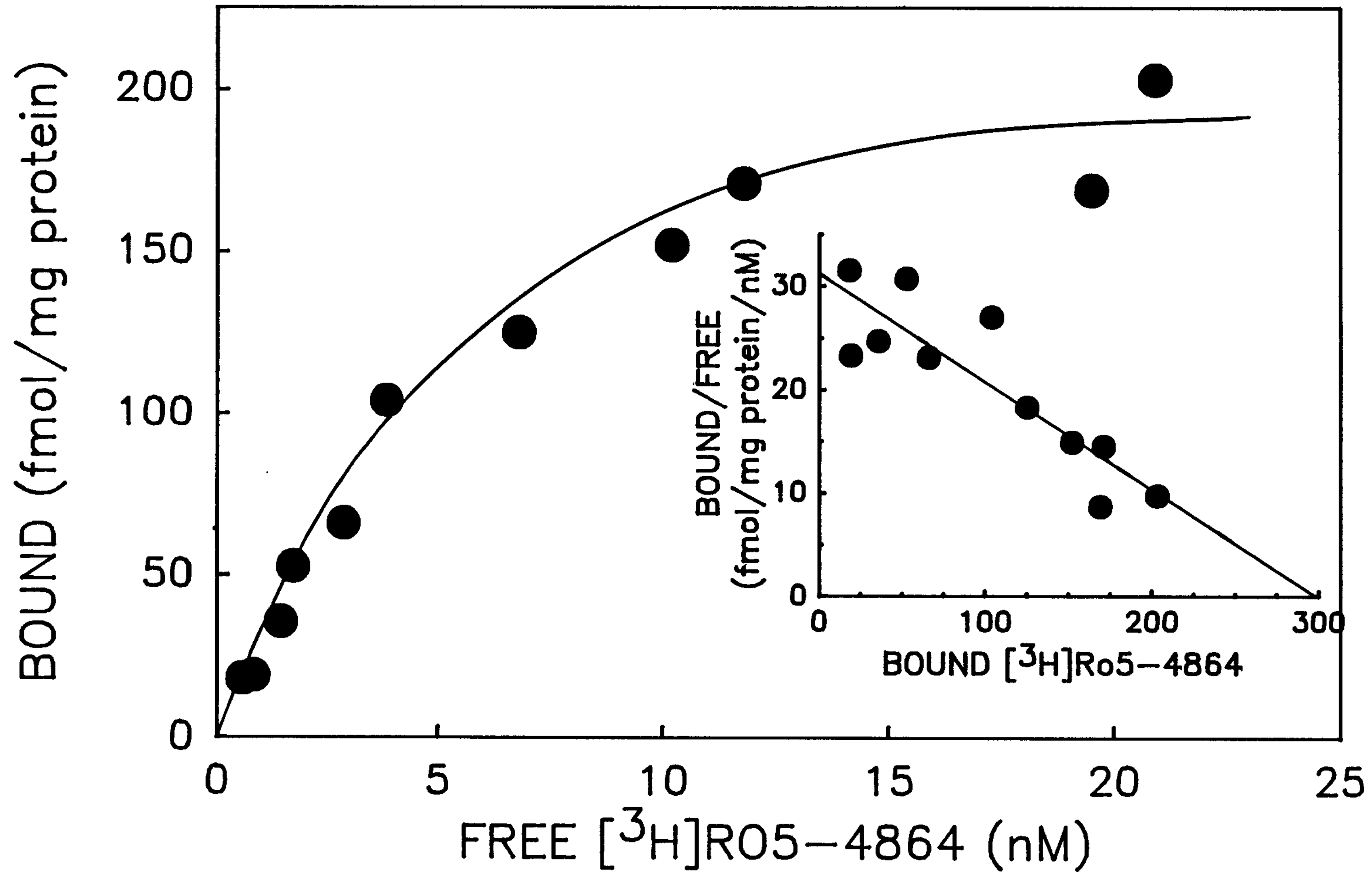


Table 2-1: Potency of PTBR, CBR, calcium channel ligands and pyrethroids as inhibitors of the specific binding of $[^3H]$PK 11195 to piscine and murine brain membranes. IC_{50} values and slope factors were determined by nonlinear least squares regression analysis as described under Methods. Values for the parameters are means +/- SEM of n experiments.

Compound	Trout IC_{50} ± s.e.m. (nM)	(n)	% maximum inhibition	slope factor	Mouse IC_{50} ± s.e.m. (nM)	(n)	% maximum inhibition	slope factor
PTBR ligands:								
PK 11195	1.64 ± 0.44	(4)	100	0.67 ± 0.14	1.66 ± 0.53	(5)	100	0.61 ± 0.07
RO5-4864	41,000 ± 5000	(3)	72	0.72 ± 0.1	18.9 ± 5.8*	(3)	95	0.79 ± 0.17
Protoporphyrin IX	4.9 ± 1.3	(3)	91	0.44 ± 0.04	149 ± 52	(3)	96	0.76 ± 0.16
CBR ligands:								
Diazepam	79,000 ± 29,	(3)	87	0.94 ± 0.09	655 ± 391	(3)	98	0.54 ± 0.08
Clonazepam	>250,000	(2)	48		19,000 ± 5,000	(2)	72	0.71 ± 0.03
CGS 9896	1,070 ± 670	(3)	78	0.61 ± 0.04	>10,000			
CGS 8216	4,000 ± 1,900	(3)	90	0.94 ± 0.03	>25,000			
RO15-1788	46,100 ± 2,800	(3)	93	1.13 ± 0.09	>250,000			
Pyrethroids								
Deltamethrin	>10,000	(3)			440 ± 220	(3)	69	0.81 ± 0.06
Permethrin	10,300 ± 6,200	(3)	55	0.48 ± 0.04	13,000 ± 10000	(2)	45	0.38 ± 0.03
Calcium Channel ligand:								
Diltiazem	10,100 ± 5,900	(3)	93	1.15 ± 0.24	>250,000			

*one site model

Compounds tested which showed no efficacy at displacement in either species include the calcium channel ligands nifedipine and verapamil, the glycine receptor antagonist strychnine and the chloride channel blockers SITS and DIDS.

Table 2-2: Inhibition of [^{3}H]PK 11195 binding by pretreatment with DEPC in rat cerebral cortex and trout brain.

	Decrease in specific binding(%)	
[DEPC] (mM)	Rat	Trout
0.5	52.1 ± 2.2	0
1.0	68.2 ± 6.6	12.7 ± 5.9
2.0	72.0 ± 0.64	19.6 ± 2.5

Results are expressed as percentage decreases from control value, which was defined as [^{3}H]PK 11195 binding measured following preincubation with 1% (vol/vol) ethanol for 5 minutes. Data presented are mean ± SEM values from a representative experiment preformed in triplicate, which was repeated twice with similar results.

Table 2-3: Parameter estimates for Ro5-4864 inhibition of [^{3}H]PK 11195 binding to mouse forebrain membranes

One Site		Two Site					
IC_{50} (M)	slope factor	IC_{50_1} (M)	$\%R_H$	IC_{50_2} (M)	$\%R_L$	F	P
1.89 X 10^{-8}	0.79	1.15 X 10^{-8}	86.5	4.6 X 10^{-5}	13.5	18.8	0.003

Parameters are derived from computer-assisted analysis of pooled data from 3 experiments as described in Methods.

Table 2-4: [^{3}H]PK 11195 and [^{3}H]Ro5-4864 binding in mouse forebrain membranes are nonadditive.

Additions	Specific labelled ligand bound (fmol/mg of protein)
[^{3}H]PK 11195 (8.3 nM)	487 ± 16
[^{3}H]Ro5-4864 (32.9 nM)	306 ± 12
[^{3}H]PK 11195 + [^{3}H]Ro5-4864	425 ± 45

The concentrations of radiolabelled ligands used were calculated to produce 93% saturation of the respective binding sites. Data are mean ± SEM values from quadruplicate determinations. The final assay concentrations of [^{3}H]PK 11195 and [^{3}H]Ro5-4864 were 8.3 nM and 32.9 nM, respectively.

Chapter 3

GABA Dependence of Pyrethroid and 4'-Chlorodiazepam Modulation of t-[^{35}S]Butylbicyclophosphorothionate Binding in Piscine Brain

Amy J. Eshleman and Thomas F. Murray

Abstract

t-[^{35}S]Butylbicyclophosphorothionate ([^{35}S]TBPS) binding sites were detected in well-washed trout brain membranes; γ-aminobutyric acid (GABA) acted as an uncompetitive inhibitor of [^{35}S]TBPS binding, decreasing both the number of binding sites and the affinity of TBPS. Inhibition of [^{35}S]TBPS binding by deltamethrin, a Type II pyrethroid, was modulated by GABA; both the affinity and the efficacy of this insecticide increased with incremental concentrations of GABA. Deltamethrin also enhanced the potency of GABA as an inhibitor of [^{35}S]TBPS binding. The interaction of 4'-chlorodiazepam (Ro5-4864) with [^{35}S]TBPS was dependent on GABA: in the absence of GABA Ro5-4864 inhibited up to 40% of binding, in the presence of 10 μM GABA Ro5-4864 enhanced binding to a maximum value of 170% of control. However, the same absolute amount of binding was observed with both these effects at micromolar concentrations of Ro5-4864. Ro5-4864 also caused a rightward shift in GABA dose-response curves, increasing the GABA IC_{50} value more than 6 fold. These results indicate the reciprocal allosteric interactions between a pyrethroid binding site, a Ro5-4864 binding site, the GABA recognition moiety and the TBPS

binding site in trout brain. The similarity of these findings to previous results in rodent brain preparations highlight the conservation of the modulation of $GABA_A$ receptor function during vertebrate evolution.

Introduction

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the CNS of vertebrates. The $GABA_A$ receptor is a transmembrane protein which forms an anion channel and contains binding sites for other compounds that affect the induced anion flow: benzodiazepines, barbiturates, halide ions, and picrotoxin and t-butylbicyclophosphorothionate (TBPS, a cage convulsant with higher affinity for the site than picrotoxin) (Barnard et al., 1987b). This receptor/ion channel complex has been shown to be the site of action for the mammalian neurotoxicity of several insecticides including endrin, endosulfan, dieldrin, and heptachlor epoxide (Abalis et al., 1986).

Pyrethroids are broad-spectrum insecticides which have been classified as either type I or type II compounds, depending on structural characteristics and signs of poisoning in insects and mammals (Gammon et al., 1981; Gray, 1985; Lawrence et al., 1985). A primary target for pyrethroid action has been identified as the neuronal sodium channel (Narahashi, 1985). However, signs of toxicity for type II, but not type I, pyrethroids can be delayed by diazepam, a positive allosteric modulator of $GABA_A$ receptors (Gammon et al., 1982). The poisoning symptoms elicited by these compounds resemble those caused by GABA antagonists such as picrotoxin and other cage convulsants (Lawrence et al, 1985). Type II pyrethroids such as cypermethrin and deltamethrin inhibited [^{35}S]TBPS

binding to rat brain membranes (Lawrence and Casida, 1983) and this inhibition was facilitated by GABA (Seifert and Casida, 1985a). These observations support the contention that, besides the sodium channel, an additional site of action at the $GABA_A$ receptor may be important for pyrethroid toxicity.

Fish are exquisitely sensitive to these insecticides: the brain levels of (1RαS)-*cis*-cypermethrin in trout required to produce signs of toxicity have been shown to be eight fold lower than those necessary to produce symptoms in the mouse (Edwards et al, 1986). Thus the trout is a useful model to investigate the mechanism of action of these insecticides. Signs of toxicity due to fenvalerate (Fig. 1-1) exposure in fish include tremors which progress to convulsions in which the head is held at a 30° angle from the body, opercular flaring, and a state of tetany (Bradbury et al., 1987). An interaction of pyrethroids with piscine [^{35}S]TBPS binding sites has been described, but limited inhibition of binding was observed under the experimental conditions employed (Cole et al., 1984).

A relationship between a site labelled by 4'-chlorodiazepam (Ro5-4864) and PK 11195, specific ligands for the peripheral-type benzodiazepine receptor (PTBR) of rodents, and pyrethroids is beginning to emerge. Pretreatment with PK 11195 antagonizes pyrethroid proconvulsant activity in the pentylenetetrazol-seizure threshold paradigm (Devaud et al., 1986). Moreover, type II pyrethroids displaced [3H]Ro5-4864 from rat brain membranes with mid-nanomolar potency (Devaud and Murray, 1988). However, in trout, neither Ro5-4864 nor type II pyrethroids have high affinity for the PTBR as labelled by [^{3}H]PK 11195 (Eshleman and Murray, 1989).

A distinct Ro5-4864 sensitive recognition site has been shown to be

associated with the $GABA_A$ receptor-chloride channel complex. Early reports indicated that Ro5-4864 inhibited $[^{35}S]$TBPS binding in a competitive manner with micromolar potency in dialyzed membranes (Weissmann et al., 1985; Ticku and Ramanjaneyulu, 1984). More recent results have emphasized the interaction of GABA and Ro5-4864 in the modulation of $[^{35}S]$TBPS binding (Gee, 1987). Moreover, one of the candidate endogenous ligands for the $GABA_A$ receptor, diazepam binding inhibitor (DBI) fragment 17-50, displaces $[^{3}H]$PK 11195 binding to synaptic membranes (Costa, 1988). Accordingly, the existence of a subtype of $GABA_A$ receptors with an allosteric modulatory site similar to that of the peripheral-type benzodiazepine receptor has been proposed (Costa, 1988). Although no high affinity binding sites for $[^{3}H]$Ro5-4864 have been measured in trout (Bolger et al., 1985; Eshleman and Murray, 1989), we now report the ability of both Ro5-4864 and pyrethroids to modulate $[^{35}S]$TBPS binding and establish the GABA dependence of this modulation in trout brain membranes.

Methods

Rainbow trout (*Oncorhynchus mykiss*), 1-2 years old, were obtained from Oregon State University Food Toxicology and Nutrition Laboratory where they were maintained on 12-hour light-dark cycles and fed Oregon Test Diet. Trout were killed by decapitation, brains were removed and immediately frozen on dry ice and stored at -70° until used.

Brains were thawed in 40:1 (vol/wt) ice cold 50 mM Tris-HCl buffer (pH 7.5) and disrupted with a Brinkman Polytron (setting 7.5, 20 sec.); the

homogenate was centrifuged at 43,000 g for 20 min. The pellet was washed with 40:1 (vol/wt) Tris buffer and the homogenate centrifuged as above. After decanting the supernatant, the pellet was frozen at -70° C for a minimum of 15 minutes. Storage of the pellet at -70° for up to 2 months had no effect on control [^{35}S]TBPS binding. The pellet was then thawed, washed two more times with Tris buffer as above and resuspended in 35:1 (vol/wt) of assay buffer (40.5 mM Na_2HPO_4, 9.5 mM KH_2PO_4, 200 mM NaCl, pH 7.4). This extensively washed preparation minimized the level of endogenous GABA.

Equilibrium binding assays were conducted at 18°C for 90 minutes. Competition assays included 200 μl membrane suspension (120 μg protein / assay), displacer in 1 μl dimethylsulfoxide or solvent alone, 0.8-2.0 nM [^{35}S]TBPS (96.5 to 100.8 Ci/mmol) and buffer to make 0.5 ml total volume. If GABA was used in an assay, it was added in 20 μl assay buffer. Protein was determined by the method of Lowry et al. (1951) following solubilization with 0.5 N NaOH. Bovine serum albumin was used as the standard. Nonspecific binding was defined as that remaining after incubation with 100 μM picrotoxinin and was typically less than 10% of total binding.

Incubation was terminated by vacuum filtration with a Brandel Cell Harvester (Gaithersburg, Md.) through Schleicher and Schuell #32 filters, followed by 4 washes of 4 ml each ice cold assay buffer. Filters were placed in minivials; Biocount scintillant (RPI, Mount Prospect, Ill.) was added and samples were counted for radioactivity six hours later.

Competition experiments and saturation isotherms performed with decreasing [^{35}S]TBPS specific activity were analyzed using the non-linear least

squares curve fitting programs of Ligand EBDA software (Munson and Rodbard, 1980). Saturation isotherms were further analyzed with the FITSAT program of the PROPHET computer system. Enhancement of [^{35}S]TBPS binding was analyzed by FITFUN of the PROPHET computer system. Linear transformations of competition binding data to Hill plot coordinates followed by calculation of the standard error of the estimate of the IC_{50} values were used to determine the significance of shifts in GABA concentration response curves.

Materials

[^{35}S]TBPS was purchased from NEN/Dupont (96.5-100.9 Ci/mmol). Deltamethrin, cismethrin, and kadethrin were gifts from Dr. P. Foulhoux (Roussel Uclaf, Romainville, France), (1RαS)-*cis*- and (1SαR)-*cis*-cypermethrin and the permethrin isomers from FMC Corporation (Princeton, N.J.), Ro5-4864 and Ro15-1788 from Dr. Peter Sorter, Hoffman-LaRoche, Inc. (Nutley, N.J.), PK 11195 from Dr. G. Le Fur (Pharmuka Laboratories, Gennevilliers, France), and (1SαS)-*cis*-cypermethrin from Ciba Geigy. TBPS was purchased from RBI (Natick, Ma.) and picrotoxinin from Sigma.

Results

Specific [^{35}S]TBPS binding was detected in well-washed trout brain membranes; the density (B_{max}) of binding sites was 1747 $\pm$ 109 fmol/mg protein and the equilibrium dissociation constant (K_d) was 20.1 $\pm$ 1.8 nM (n=3) (Fig. 1). Preliminary experiments demonstrated that [^{35}S]TBPS binding was suppressed by the high levels of endogenous GABA present in a standard synaptosomal membrane preparation. The extensive washing and freeze/thaw cycle of this membrane preparation resulted in an enhanced signal to noise ratio with specific binding representing >90% of the total. In this preparation bicuculline did not increase specific binding at any concentration tested (0.1-100 μM) confirming that the levels of endogenous GABA in the final membrane preparation were very low (data not shown). Exogenous GABA was able to inhibit up to 95% of specific [^{35}S]TBPS binding with an IC_{50} of 3.34 $\pm$ 0.36 μM. GABA inhibition was uncompetitive, increasing the K_d of [^{35}S]TBPS to 30.4 $\pm$ 4.9 nM and decreasing the density of receptors to 1176 $\pm$ 122 fmol/ mg protein (Fig. 1).

Deltamethrin, a Type II pyrethroid, was able to inhibit only 20% of specific [^{35}S]TBPS binding in the absence of GABA (Fig.2). However, with increasing concentrations of GABA both the potency and the efficacy of deltamethrin increased, reaching a maximum inhibition of 65% with an IC_{50} of 4.7 $\pm$ 0.3 μM in the presence of 5 μM GABA (Table 1).

The stereoselectivity of this pyrethroid effect was assessed with 3 stereoisomers of cypermethrin: (1RαS)-*cis*-, (1SαR)-*cis*-, and (1SαS)-*cis*-cypermethrin, representing the most insecticidally active and most potent proconvulsant, a 200-fold less insecticidally active, and the least active isomer,

respectively. As shown in Figure 3, a similar shift in potency with increasing concentrations of GABA was observed with all isomers. Although the level of inhibition of [^{35}S]TBPS binding was similar for the isomers, ranking them by maximum efficacy, or affinity (Table 2) results in the predicted rank order of potency by other indices: (1RαS)-*cis*- (74.4%, 4.9 μM), (1SαR)-*cis*- (69.6%, 7.7 μM), and (1SαS)-*cis*- (59.7%, 11.5 μM).

To further define the allosteric interaction of the $GABA_A$ receptor and a pyrethroid binding site, the ability of deltamethrin to influence GABA inhibition was examined. As shown in Table 3, deltamethrin caused a dose-dependent leftward shift of the GABA concentration response curves. The IC_{50} decreased from 3.05 to 1.83 μM ($p<0.05$) with the addition of 3 μM deltamethrin with no change in GABA efficacy or Hill slope.

An array of selected pyrethroids was used to pharmacologically characterize the pyrethroid binding site. All of the pyrethroids tested effected a concentration dependent inhibition of [^{35}S]TBPS binding (Table 4). The rank order of potency was (1RαS)-*cis*-cypermethrin = deltamethrin = allethrin > kadethrin = cismethrin = (1SαS)-*cis*-cypermethrin > (1R)-*cis*-permethrin > (1S)-*cis*- permethrin. Allethrin was the most efficacious compound, followed by cismethrin, the cypermethrin isomers, and deltamethrin.

The peripheral-type benzodiazepine receptor ligand, [^{3}H]Ro5-4864, has no detectable high-affinity binding sites in trout brain (*vide supra*). However, this ligand had a conspicuous effect on TBPS binding in trout brain membranes at micromolar concentrations, the nature of which was dependent on the concentration of GABA in the incubate. As illustrated in Figure 4, in the

absence of exogenous GABA, Ro5-4864 acted as an inhibitor of [^{35}S]TBPS binding, decreasing binding by more than 40% at concentrations above 3 μM. The addition of increasing concentrations of GABA alone inhibited binding. Furthermore in the presence of 10 μM GABA, Ro5-4864 produced a concentration dependent stimulation of [^{35}S]TBPS binding. The maximum enhancement of [^{35}S]TBPS binding obtained was 170% at a Ro5-4864 concentration of 10 μM. As can be seen from Figure 4, the increase in binding reached a level that was virtually identical to that obtained in the absence of GABA. Thus the Ro5-4864-induced enhancement of [^{35}S]TBPS binding most plausibly represents a reversal of the inhibition exerted by GABA.

In an effort to further address the involvement of central- or peripheral-type benzodiazepine receptors in the Ro5-4864 enhancement of [^{35}S]TBPS binding, the influence of Ro15-1788 and PK 11195 on this response was investigated. In the presence of 10 μM GABA, Ro15-1788 (3 and 30 μM) alone had no effect on [^{35}S]TBPS binding nor did it reverse the enhancement by Ro5-4864 at either concentration. In contrast to the biphasic nature of Ro5-4864 modulation of [^{35}S]TBPS binding, PK 11195 inhibited [^{35}S]TBPS binding in a concentration-dependent manner in the presence and absence of GABA, as shown in Figure 5, with IC_{50} values ranging from 26 to 11 μM (Table 5). Inasmuch as no inhibition was observed with 1 μM PK 11195, this concentration was used to examine whether PK 11195 could reverse the enhancement by Ro5-4864 in the presence of 10 μM GABA. As shown in Figure 6, addition of 1 μM PK 11195 had no effect on the activity of Ro5-4864; the Ro5-4864 EC_{50} values in the absence and presence of PK 11195 were 2.9 $\pm$ 1.0 μM and 2.8 $\pm$ 0.8 μM, respectively.

Given the GABA dependence of the interaction of Ro5-4864 with the TBPS binding site, we investigated the ability of Ro5-4864 to modulate GABA-induced inhibition of [^{35}S]TBPS binding. At GABA concentrations greater than 1 μM, Ro5-4864 produces an apparent enhancement of binding. However, examination of Figure 7 reveals that, consistent with the presence of a reciprocal allosteric inhibition, Ro5-4864 shifted the GABA concentration-response curves to the right. The IC_{50} of GABA increased more than 6 fold with the addition of 10 μM Ro5-4864, but there was no change in the maximum level of inhibition (Table 6).

Discussion

In the present study, the observed interactions between pyrethroid insecticides and the $GABA_A$ receptor of piscine brain are consistent with a modulation of $GABA_A$ receptor function contributing to pyrethroid neurotoxicity in vertebrates. All type II pyrethroids tested produced a concentration-dependent inhibition of the specific binding of [^{35}S]TBPS. The inhibition was GABA dependent: the presence of micromolar concentrations of GABA increased both the efficacy, up to a maximum of 60-70%, and potency, to a range of IC_{50} values of 5-12 μM, of these insecticides to inhibit [^{35}S]TBPS binding. Consistent with this enhancement by GABA of pyrethroid inhibition, deltamethrin significantly increased the potency of GABA as an inhibitor of [^{35}S]TBPS binding. This reciprocal positive cooperativity between pyrethroid and GABA inhibition provides evidence for an allosteric coupling of the binding sites for these compounds. Seifert and Casida (1985a) reported similar GABA-

mediated enhancement of the pyrethroid interaction with the TBPS site of rodents, increasing the efficacy of 10 μM (1RαS)-*cis*-cypermethrin from 10% to 83% inhibition with the addition of 5 μM GABA. They reported no facilitation by GABA for the non-toxic isomer (1SαS)-*cis*- cypermethrin at concentrations of GABA of 1-4 μM, while in trout brain membranes GABA produced an enhancement of the inhibitory actions of this isomer.

Although it is evident that pyrethroids are able to modulate binding to the TBPS site of the chloride ionophore in the trout model, the ability to interact with the [^{35}S]TBPS receptor may not be a good predictor of toxicity for this species. Limited stereoselectivity was observed between 3 isomers of cypermethrin, which have potencies ranging from the most potent both insecticidally and as a proconvulsant to the least potent insecticidally. The largest affinity ratio was 3.3, measured in the absence of exogenous GABA (Table 3). The low stereoselectivity is not in agreement with the original report by Lawrence and Casida (1983) who observed absolute stereospecificity of inhibition of binding in rodent membranes, with only toxic isomers exhibiting inhibitory potency. However, the results reported herein are not as disparate from the results reported by Seifert and Casida (1985b) as their inference would suggest. This group reported 65% vs 22% inhibition of [^{35}S]TBPS binding by 5 μM concentrations of (1RαS)-*cis*- and (1SαS)-*cis*-cypermethrin, respectively, while in the trout the corresponding displacements were 53% and 35% at a 6 μM concentration of the two isomers. The stereoselectivity of pyrethroid inhibition of [^{35}S]TBPS binding is therefore comparable in piscine and murine brain membranes.

The two Type I stereoisomers tested, (1R)-*cis*- and (1S)-*cis*-permethrin, had an affinity ratio of approximately 1.5, while *in vivo* these two isomers have a 1000 fold difference in their toxicities to teleosts (Miyamoto, 1976). All of the Type I pyrethroids tested inhibited binding by more than 50% and allethrin was the most efficacious pyrethroid tested, indicating that this site in trout brain recognizes both Type I and II pyrethroids.

A primary advantage of this extensively washed and freeze/thawed membrane preparation was that it minimized the levels of endogenous GABA, thereby permitting a thorough investigation of the allosteric interactions of the GABA recognition site of this receptor with other allosteric modulator sites. The inability of bicuculline to enhance [^{35}S]TBPS binding at any concentration confirmed that the levels of endogenous GABA were minimal. Saturation isotherms performed in the absence and presence of GABA revealed that GABA inhibited [^{35}S]TBPS binding uncompetitively, decreasing both density and affinity. This uncompetitive inhibition may help to explain the discrepancies between our results and those of Corda et al. (1989). Corda et al., using eel brain membranes, reported a low efficacy of GABA to inhibit, and a high efficacy of bicuculline to enhance, [^{35}S]TBPS binding, which was unaffected by repeated washings. The wash buffer they used had a 5 fold greater osmolarity, thus the washing may not have been as effective at removing GABA from membrane vesicles. The high osmolarity and lack of a freeze-thaw cycle may have contributed to intact vesicles which contained high levels of GABA. Higher levels of endogenous GABA would have decreased the efficacy of added GABA

and would cause a decrease in both affinity and density of [^{35}S]TBPS binding sites which was reported by this group.

Ro5-4864, for which no high affinity sites have been detected in trout brain (Eshleman and Murray, 1989; Bolger et al, 1985), was able to modulate [^{35}S]TBPS binding in a manner similar to the activity in rodent brain membranes ($EC_{50\ trout}$=3 μM, $EC_{50\ rodent}$=0.25 μM (Gee, 1987)). Inhibition of binding in the absence of GABA and enhancement of binding in the presence of GABA were produced by similar concentrations of Ro5-4864. As shown in Figure 5, the apparent enhancement of [^{35}S]TBPS binding by Ro5-4864 in the presence of GABA was due to a rightward shift of the GABA concentration-response curve, decreasing the affinity of GABA for its receptor. Consistent with the contention that Ro5-4864 produces a decrease in the affinity of GABA for its receptor, Ro5-4864 reversed the inhibitory effects of 1 and 5 μM GABA on [^{35}S]TBPS binding in rodent membranes, (Squires and Saederup, 1987) and increased the IC_{50} of GABA approximately 2 fold (Gee, 1987).

The PTBR of trout brain is not likely to be the site of Ro5-4864 or PK 11195 modulation of [^{35}S]TBPS binding. PK 11195 exhibited nanomolar affinity for the trout brain PTBR (Eshleman and Murray, 1989) but inhibited [^{35}S]TBPS binding only at micromolar concentrations and, at a 1 μM concentration, was unable to alter the Ro5-4864-induced enhancement of binding. Moreover, Ro5-4864 has lower affinity for the trout brain PTBR (IC_{50}= 41 μM) than the effective concentrations used in the present study.

These results lend further support to the proposal that a Ro5-4864 sensitive recognition site is associated with a subtype of the $GABA_A$ receptor

which may be responsible for its convulsant activity. This site is distinguished from the classical central type benzodiazepine receptor by the inability of micromolar concentrations of Ro15-1788 (flumazenil) to reverse the modulation by Ro5-4864. Rodent studies have also shown that this Ro5-4864 site is not affected by central benzodiazepine receptor compounds: the irreversible labelling of the central recognition site by Ro15-4513 did not change the ability of Ro5-4864 to enhance the binding of [^{35}S]TBPS (Gee et al., 1988), and Ro15-1788 (3 and 10 μM) did not antagonize the Ro5-4864 modulation of [^{35}S]TBPS binding (Ticku and Ramanjaneyulu, 1984; Gee, 1987).

Our demonstration of the inability of PK 1195 to antagonize the Ro5-4864 enhancement of [^{35}S]TBPS binding is consonant with recent reports of the electrophysiological actions of Ro5-4864. The Ro5-4864-induced increase in the spontaneous firing of cerebellar purkinje neurons was reported to be insensitive to PK 11195 (Basile et al., 1989). In addition, Puia et al. (1989) demonstrated that the Ro5-4864 reduction of GABA-activated Cl^- currents in cortical neurons was similarly resistant to PK 11195 antagonism. Collectively, these results indicate Ro5-4864 modulates $GABA_A$ receptor function through an interaction with a recognition site distinct from the peripheral-type benzodiazepine receptor.

Receptors which bind GABA, TBPS and Ro5-4864 have been termed $GABA_{A3}$ and are considered to represent a subset of the total population of $GABA_A$ receptors (Barbaccia et al., 1988). In the present study, PK 11195 was able to displace 85% of [^{35}S]TBPS binding which may indicate that the majority of [^{35}S]TBPS recognition sites in trout also contain binding sites for PK 11195.

Evidence for the regulation of $GABA_{A3}$ receptor function by ligands of the PTBR was provided by the demonstration that the proconflict response elicited by the fragment DBI 17-50 was antagonized by PK 11195 (Costa, 1988). Moreover, DBI 17-50 inhibited the binding of [^{3}H]PK 11195 to synaptic membranes (Costa, 1988).

Considered together, the results reported herein indicate that pyrethroids and Ro5-4864 have binding sites on the $GABA_A$ receptor complex of piscine brain which interact allosterically with the GABA and the [^{35}S]TBPS recognition sites. The allosteric modulation of [^{35}S]TBPS binding by Ro5-4864 and pyrethroids in trout and rodent brain membranes are parallel, suggesting that this subtype of the $GABA_A$ receptor has been conserved over vertebrate evolution. These results suggest that a modulation of GABAergic synaptic signaling may be operative in the neurotoxicological action of Ro5-4864 and pyrethroids.

Acknowledgements. This work was supported by National Insititutes of Health Grant ES04891. Permission to include this paper in the thesis was granted by Pergamon Press.

Figure 3-1. Equilibrium binding of [^{35}S]TBPS to trout brain membranes: uncompetitive inhibition by GABA. Increasing concentrations of unlabeled TBPS were added to 1 nM [^{35}S]TBPS to decrease the specific activity of the radioligand. A. Saturation isotherms. The densities of binding sites were 1747 $\pm$ 109 and 1176 $\pm$ 122 fmol / mg of protein with 0 and 5 μM GABA respectively (n=3). The K_D shifted from 20.1 $\pm$ 1.8 to 30.4 $\pm$ 4.9 nM with the addition of GABA. B. Scatchard coordinate plot of the same data. Data shown are the average of three separate experiments (mean $\pm$ SEM).

Figure 3-1A.

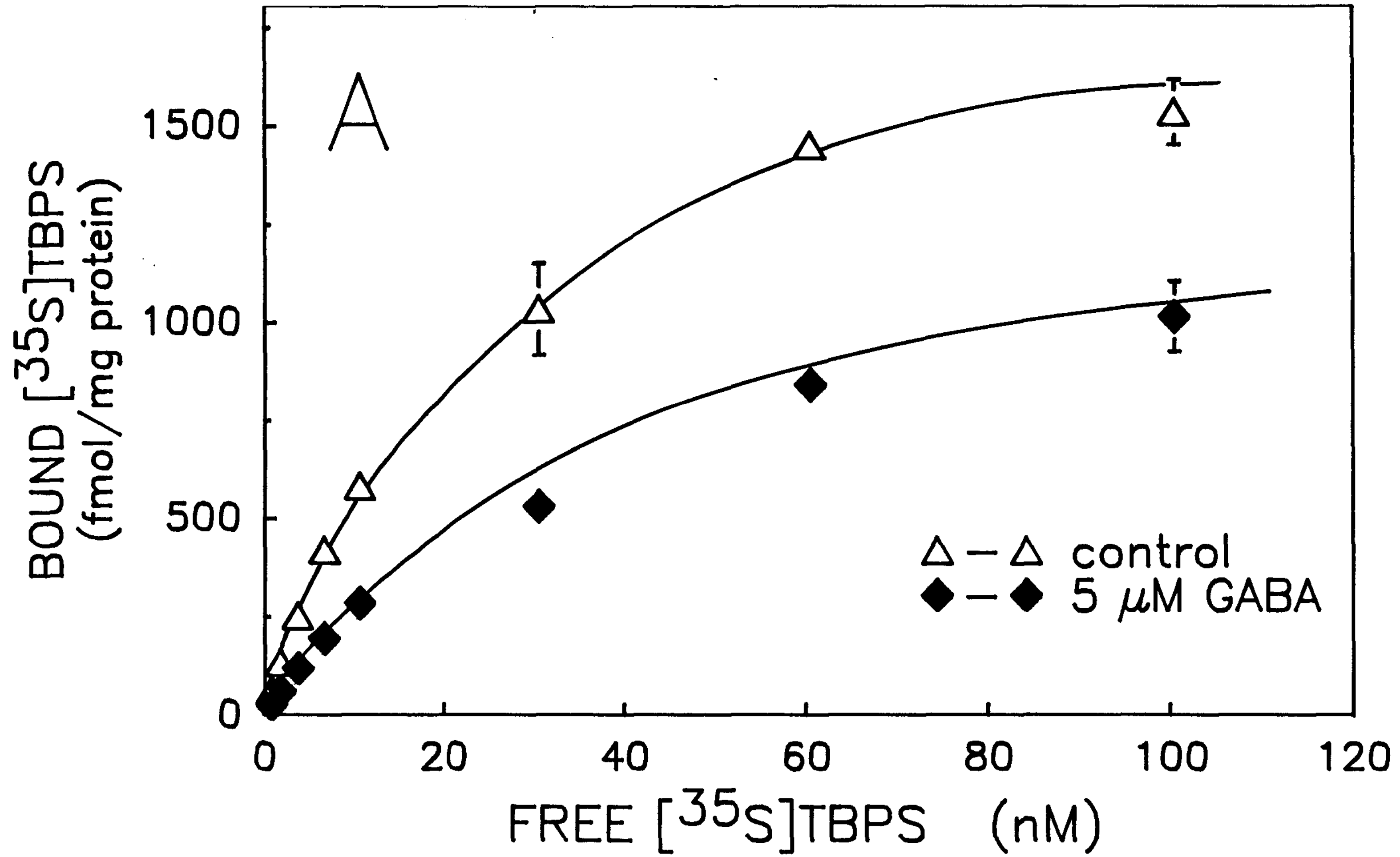


Figure 3-1B.

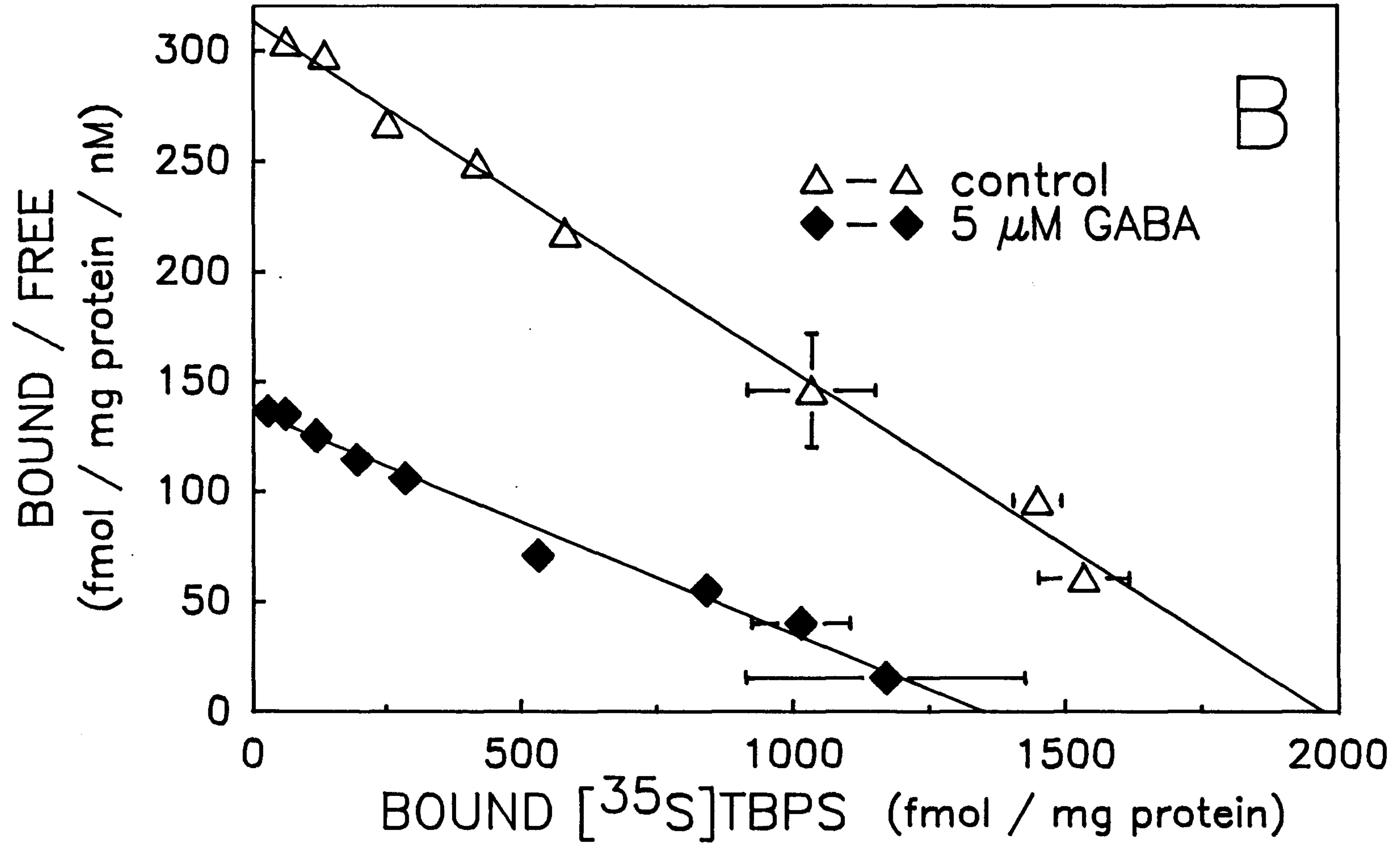


Figure 3-2. GABA modulation of deltamethrin concentration-response curves of inhibition of [^{35}S]TBPS binding. Data shown are the average of 3 experiments (mean $\pm$ SEM). The estimates of parameters derived from these curves are given in Table 1. Control binding (100%) is defined for each curve as the amount bound at that concentration of GABA in the absence of deltamethrin. In a typical experiment, the amount of [^{35}S]TBPS bound was 165, 151, 88, and 53 fmol/mg protein with 0, 1, 5, and 10 μM GABA respectively.

Figure 3-2.

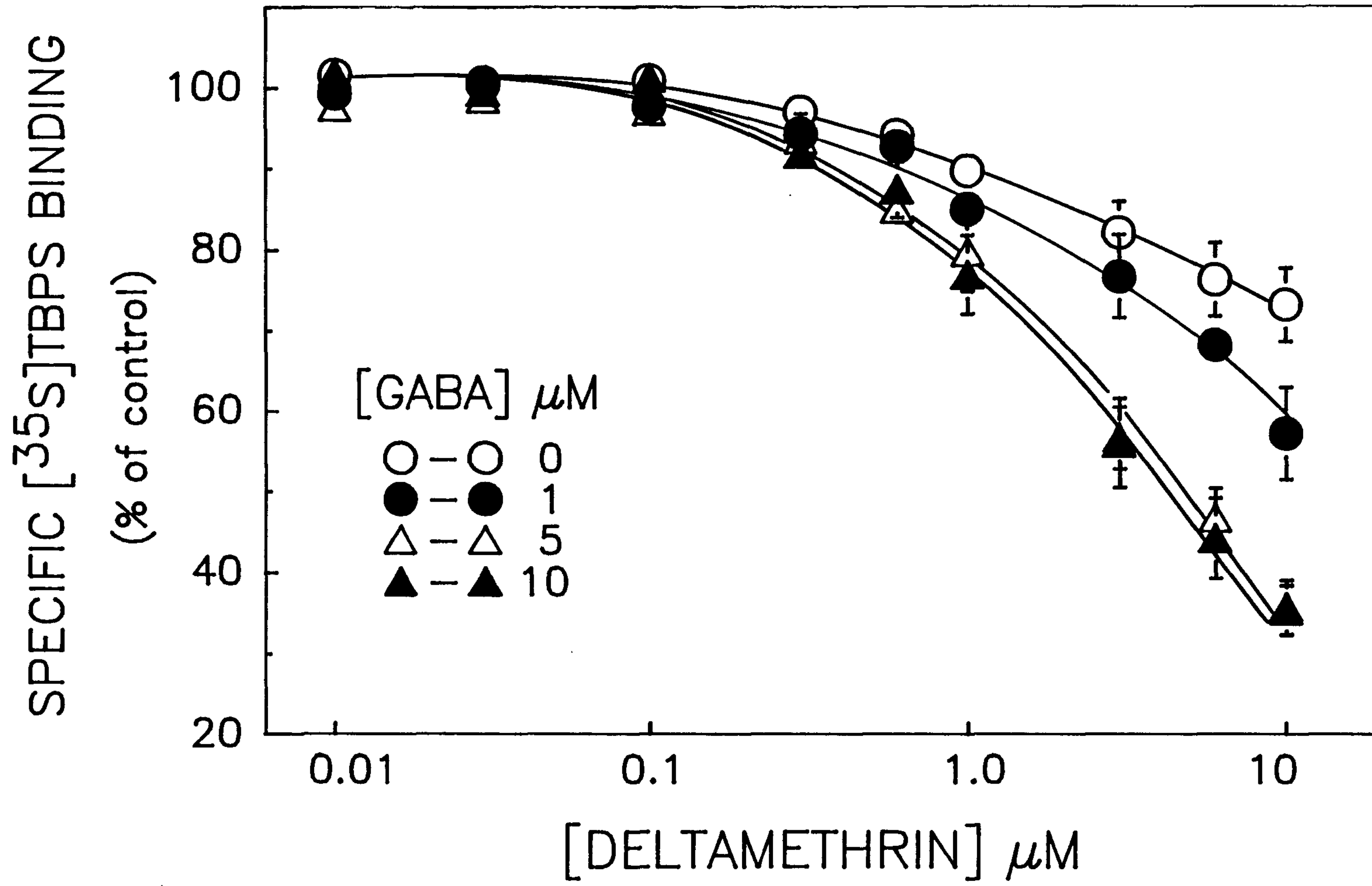


Figure 3-3. Inhibition of [^{35}S]TBPS binding by three cypermethrin isomers. Data plotted are the results of 5, 3, and 3 experiments (mean $\pm$ SEM) for (1RαS)-*cis*-, (1SαR)-*cis*-, and (1SαS)-*cis*-cypermethrin, respectively. GABA increased the efficacy and potency of all three isomers in a similar manner.

Figure 3-3.

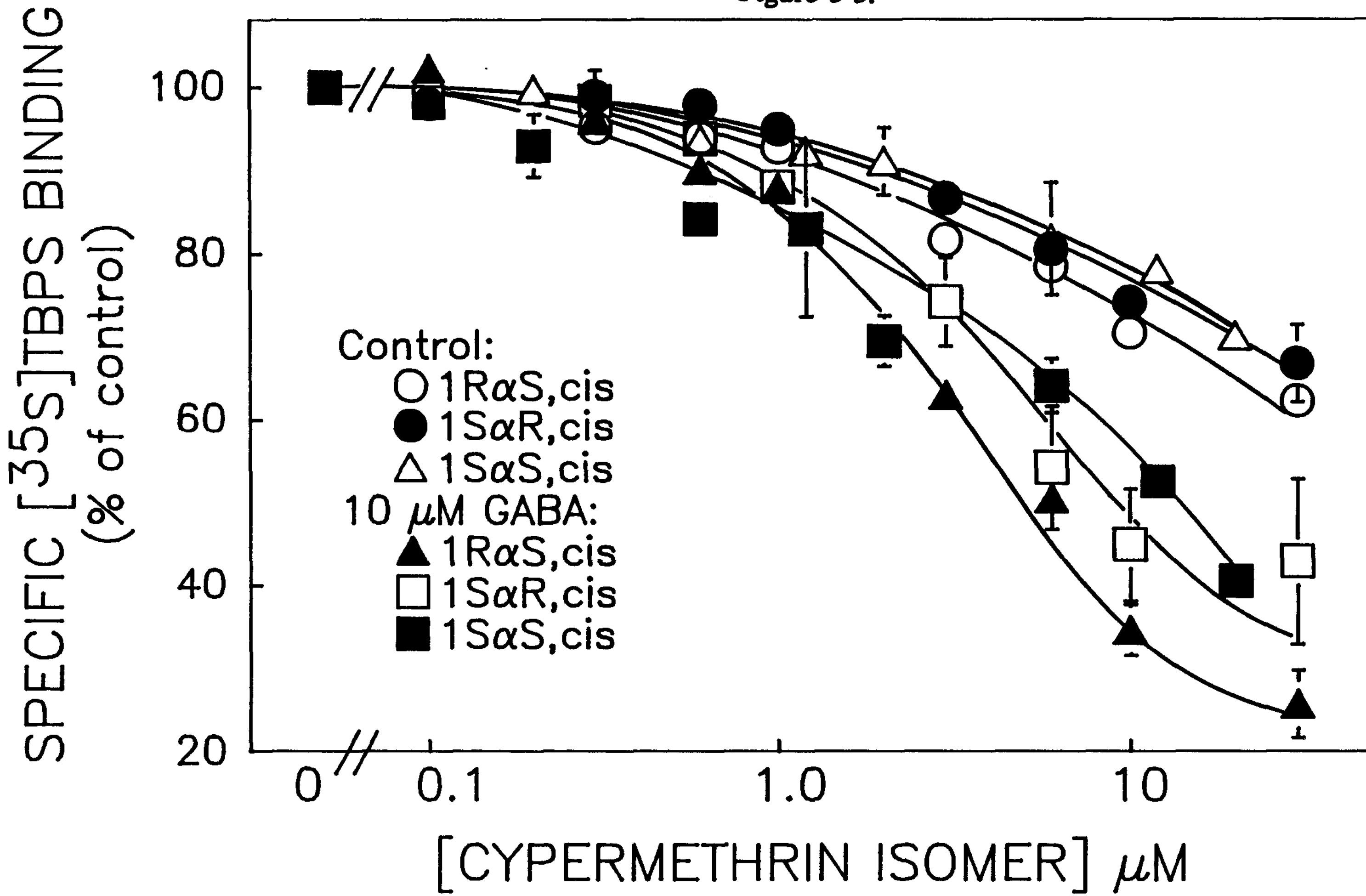


Figure 3-4. The influence of GABA on Ro5-4864 modulation of specific [^{35}S]TBPS binding. Data shown are the results of a representative experiment which was repeated with similar results. The maximum inhibition of [^{35}S]TBPS binding by Ro5-4864 was 41% and 33% in the presence of 0 and 1 μM exogenous GABA. The maximum enhancement of [^{35}S]TBPS binding was 36% and 71% in the presence of 5 and 10 μM GABA. The amount of [^{35}S]TBPS bound at 10 μM Ro5-4864 was similar with all treatments.

Figure 3-4.

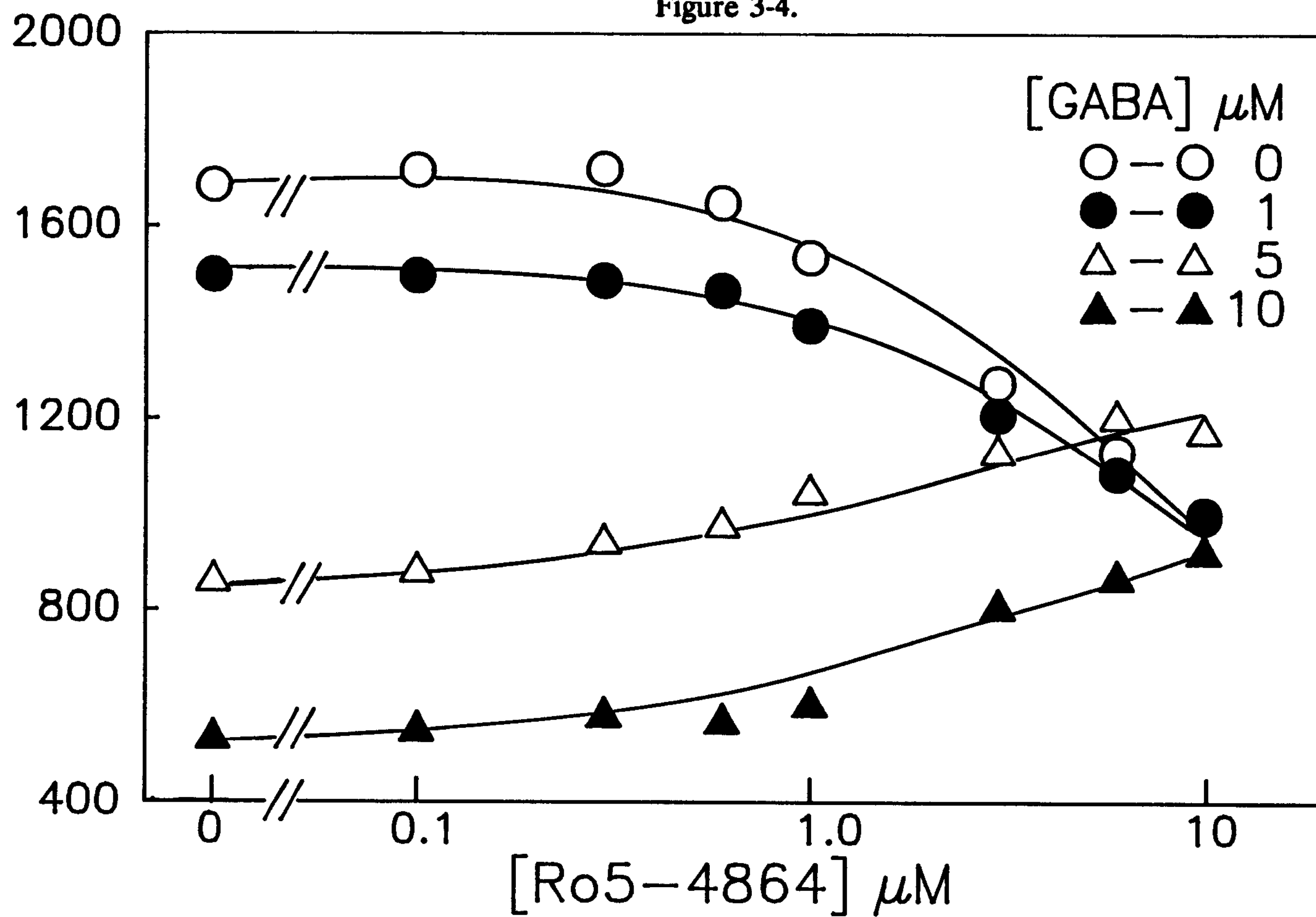


Figure 3-5. PK 11195 inhibition of specific [^{35}S]TBPS binding. Data shown are the results of a representative experiment performed in duplicate, which was repeated with similar results. The symbols represent binding in control (○), 1 μM (●), 5 μM (△), and 10 μM (▲) GABA. Estimates for the parameters of PK 11195 inhibition are given in Table 5.

Figure 3-5.

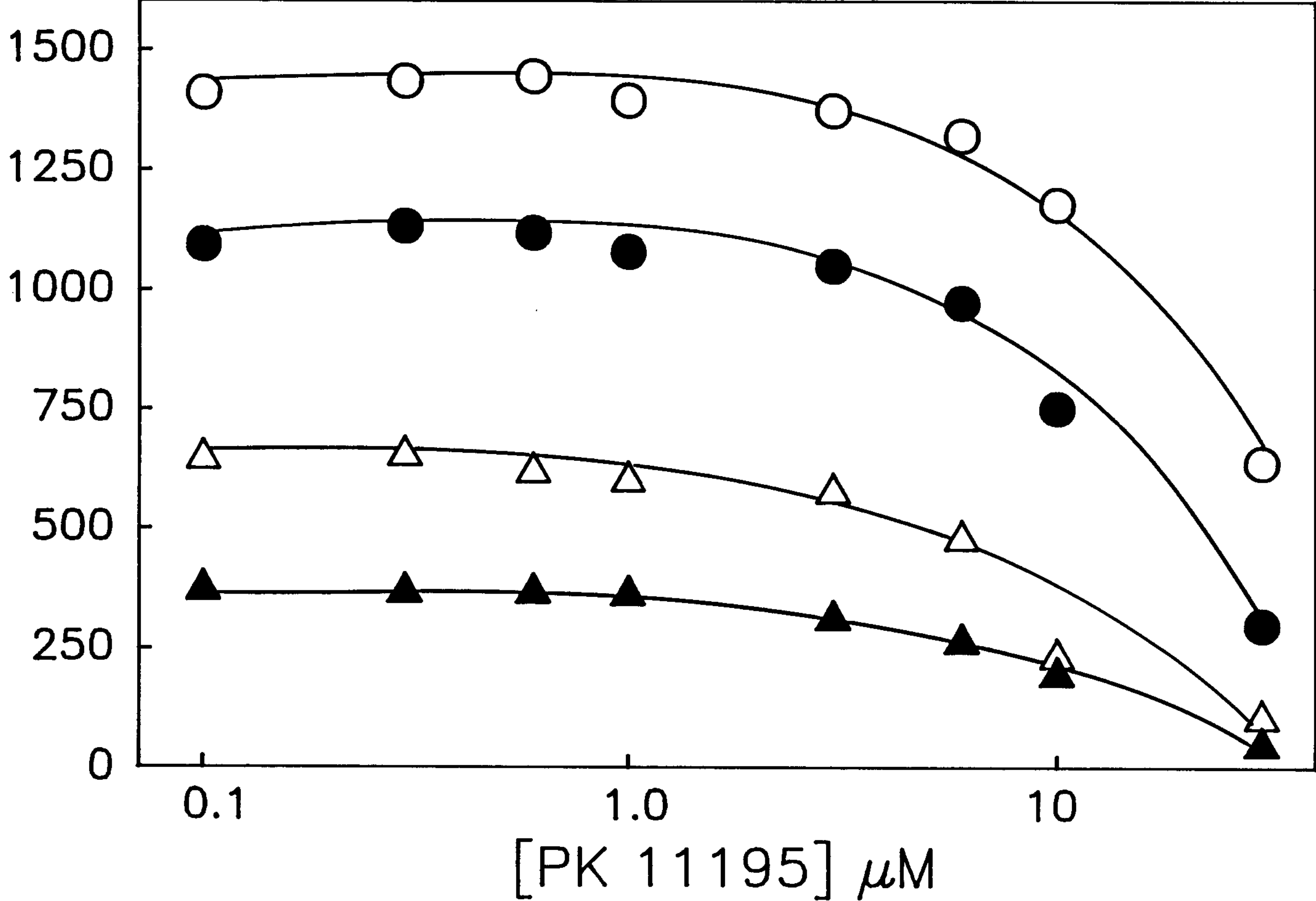


Figure 3-6. Lack of effect of PK 11195 on Ro5-4864-induced enhancement of [^{35}S]TBPS binding in the presence of 10 μM GABA. Data are the results of a representative experiment conducted in triplicate (mean $\pm$ SEM), which was repeated with similar results. PK 11195 (1 μM) had no effect on the potency or efficacy of Ro5-4864 at this concentration.

Figure 3-6.

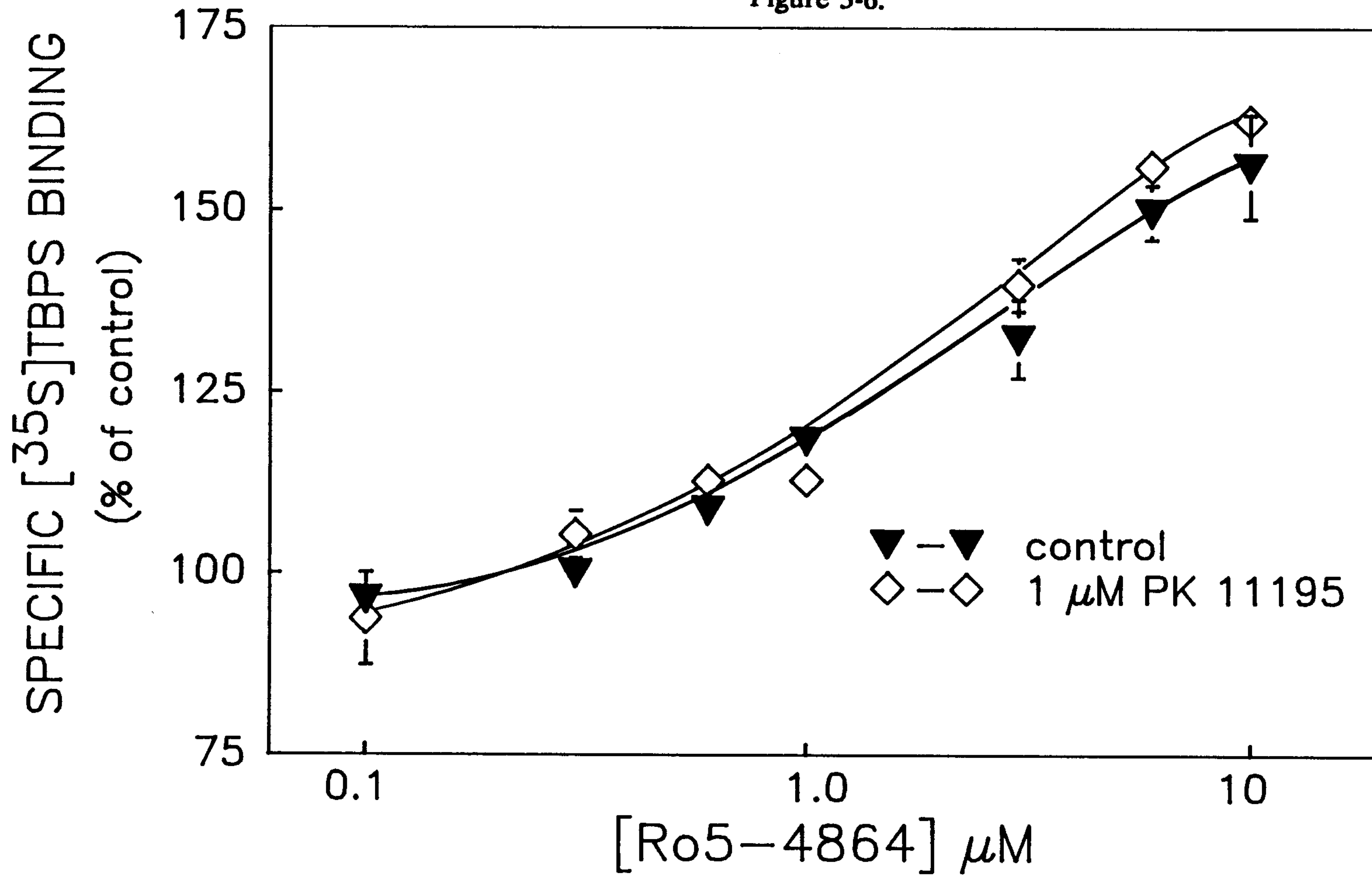


Figure 3-7. Ro5-4864-induced shift of GABA concentration-response curves for inhibition of [^{35}S]TBPS binding. Data are the average ($\pm$SEM) of 3 separate experiments. The estimates for the parameters of GABA inhibition of [^{35}S]TBPS binding are given in Table 6.

Figure 3-7.

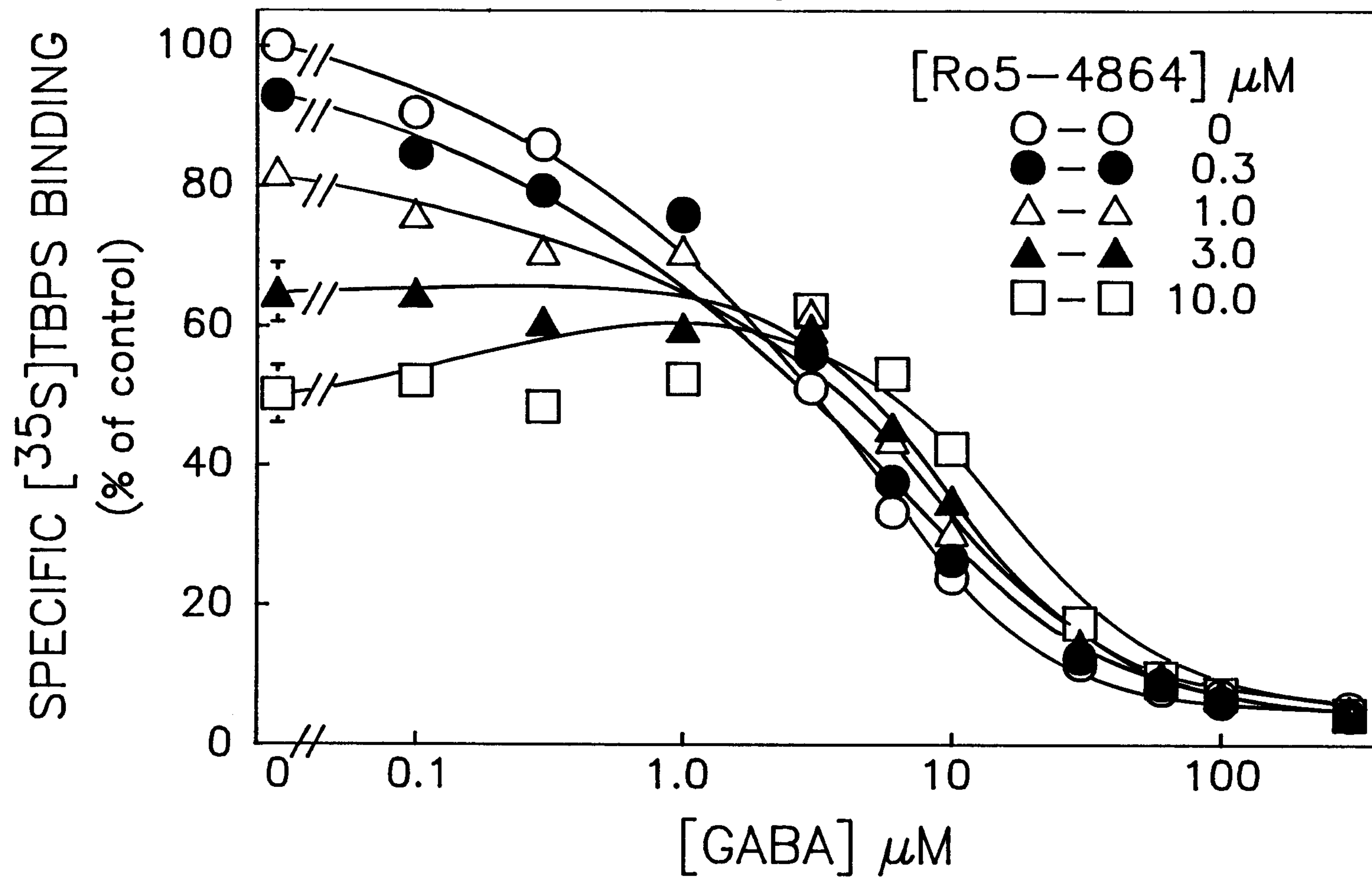


Table 3-1. Influence of GABA on the deltamethrin inhibition of [^{35}S]TBPS binding to trout brain membranes

[GABA] (μM)	Deltamethrin IC$_{50}$ (μM)	Deltamethrin Slope factor	Deltamethrin Maximum inhibition (%)
0	50.1±10.4	0.53±0.06	26.8±4.6
1	15.3±1.9	0.71±0.06	42.8±5.8
5	4.7±0.3	0.83±0.04	64.7±3.1
10	4.1±0.3	0.78±0.04	64.3±3.4

Mean values (±SEM) for parameter estimates were derived from the simultaneous fit of 3 separate experiments. GABA increased both the efficacy and potency of deltamethrin.

Table 3-2. Inhibition of [^{35}S]TBPS binding to trout brain membranes by 3 cis-cypermethrin isomers.

[GABA]	IC_{50} (μM)			Slope factor			Maximum inhibition (%)		
(μM)	1RαS	1SαR	1SαS	1RαS	1SαR	1SαS	1RαS	1SαR	1SαS
0	68 ±11	103±30	224±73	0.5±0.1	0.5±0.1	0.4±0.1	37.9	33.4	33.3
1	22.1±2.5	21.0±2.3	48.4±6.9	0.6±0.1	0.6±0.1	0.6±0.1	52.8	53.1	39.4
5	4.9±1.5	8.2±30	11.5±1.2	0.6±0.1	0.7±0.1	0.8±0.1	71.2	69.6	59.5
10	5.0±1.2	7.7±0.4	12.7±2.4	0.7±0.1	0.9±0.1	0.6±0.1	74.4	57.3	59.5

Mean values (±SEM) for the parameter estimates were derived from the simultaneous fit of 5, 3, and 3 separate experiments for (1RαS)-cis-, (1SαR)-cis- and (1SαS)-cis-cypermethrin, respectively.

Table 3-3. Deltamethrin modulation of GABA inhibition of [^{35}S]TBPS binding.

[Deltamethrin] (μM)	GABA	
	IC_{50} (μM)	(95% confidence limits)
Control	3.05	(2.67-3.40)
0.3	2.69	(2.11-3.27)
1.0	2.69	(2.36-3.02)
3.0	1.83*	(1.42-2.24)

Parameters are derived from simultaneous fit of data from 2 separate experiments to Hill plot coordinates.
*Significantly different from control at $p<0.05$.

Table 3-4. Pyrethroid inhibition of [^{35}S]TBPS binding in the presence of 5 μM GABA.

Pyrethroid	(n)	IC_{50} (μM)	Slope factor	% maximum displacement
Deltamethrin	(7)	5.3±0.4	0.90±0.07	64.3±1.7
Cypermethrin				
(1RαS)-cis	(5)	4.6±1.9	0.58±0.10	67.6±3.0
(1SαR)-cis	(5)	7.6±0.9	0.71±0.05	69.8±2.3
(1SαS)-cis	(3)	11.5±1.2	0.82±0.08	59.5±2.6
Permethrin				
(1R)-cis	(3)	20.1±1.0	0.96±0.06	58.6±4.7
(1S)-cis	(3)	24.7±1.50	0.92±0.06	50.6±11.1
Allethrin	(4)	5.7±0.5	1.78±0.35	95.8±3.2
Kadethrin	(3)	7.3±0.9	0.68±0.09	85.5±2.1
Cismethrin	(3)	7.6±0.6	1.25±0.09	85.5±2.1

Mean values (±s.e.) for parameter estimates were derived from the simultaneous fit of n separate experiments. For all pyrethroids tested, eight concentrations were used ranging from 0.1 to 30 μM.

Table 3-5. Influence of GABA on PK11195 inhibition of [^{35}S]TBPS binding.

[GABA] (μM)	PK11195 IC$_{50}$ (μM)	Slope factor	Maximum inhibition (%)
0	26.4±0.4	1.66±0.06	55.3
1	16.3±0.5	1.66±0.07	73.7
5	11.1±0.4	1.59±0.07	82.8
10	10.7±0.3	1.65±0.06	85.0

Mean values (± SEM) for parameter estimates were derived from the fit to a competition experiment performed in duplicate and repeated with identical results. The highest PK 11195 concentration used was 30 μM.

Table 3-6. Ro5-4864 modulation of GABA inhibition of [^{35}S]TBPS binding.

[Ro5-4864] (μM)	GABA		
	IC_{50} (μM)	Slope factor	Maximum inhibition (%)
Control	2.98±0.18	1.09±0.04	94.6±0.2
0.3	4.12±0.36	1.05±0.05	95.7±0.3
1.0	6.64±0.61	1.22±0.08	95.0±0.4
3.0	10.38±1.11	1.31±0.10	95.5±1.3
10.0	18.50±2.67	1.82±0.28	95.6±1.7

Mean values (±SEM) for parameter estimates were derived from the simultaneous fit of data from 3 separate experiments.

Chapter 4

Pyrethroid Insecticides Indirectly Inhibit GABA-Dependent $^{36}Cl^-$ Influx in Trout Brain Synaptoneurosomes

Amy J. Eshleman and Thomas F. Murray

Abstract

Rainbow trout (*Oncorhynchus mykiss*) are extremely sensitive to the neurotoxic activity of pyrethroid insecticides. One possible target for pyrethroids is the $GABA_A$ receptor of trout brain, the function of which can be tested by measurement of $^{36}Cl^-$ influx into synaptoneurosomes in response to the application of agonists. GABA produced a time- and concentration- dependent increase in $^{36}Cl^-$ influx in trout brain synaptoneurosomes which exhibited the pharmacology characteristic of a response mediated by activation of the $GABA_A$ receptor. Deltamethrin, (1RαS)-*cis*-cypermethrin and permethrin produced a dose-dependent increase in basal uptake and a corresponding decrease in GABA-dependent influx with a maximal inhibition of 70-82%. This effect was stereospecific, of high potency and inhibited by tetrodotoxin (TTX) and t-butylbicyclophosphorothionate (TBPS). The sensitivity of the pyrethroid effect to TTX suggested a pyrethroid activation of the voltage-dependent sodium channel. Veratridine, a sodium channel activator, elicited similar changes in basal chloride uptake which were TTX-sensitive. Neither deltamethrin nor veratridine had measurable effect on $^{36}Cl^-$ efflux from synaptoneurosomes. Thus, pyrethroid insecticides may interfere with $GABA_A$ receptor function indirectly via

an interaction with the voltage-dependent sodium channel in trout brain and consequently perturb chloride influx, possibly through a voltage-dependent chloride channel (VDCC).

Introduction

Pyrethroid insecticides are broad spectrum, photostable analogs of natural pyrethrins which are widely used in agriculture, home pest control, protection of stored foodstuffs, and disease vector control (Herve, 1985). These neurotoxic pesticides have been shown to have a mechanism of action in the central nervous system of vertebrates by intracerebroventricular injections (Gammon et al., 1982). Several molecular targets have been proposed as the site of action of these compounds including the voltage-gated sodium channel (Narahashi, 1985), the peripheral-type benzodiazepine receptor (Devaud and Murray, 1988; Ramadan et al., 1988b), the Ca^{2+}-dependent ATPase (Clark and Matsumura, 1982), the voltage-dependent Ca^{2+} channel (Clark and Brooks, 1989), and the $GABA_A$ receptor (Lawrence and Casida 1983, Ramadan et al., 1988a). This latter target was the focus of the present investigation.

Fish are extremely sensitive to the neurotoxic action of pyrethroids; the brain levels that elicit toxic symptoms in trout are 1/8 that measured in rats at the initiation of toxic activity (Edwards et al., 1986). We have observed seizure activity in trout with doses as low as 30 ug deltamethrin / kg body weight (unpublished observations). Convulsions are manifested by wide opercular flaring, increased ventilation rate, and myoclonic head swaying.

As the primary inhibitory neurotransmitter system in the vertebrate central

nervous system, the $GABA_A$ receptor performs a critical role in normal brain functioning. Compromising the function of this system is the mechanism of toxicity for cage convulsants, picrotoxin, bicuculline, and insecticides such as dieldrin and endrin (Eldefrawi and Eldefrawi, 1987). Evidence for pyrethroid insecticide interaction with the $GABA_A$ receptor of rodent brain was shown initially by ligand binding studies. [^{35}S]TBPS, a cage convulsant which labels a site within the chloride channel of the $GABA_A$ receptor, has been employed as a ligand to detect allosteric interactions of binding sites on this receptor. Low micromolar quantities of several type I and II pyrethroids displaced 70-90% of specific [^{35}S]TBPS binding from trout brain membranes in the presence of GABA (Eshleman and Murray, in press) and rodent brain membranes (Seifert and Casida, 1985b).

Ligand binding studies employing [^{3}H]batrachotoxin (BTX) have also revealed an interaction of pyrethroids with the voltage-dependent sodium channel of rodent brain membranes and mouse neuroblastoma cells (Jacques et al., 1980; Lombet et al.,1988; Brown et al., 1988). The affinities exhibited by pyrethroids in this system were similar to the affinities of the pyrethroid interaction with the $GABA_A$ receptor as measured by [^{35}S]TBPS binding (Seifert and Casida, 1985a).

A biochemical index of $GABA_A$ receptor function is the measurement of flux of chloride ions through channels in response to the application of $GABA_A$ agonists to brain synaptoneurosomes. This assay has been used to measure the influence of a variety of compounds including alcohols (Allan and Harris, 1987), barbiturates (Schwartz et al., 1985), cage convulsants (Obata et al., 1988), and

steroids (Morrow et al., 1987) on $GABA_A$ receptors in rodent brain membranes. Here we characterize the agonist-dependent $^{36}Cl^-$ influx into rainbow trout synaptoneurosomes and the effect of pyrethroids on this influx. In addition, pyrethroid influence on $^{36}Cl^-$ efflux was characterized using avermectin (AVA), an anthelmintic, and pentobarbital as effectors.

Methods

Rainbow trout, 1-2 years old, were obtained from the Food Toxicology and Nutrition Laboratory of Oregon State University (Corvallis, OR) and were fed Purina trout chow and maintained in flow-through tanks at ambient lighting. Trout were sacrificed by decapitation, brains quickly removed and placed in ice cold saline.

Synaptoneurosome preparation was similar to that of Harris and Allen (1985) with minor modifications. The brains were homogenized in 10 volumes (vol/wt) ice cold buffer (145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 10 mM D-glucose, adjusted to pH 7.5 with Tris base) with a loose fitting Dounce homogenizer, filtered through 2 layers of nylon mesh (160 μm, Tetko, NY), and centrifuged at 1000 g for 15 min. The supernatant was carefully decanted and the pellet washed twice with 10 volumes (vol/wt) assay buffer and centrifuged as above.

$^{36}Cl^-$ Uptake: Synaptoneurosomes were resuspended in 2 volumes (vol/wt) assay buffer. Aliquots (200 μl, 1.7-2.1 mg of protein) of membrane preparation were pre-incubated alone, with inhibitor in 2 μl dimethylsulfoxide (DMSO), or vehicle alone for 12 minutes. Influx was initiated by addition of ^{36}Cl (0.095-0.113

μCi / 200 μl of assay buffer) with or without agonist and terminated 30 seconds later except where noted. Influx was terminated by dilution with 4 ml ice cold assay buffer and vacuum filtration with a Hoefer single filtration unit onto Schleicher and Schuell (S&S) #32 glass fiber filters. Filters were then washed with 8 ml of ice cold assay buffer.

$^{36}Cl^-$ Efflux: The pellet was resuspended in 1 volume (vol/wt) assay buffer. ^{36}Cl was added to the suspension and gently stirred at 4° C for 1 hour. Efflux was initiated by dilution of a 100 μl aliquot of membranes with buffer containing modulators in DMSO or vehicle alone for a final volume of 5 ml. At times ranging from 0 to 480 seconds, efflux was terminated by filtration onto S&S filters and filters were washed with 5 ml of ice cold assay buffer.

Cytoscint (INC Biomedicals, Irvine, CA) was added to filters in minivials, and radioactivity was determined 6 hours later with a Beckman liquid scintillation counter.

Protein determination: The method of Lowry et al. (1951) was used to determine protein levels following solubilization in 0.5 N NaOH. Bovine serum albumin was used as the standard.

Data analysis: Basal or GABA-independent uptake was defined as uptake occurring in the absence of GABA at every concentration of modulator tested. GABA-dependent influx was defined as the difference between the uptake in the presence and absence of GABA for each experimental condition. Values for the parameters of maximum enhancement (Emax) and the concentration that elicited 50% of maximal enhancement (EC_{50}) were calculated by the fit of influx data to the logistic equation $e = e_{max}/(1 + (k/x)^{**}n)$, where e is the enhancement of influx

at a given concentration of agonist, k is the EC_{50} value for the agonist and n is the slope factor, using the iterative routine FITFUN on the PROPHET computer system. The statistical comparisons of EC_{50} or maximum flux values were performed with the Student's t-test. Efflux data were fitted using the equation $p=p_1*e^{-k1t} + p_2*e^{-k2t}$, where p is the percent of control cpm retained at time 0, p_1 and p_2, the fraction of efflux in each component, and k_1 and k_2, the rate constants for the rapid and slow phases of efflux, respectively. Efflux data were analyzed by iterative curve fitting using FITFUN on the PROPHET computer system.

Materials: $^{36}Cl^-$ (specific activity 15.06-18.52 mCi / g) was purchased from NEN Dupont. Deltamethrin was a gift from Dr. P. Foulhoux (Roussel Uclaf, Romainville, France) and the cypermethrin isomers and permethrin were donated by FMC Corporation (Princeton, N.J.). Muscimol and TBPS were purchased from RBI and veratridine (VTD) from Sigma. Avermectin was a gift from Merck.

Results

The time course of GABA-dependent $^{36}Cl^-$ uptake in trout brain synaptoneurosomes was determined using 300 μM GABA. As shown in Figure 1, GABA produced a time-dependent increase in $^{36}Cl^-$ influx that was linear up to 30 seconds. This time point was used in all other assays. The concentration-dependence of both GABA and muscimol was examined, as shown in Figure 2A. Both agonists produced similar levels of maximal influx: 18.1 $\pm$ 2.1 and 18.7 $\pm$ 1.2 nmol/ mg of protein / 30 sec., for muscimol and GABA, respectively.

Muscimol was more potent with an EC_{50} value of 0.95 $\pm$ 0.44 μM as compared to the EC_{50} value of 3.67 $\pm$ 0.89 μM for GABA. Bicuculline (1 mM) inhibited all of the GABA-dependent influx and 15% (n=4) of the basal uptake, indicating that some endogenous GABA was present in the incubate. TBPS at 1 μM inhibited 85% of GABA-dependent influx. Pentobarbital (PB) acted as an agonist in this system, producing a bi-phasic concentration-response curve with maximal influx at 0.3-1 mM (Fig. 2B) and decreasing uptake with concentrations greater than 1 mM. Maximal PB uptake was 19.8 $\pm$ 4.3 nmol / mg of protein / 45 sec. at 300 μM. Thus the pharmacology of this $^{36}Cl^-$ influx was consistent with a specific response mediated by the $GABA_A$ receptor.

Deltamethrin produced a dose-dependent inhibition of GABA-dependent $^{36}Cl^-$ uptake. GABA-dependent $^{36}Cl^-$ uptake was defined as the difference between uptake in the presence and absence of GABA at every concentration of pyrethroid tested. This definition is critical because deltamethrin's effect was primarily an increase of the GABA-independent or basal $^{36}Cl^-$ uptake. The maximal inhibition produced by deltamethrin was 80% (Fig.3) and the IC_{50} value for deltamethrin was 210 $\pm$ 50 nM (Table 1).

To investigate the nature of this inhibition, GABA concentration-response experiments were conducted in the absence and presence of 500 nM deltamethrin. Deltamethrin inhibition was noncompetitive (Fig. 4), as indicated by the decrease in maximum influx (17.9 $\pm$ 2.3 and 12.7 $\pm$ 1.3 nmol / mg of protein / 30 sec, control and deltamethrin-treated, respectively, $p<0.05$) with no significant change in the EC_{50} value (2.9 $\pm$ 1.6 and 4.9 $\pm$ 1.8 μM).

To further characterize this pyrethroid effect on GABA-dependent $^{36}Cl^-$

influx, two cypermethrin isomers, (1RαS)-*cis* and (1SαR)-*cis*, and permethrin, which is structurally similar to cypermethrin but without the α-cyano moiety, were also tested. (1RαS)-*cis*-cypermethrin caused a similar increase in basal flux and concurrent decrease in GABA-dependent influx as deltamethrin, with an IC_{50} value of 151 $\pm$ 16 nM and maximal inhibition of 82% (Fig. 5A and Table 4-1). (1SαR)-*cis*-cypermethrin, a less toxic isomer in insect and mammalian assays, produced no inhibition over the range of 0.3 to 30 μM, indicating essentially absolute stereoselectivity. Permethrin was much less potent than the Type II pyrethroids, with an IC_{50} value of 9.8 $\pm$ 3.5 μM, but it inhibited a similar level of GABA dependent influx: 72% at 30 μM (Fig. 5B and Table 4-1). The limited solubility of these compounds precluded investigation of the effect of higher concentrations.

To determine if an interaction with the voltage-dependent sodium channel was necessary for the deltamethrin-induced increase in basal $^{36}Cl^-$ uptake, tetrodotoxin (TTX, 1 μM), a sodium channel blocker, was co-incubated with deltamethrin. Under these conditions, TTX effected essentially a complete inhibition of the deltamethrin-induced increase in basal uptake and returned GABA-dependent influx to normal levels (Fig. 6). TBPS (1 μM) also inhibited 80% of the deltamethrin-induced increase in basal $^{36}Cl^-$ uptake, demonstrating the involvement of chloride channels, possibly voltage-dependent chloride channels, in the deltamethrin effect. The efficacy of veratridine (VTD), an alkaloid which activates voltage-gated sodium channels, to produce a deltamethrin-like effect was investigated to further define the mechanism of the deltamethrin-induced increase in basal flux. VTD produced a significant increase in basal flux to 26 nmol / mg of protein / 30 sec. and inhibited GABA-

dependent influx by 86 ± 3%, with an IC_{50} value of 0.44 ± 0.11 μM. The veratridine-induced increase in basal uptake was inhibited by 80% by 1 μM TTX (Fig. 7). The enhancement of $^{36}Cl^-$ uptake by VTD supports the contention that the pyrethroid-stimulated $^{36}Cl^-$ uptake involves interaction at voltage-dependent sodium channels.

Concentrations of deltamethrin and VTD that produced maximal increases were used separately, in combination and with TTX to determine if the effects of these two neurotoxic compounds were additive. Both compounds increased basal levels of chloride uptake (24.6 ± 2.3% and 21.3 ± 2.7% increase above basal, VTD and deltamethrin, respectively, n=3. Fig. 8). With the addition of both compounds to the incubation medium, no increase above the level measured with VTD alone was observed (27.3 ± 2.3%). TTX, when coincubated with both compounds, significantly decreased the magnitude of toxicant-stimulated uptake (9.3 ± 2.7% of basal uptake), indicating that blockade of voltage-dependent sodium channels inhibited the enhancement of basal uptake by both compounds.

$^{36}Cl^-$ efflux from preloaded synaptoneurosomes was measured in an attempt to further define the deltamethrin modulation of chloride movement. For all compounds, the time course of efflux was measured at intervals over an eight minute period. Basal efflux was best described by a two component model, with 33% in the rapid phase with a k_1 of 1.01 min^{-1} and 68% in the slow phase with a k_2 of 0.04 min^{-1} ($F_{(4,2)}=19.3$, $p<0.05$). GABA did not cause a measurable increase in $^{36}Cl^-$ efflux. In contrast, avermectin, an anthelmintic that increased chloride efflux from rat synaptoneurosomes, and pentobarbital produced a time-

dependent increase in efflux from trout brain vesicles (Fig.9A and B). Analysis of the pooled data from three experiments indicated that avermectin increased the rate of the fast phase (1.68 min^{-1}, Table 4-2). Deltamethrin caused no measurable change from control nor did it alter AVA-dependent or pentobarbital-dependent rates. VTD was also without detectable effect in this system. Thus this assay appears to lack the sensitivity to detect the changes in chloride movement by GABA, pyrethroids or VTD.

Discussion

The data presented herein indicate that pyrethroid insecticides may interfere with $GABA_A$ receptor function indirectly via an interaction with voltage dependent sodium channels in trout brain. This indirect inhibition was stereospecific, generalized to at least 3 pyrethroids, of high potency (nanomolar range for type II pyrethroids) and TTX-sensitive. Furthermore, veratridine, an alkaloid which increases sodium conductance, elicited similar changes in basal chloride uptake. A pyrethroid interaction with sodium channels may cause an increase in sodium ion flux into the vesicles, and consequently, an increase in basal uptake of chloride ions through VDCC, thereby decreasing the chloride electrochemical gradient. This mechanism may be operative in the pyrethroid-induced attenuation of GABA-dependent $^{36}Cl^-$ influx.

As verification of the methodology, GABA and muscimol produced a time and concentration-dependent increase in chloride uptake in trout synaptoneurosomes which exhibited the pharmacology consistent with activation of the $GABA_A$ receptor. Bicuculline, a competitive inhibitor of the GABA site,

and TBPS, which binds to a site associated with the ion channel, both inhibited GABA-dependent flux. Guinea pig brain vesicles have been shown to establish a transmembrane potential of -58 to -78 mV at physiological K^+ concentrations (Creveling et al., 1980) which indicates that synaptoneurosomes establish normal chloride and sodium gradients. Our results suggest that trout synaptoneurosomes establish a membrane potential as well.

The magnitude of GABA-dependent influx was slightly lower than that reported in rodent studies: 18-20 nmol/ mg of protein/ 30 sec vs. 25-40 nmol / mg of protein / 5 sec for rodent vesicles (Ramadan, 1988a). GABA and muscimol were more potent in trout brain, with EC_{50} values of 3.67 and 0.95 μM respectively, which is approximately 4-fold more potent than reported values in rodent preparations. Electron microscopic examination of the rodent preparation revealed many vesicles and intact synaptic elements (Allan and Harris, 1986). The slightly lower level of maximal GABA-dependent influx in trout could be due to the differences in membrane lipids between fish brain and rodent brain, which may influence the tendency of cellular membranes from these two species to form vesicles upon gentle homogenization. The difference in the maximal level of GABA-dependent influx is not due to differences in receptor number, as both species have approximately equal density of $GABA_A$ receptors as measured by [^{35}S]TBPS binding (Eshleman and Murray, in press; Marvizon and Skolnick, 1988) The rate of influx was much slower than for rodent, reaching a maximum at 30 seconds instead of 3-5 sec. This slower GABA-dependent influx is presumably due to the temperature at which the trout assays were conducted: 10° C (a physiologically relevant temperature inasmuch

as the trout were maintained in water which varied between 10 and 13°C) vs. 30° for rodent studies.

We have reported recently (Eshleman and Murray, in press) that several type I and II pyrethroids inhibited the binding of [^{35}S]TBPS to trout brain membranes. GABA and deltamethrin were positive allosteric modulators of each other's inhibitory activity toward [^{35}S]TBPS binding: each increased the potency of the other and GABA increased the efficacy of deltamethrin as well. Several type I and II pyrethroids were tested and the IC_{50} values were all in the micromolar range. Thus, there is an established interaction of pyrethroid insecticides with the $GABA_A$ receptor of trout brain.

Pyrethroid interaction with the $GABA_A$ receptor has been detected in several species: studies using rodents have given support to the hypothesis that the $GABA_A$ receptor is involved in pyrethroid toxicity. This evidence was on several levels: diazepam, which interacts with the benzodiazepine site of the $GABA_A$ receptor, delayed signs of toxicity of type II pyrethroids (Gammon et al., 1982); convulsions elicited by type II pyrethroids were similar to those elicited by $GABA_A$ antagonists such as picrotoxin (Lawrence et al., 1985); and type II pyrethroids were reported to inhibit [^{35}S]TBPS binding in a stereospecific manner (Lawrence and Casida, 1983; Seifert and Casida, 1985a).

Using $^{36}Cl^-$ uptake methodology, several groups have investigated the ability of pyrethroids to perturb this system in rodent membranes. Abalis et al. (1986) observed stereospecific inhibition of chloride flux by cypermethrin isomers. Ramadan et al. (1988a) measured a decrease in GABA-dependent uptake that was not due to an increase in basal flux. This effect was

stereospecific among cypermethrin isomers and type II compounds were generally more potent than type I, though in the micromolar range. Inhibition of GABA-dependent influx by pyrethroids was incomplete and little correlation between inhibitory potency and LD_{50} levels was observed. Bloomquist et al. (1986) measured a 50% inhibition of GABA dependent uptake by high concentrations of deltamethrin and also observed less-potent inhibition by a non-toxic enantiomer. Devaud (1988) reported that deltamethrin and (1RαS)-*cis*-cypermethrin increased basal uptake by a third, but decreased GABA-dependent influx by more than 90% with potency in the nanomolar range; both effects were inhibited by TTX. These reports give support to an allosteric interaction of pyrethroids with the $GABA_A$ site, at concentrations similar to sodium channel interactions (vide infra). Unlike these results obtained with rodent membranes, the effects reported herein were primarily on the basal uptake of $^{36}Cl^-$. The affinities of pyrethroids for the $GABA_A$ receptor reported in piscine and rodent preparations were generally in the micromolar range, thus a direct inhibition of GABA-dependent influx may have been obscured by the higher potencies of pyrethroids in the present investigations.

Evidence for an interaction with sodium channels in pyrethroid poisoning has been accumulating since early experiments with the first synthetic pyrethroids. Electrophysiological studies conducted with arthropod nerve preparations showed that type I pyrethroids produced a slow tail current by modifying a fraction of sodium channels which returned to resting state much slower than control sodium channels (Lund and Narahashi, 1981). These modified channels caused repetitive firing upon electrical stimulation of the

nerves. Type II pyrethroids were found to depolarize without repetitive firing; the tail current time constants extended to several minutes in crayfish (Salgado and Narahashi, 1982). Fenvalerate induced a steady state sodium current at potentials less negative than -100 mV. In vertebrates, α-cyano pyrethroids produced tail current time constants that were much shorter than in insects; thus the open state of these sodium channels was not stabilized as effectively (Vijverberg, 1983). The sodium channel has binding sites for many compounds which modulate sodium flux, including tetrodotoxin, batrachotoxin, aconitine, scorpion toxins and DDT (Catterall, 1988). Many of these sites have been shown to interact allosterically via binding studies.

Since reports investigating the interaction between pyrethroids and alkaloid activators of sodium channels in rodent brain membranes reveal a synergy for sodium channel activation, the lack of additivity between VTD and deltamethrin was unexpected. Lombet et al., (1988) reported that Ru 39568, a potent pyrethroid, increased [^{3}H]batrachotoxin A 20α-benzoate (BTX) by up to 30 fold. BTX and VTD bind to the same site of the sodium channel inducing an increase in sodium flux, but with differing affinities. Although the pyrethroid had no measurable effect on $^{22}Na^{+}$ uptake into neuroblastoma cells when applied alone (Jacques et al., 1980), it increased the BTX-dependent $^{22}Na^{+}$ influx in these cells by 4 fold, possibly by increasing the probability that sodium channels would be in the open form thus favoring the binding of BTX. The pyrethroid effect was additive with the enhancement by sea anemone toxin and brevetoxin of the BTX site, showing that it interacted with a unique site on the protein. Deltamethrin was less potent than Ru 39568, but its K_d for BTX binding enhancement and $K_{0.5}$

for increasing $^{22}Na^+$ flux in the presence of BTX were both between 1 and 10 μM. Thus the concentration of deltamethrin used in our study should have been sufficient to produce significant occupancy of its binding site on the sodium channel of trout brain.

Although electrophysiological experiments have shown that pyrethroids are active at the sodium channel of mammalian neurons by prolonging the action potential at concentrations greater than 0.1 μM, several reports confirm the inability of $^{22}Na^+$ flux experiments to detect any modulation of $^{22}Na^+$ movement by pyrethroids in the absence of other active compounds (Jacques et al., 1980; Ghiasuddin and Soderlund, 1985; Lombet et al., 1988). Similarly most chloride influx studies indicate that pyrethroids have little or no effect on basal $^{36}Cl^-$ uptake in mammalian vesicles. The increase in $^{36}Cl^-$ basal uptake in trout brain was inhibited by TTX and mimicked by VTD, suggesting that the ability of pyrethroids to generate a sodium flux is greater in the trout brain, since the chloride entry is dependent on sodium ion movement as evidenced by the blockade by TTX.

Sodium channel diversity both between organs of a single species and among different species has been detected by sequence analysis of cDNA clones. The molecular structure of the sodium channel is a large α-subunit of ~200,000 daltons and, when present, two smaller subunits, $\beta1$ and $\beta2$ (reviewed in Trimmer and Agnew, 1989). Analysis of cDNA clones of α-subunits has detected variations in a putative cytoplasmic loop between electroplax and mammalian brain sodium channels. Degrees of glycosylation vary as does the type of carbohydrate between sodium channels of brain, muscle and electroplax. Distinct Na channel cDNAs have been identified from rat skeletal muscle (Trimmer et

al., 1988) and four from mammalian brain (Noda et al., 1986) Pharmacological evidence for a difference between mammalian and piscine sodium channel modulation by BTX, VTD and DDT (Stuart et al., 1987) gives support to the contention that the piscine sensitivity to pyrethroids may result from a difference in structure of trout brain sodium channels. A difference in channel structure would allow pyrethroids to modulate sodium flux to a greater extent than detectable in rodent assays.

The route of entry of chloride ions after pyrethroid application would appear to be channel-mediated since the uptake is blocked by TBPS. The $GABA_A$ receptor has been shown to be voltage-sensitive only at hyperpolarizing membrane potentials (Akaike et al., 1986), thus an influx of chloride ions through this protein would not be sensitive to blockade by TTX. A second possible channel is the voltage-dependent chloride channel (VDCC). VDCC has been characterized from several tissues, including electric ray, squid giant axon and cultured rat skeletal muscle (Eldefrawi and Eldefrawi, 1987). These channels have some characteristics in common with the $GABA_A$ receptor such as blockade by TBPS, picrotoxin, lindane and cyclodiene insecticides. Their function is to determine and maintain the resting membrane potential. Thus VDCC in brain membranes would be sensitive to changes in membrane potential, an effect blocked by TTX, and the chloride influx through this protein would be blocked by TBPS. The possible sequence of events is a pyrethroid-dependent increase of sodium uptake which causes opening of VDCC. Chloride would then flow down its concentration gradient, thus attenuating GABA-dependent $^{36}Cl^-$ influx.

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Figure 4-1. Time course of GABA-dependent $^{36}Cl^-$ influx.

GABA, at a final concentration of 300 μM, and $^{36}Cl^-$ were added to the membrane homogenate; influx was terminated at the indicated time intervals. Results shown are the average of three experiments, each performed in triplicate (mean $\pm$ SEM). Based on this time course, 30 seconds was chosen as the end point for further studies.

Figure 4-1.

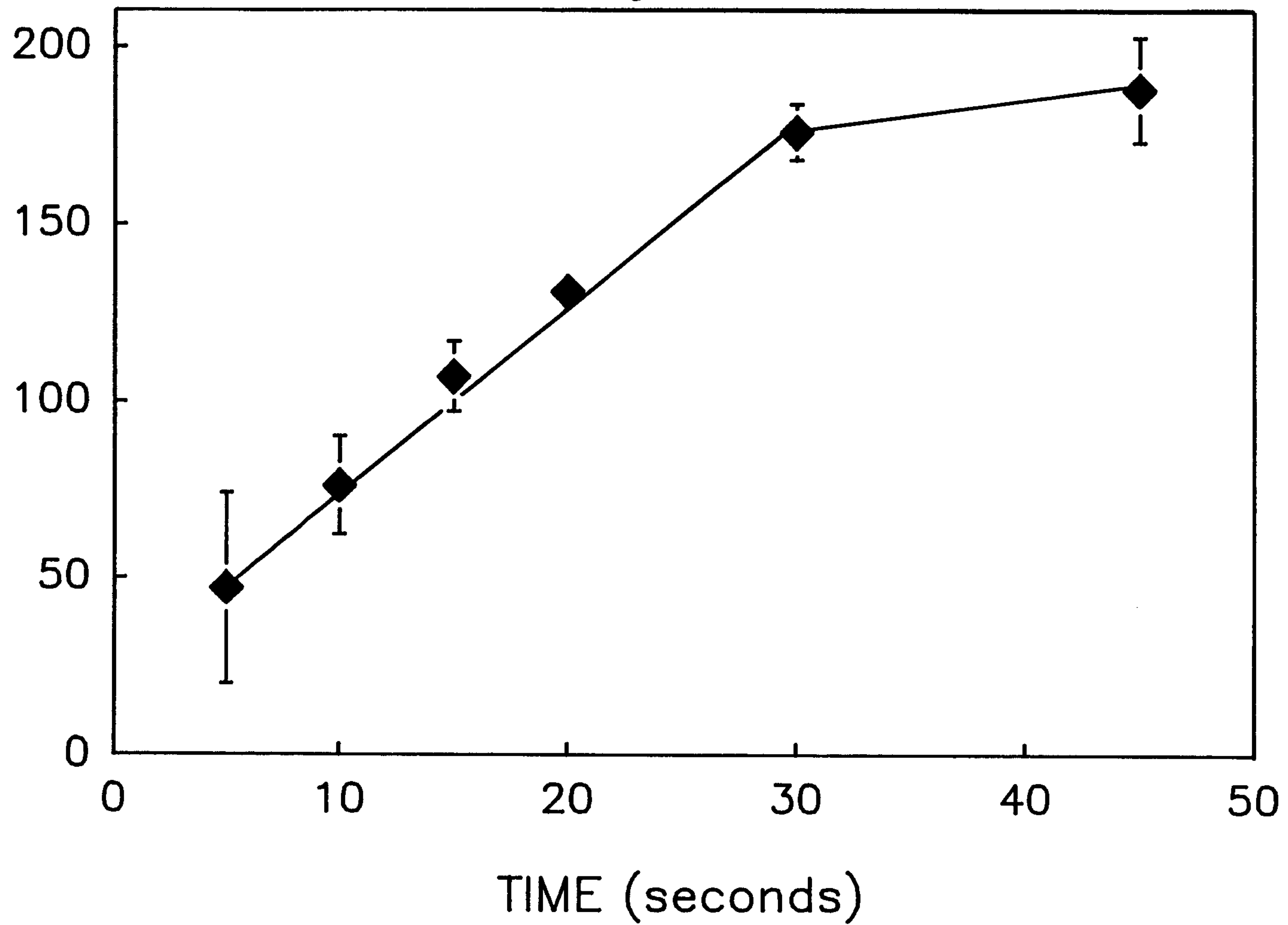


Figure 4-2. GABA, muscimol and pentobarbital concentration-response curves. A. GABA and muscimol were added simultaneously with the $^{36}Cl^-$solution and the influx terminated 30 sec. later. Data presented are the average ($\pm$ SEM) of 10 and 3 experiments each performed in triplicate, GABA and muscimol respectively. Non-linear least squares analysis of this data computed an E_{max} of 18.7 $\pm$ 1.2 and 18.1 $\pm$ 2.1 nmol / mg of protein / 30 sec. and EC_{50} values of 3.67 $\pm$ 0.89 and 0.95 $\pm$ 0.44 μM for GABA and muscimol, respectively. B. Pentobarbital was added simultaneously with $^{36}Cl^-$ and the influx terminated 45 seconds later. PB produced a biphasic response with maximum enhancement between 0.3 and 1 mM.

Figure 4-2A.

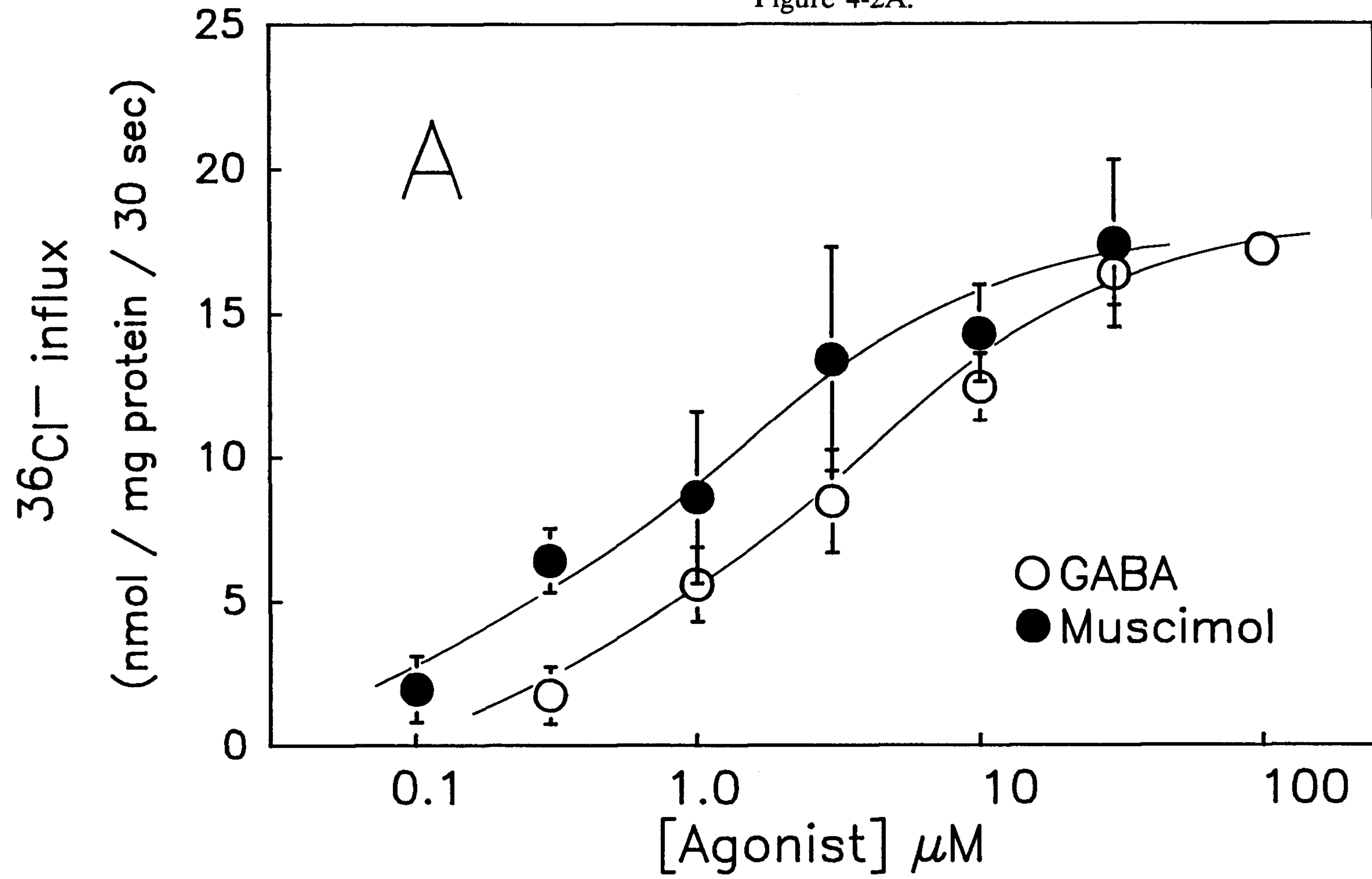


Figure 4-2B.

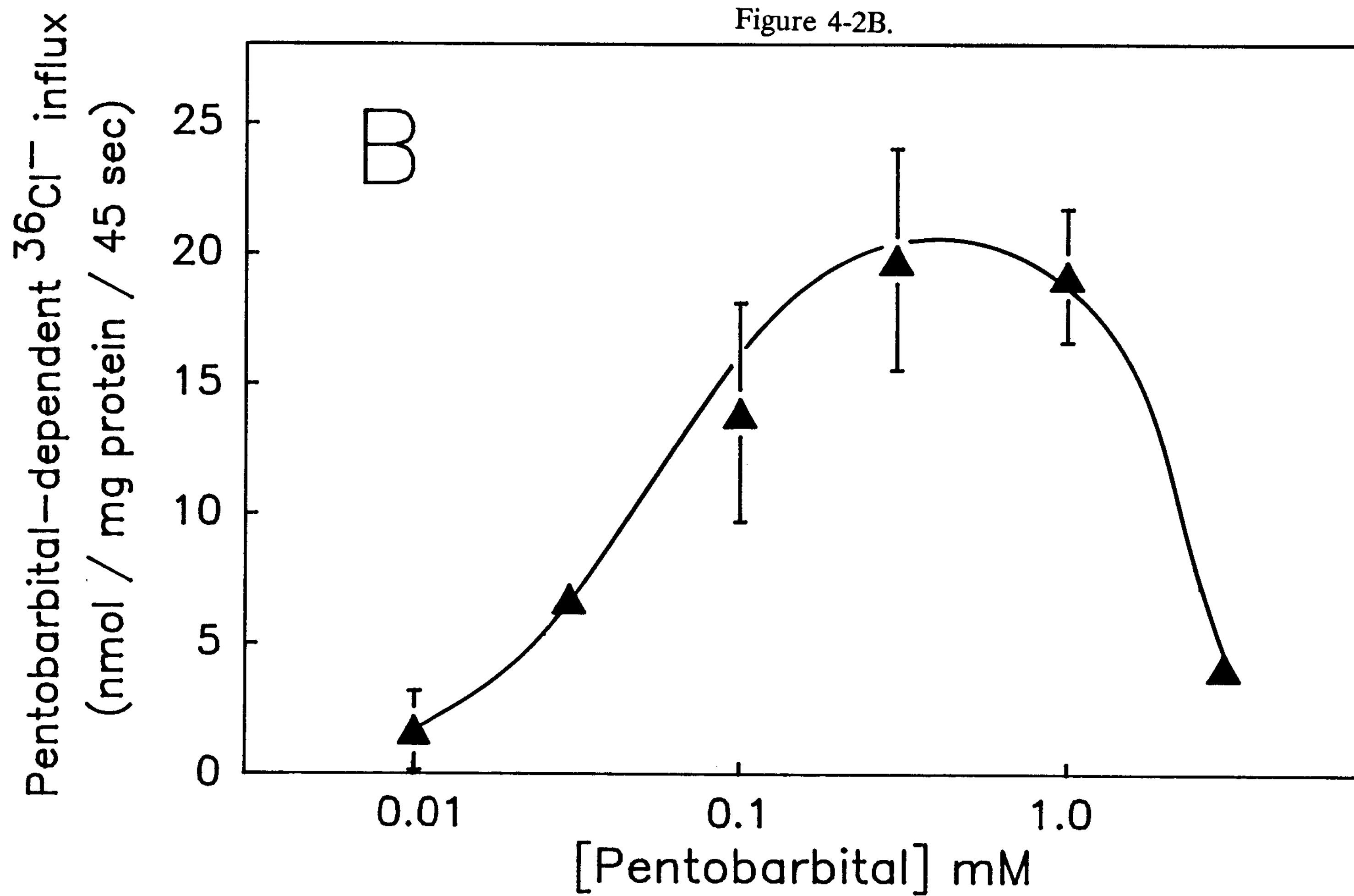


Figure 4-3. Concentration-response curve for deltamethrin. Basal uptake was measured in the absence of GABA at each concentration of deltamethrin and subtracted from total influx in the presence of GABA to determine GABA-dependent influx. Data shown are the average (± SEM) of 3 experiments performed in triplicate. Parameters derived from this data are shown in Table 1.

Figure 4-3.

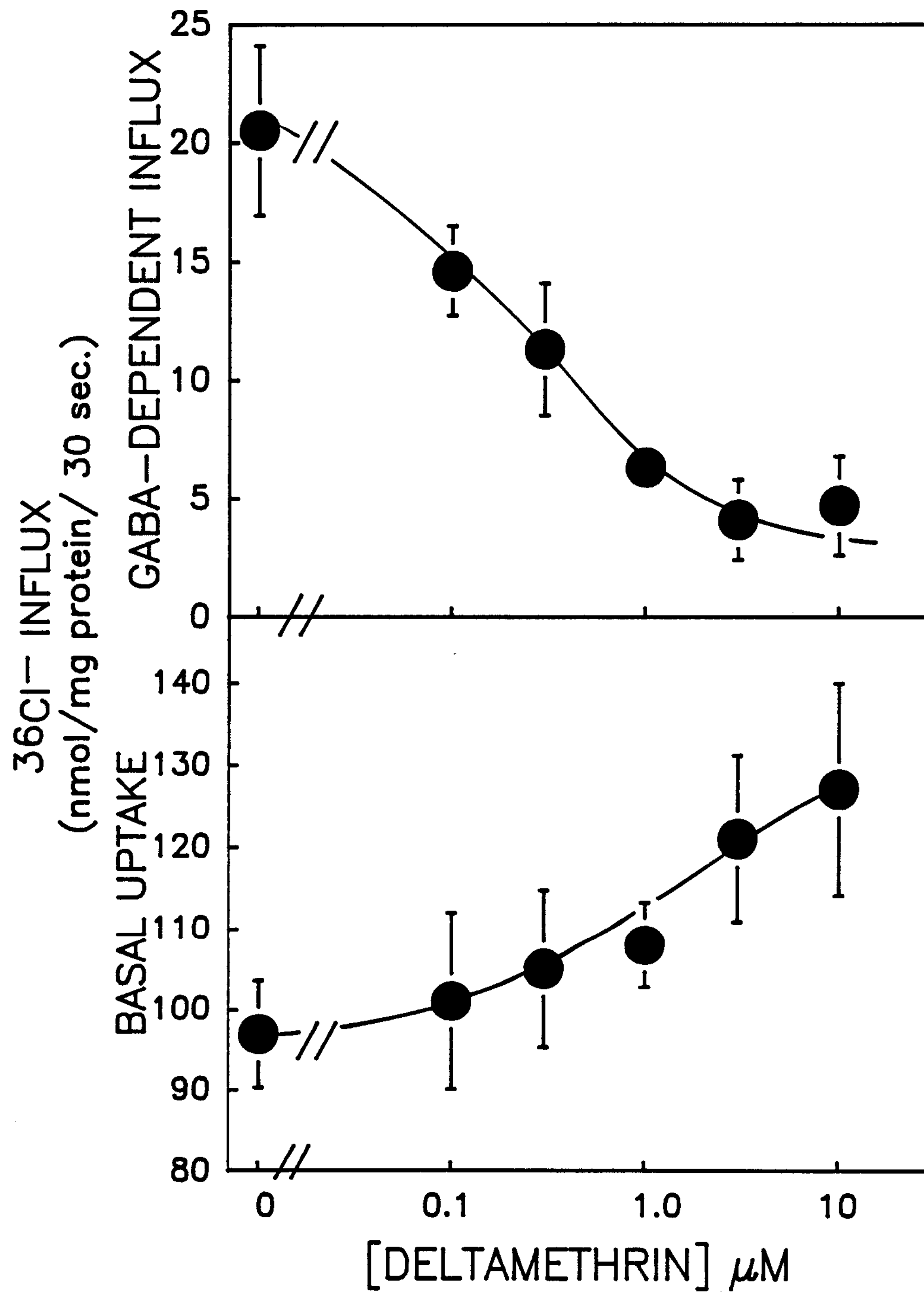


Figure 4-4. Noncompetitive inhibition by deltamethrin of GABA concentration-response curve. Deltamethrin, 500 nM, was added 10 minutes prior to addition of GABA. Data shown represent the mean ± SEM of 4 separate experiments each performed in triplicate. Deltamethrin significantly inhibited the GABA-dependent influx at both 30 and 100 μM GABA. Deltamethrin produced a noncompetitive inhibition as evidenced by the decrease in Emax (17.9 ± 2.3 and 12.7 ± 1.3 nmol / mg of protein / 30sec, control and deltamethrin-treated, respectively. In contrast, the addition of deltamethrin had no effect on the potency of GABA: GABA EC_{50} values were 2.9 ± 1.6 and 4.9 ± 1.8 for control and treated.

Figure 4-4.

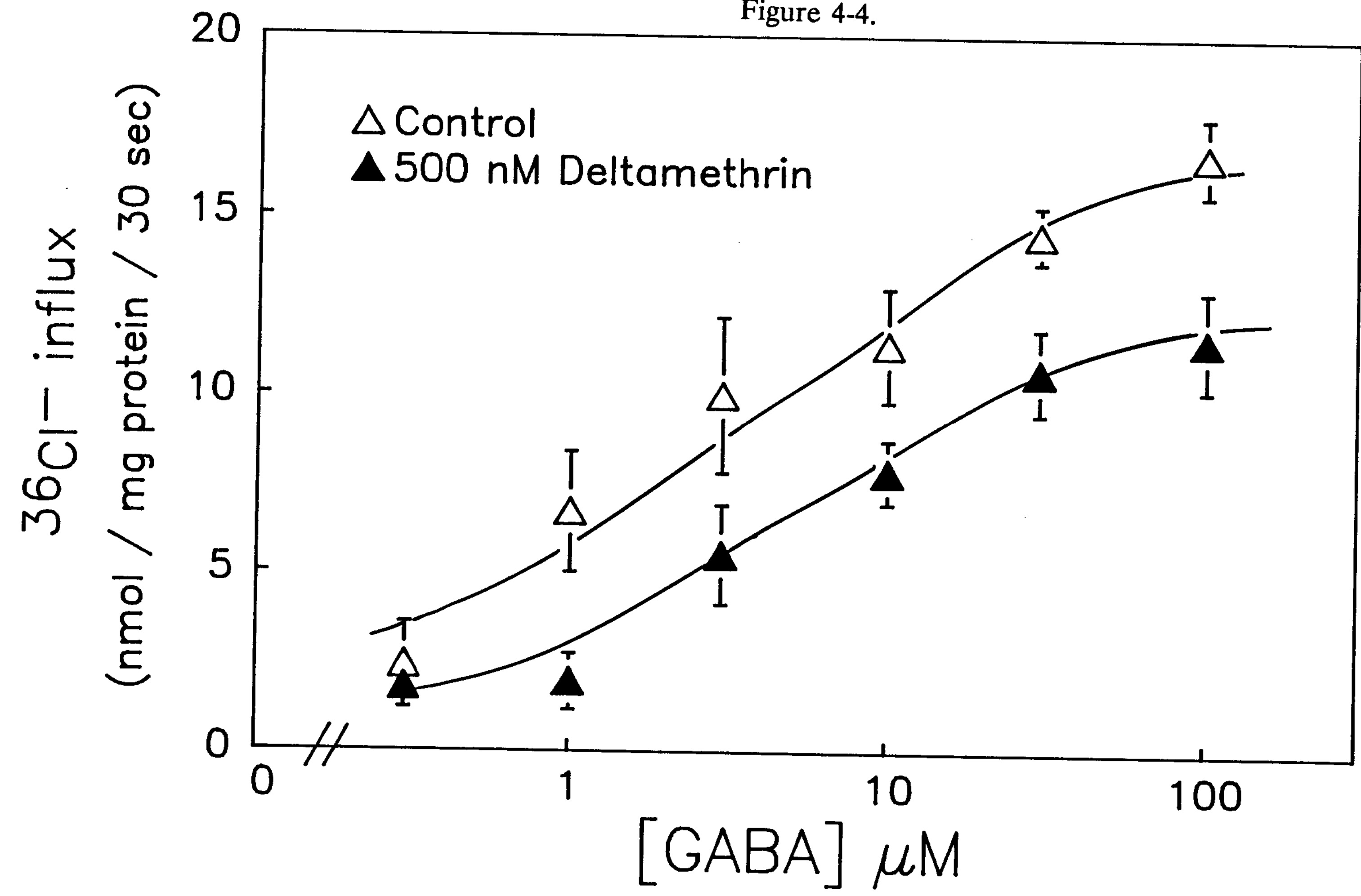


Figure 4-5. Concentration-response curves for (1RαS)-*cis* and (1SαR)-*cis*-cypermethrin and 1R-*cis*-permethrin. A. Cypermethrin isomers. B. 1R-*cis*-Permethrin. Basal uptake was measured in the absence of GABA at each concentration of pyrethroid and subtracted from total influx in the presence of GABA to determine GABA-dependent influx. (1RαS)-*cis*-cypermethrin and 1R-*cis*-permethrin increased basal uptake. Data shown are the average (± SEM) of 4, 2, and 3 experiments for (1RαS)-*cis*-cypermethrin (●), (1SαR)-*cis*-cypermethrin (○), and 1R-*cis*-permethrin, respectively. Parameters derived from this data are shown in Table 1.

Figure 4-5A.

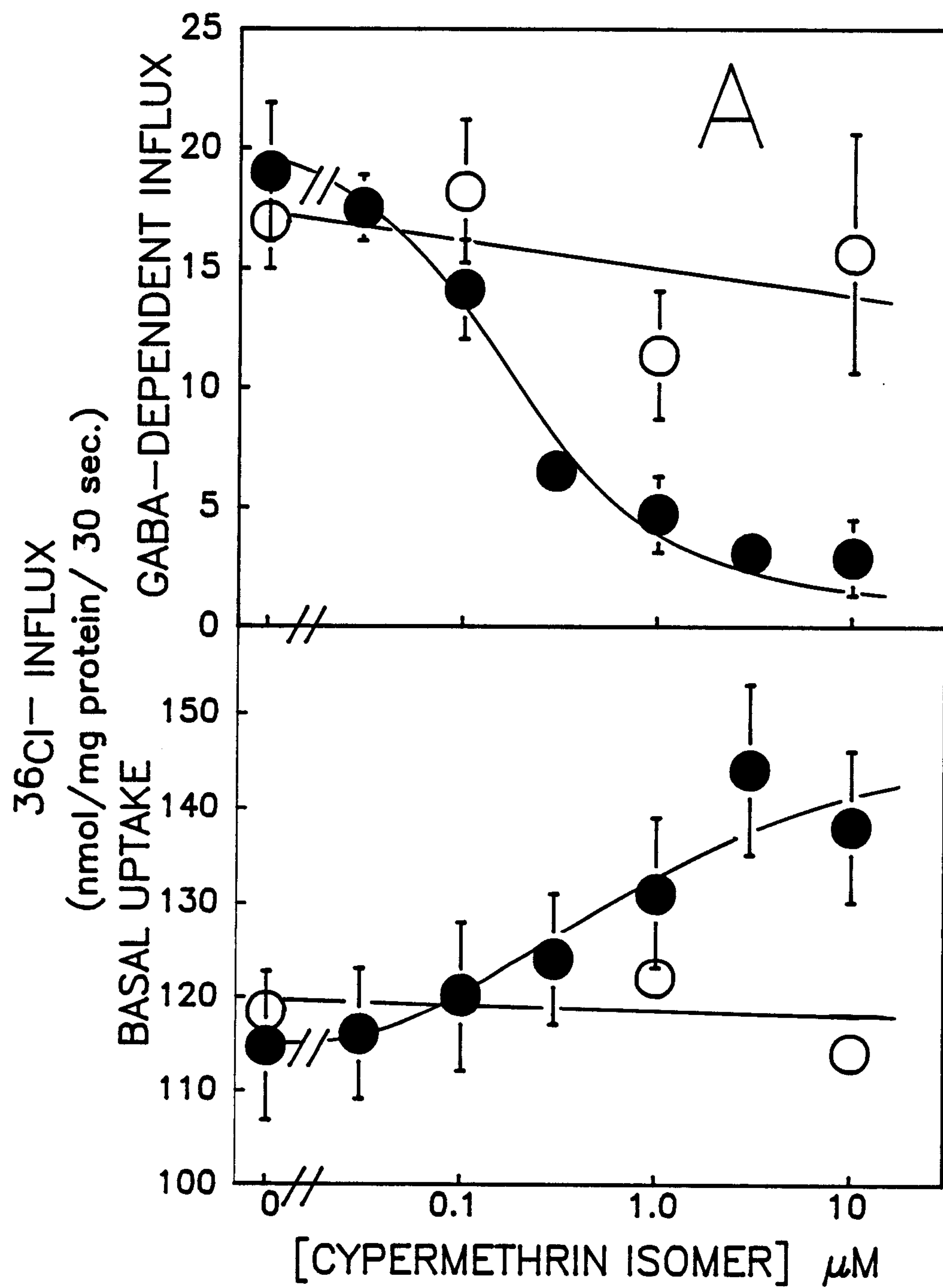


Figure 4-5B.

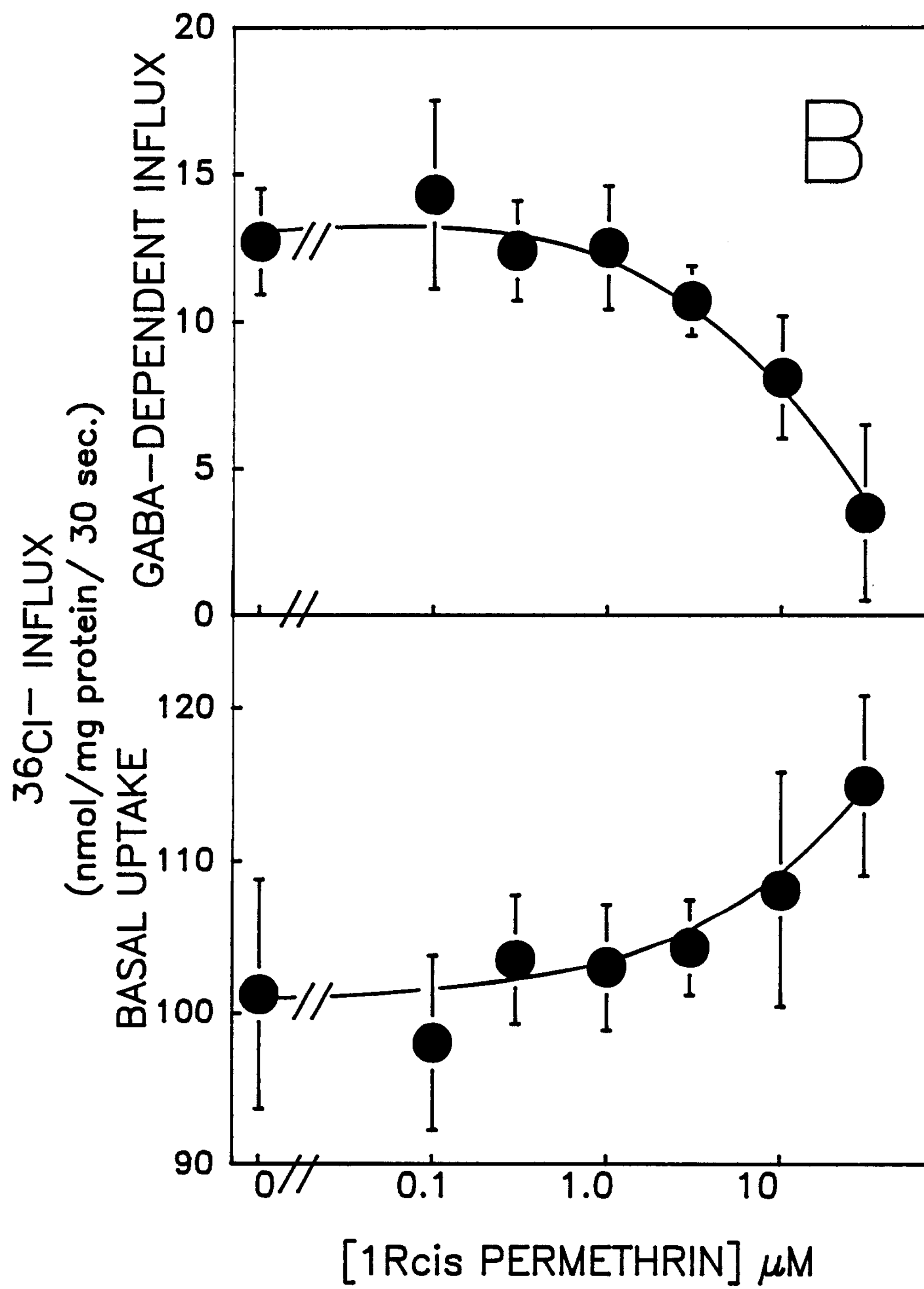


Figure 4-6. Tetrodotoxin (TTX) inhibition of the deltamethrin-induced increase in basal uptake. Tetrodotoxin and deltamethrin were added before the 12 minute preincubation. Deltamethrin caused a maximal increase in basal uptake of 12 and 3.5 nmol/mg of protein / 30 sec. and maximal inhibition of GABA dependent flux of 89% and 42% in the absence and presence of TTX, respectively. Data are the mean ($\pm$ SEM) of 3 experiments conducted in triplicate.

Figure 4-6.

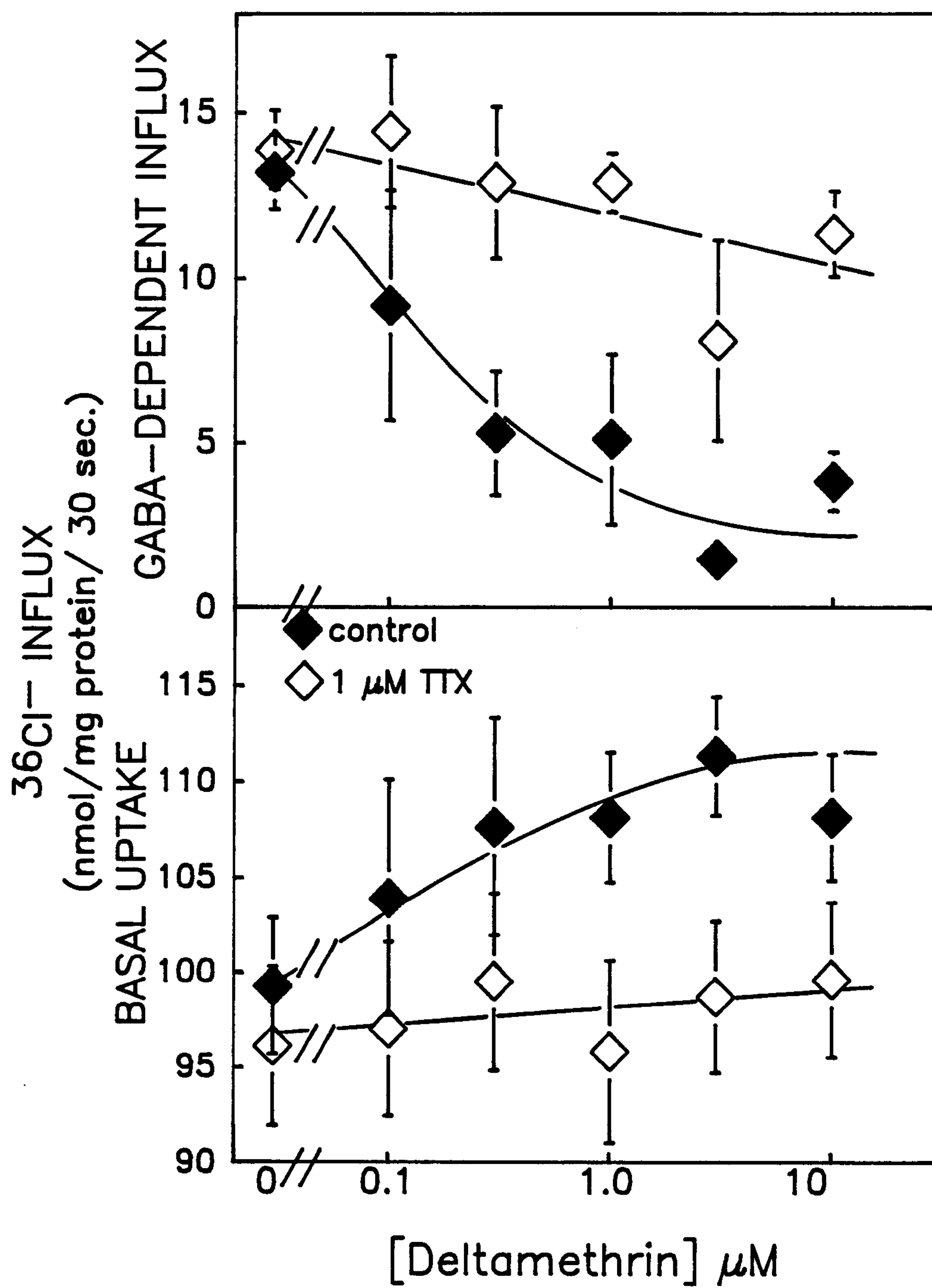


Figure 4-7. Veratridine-induced increase in basal uptake is inhibited by TTX. Veratridine, a voltage-dependent sodium channel activator, produced a dose-dependent increase in basal chloride flux, causing a decrease in GABA-dependent $^{36}Cl^-$ influx. Both the increase in basal uptake and a majority (80%) of the decrease in GABA-dependent influx were inhibited by TTX. Data shown are the mean (± SEM) of three experiments conducted in triplicate.

Figure 4-7.

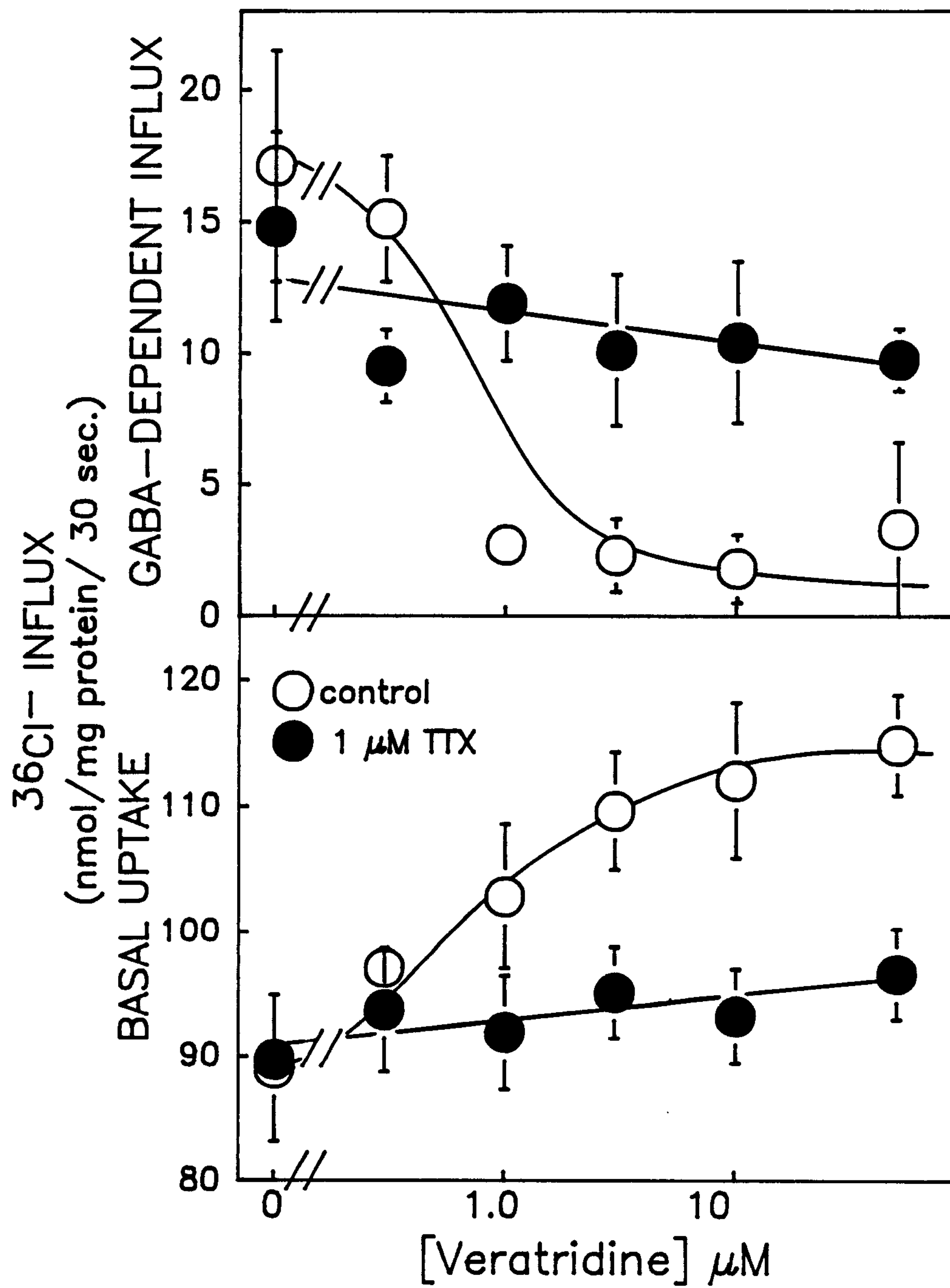


Figure 4-8. Lack of additivity of basal uptake of deltamethrin and veratridine. Maximal concentrations of both toxicants were applied, both alone and together. Basal uptake in the presence of both was not significantly different from basal with either alone. TTX was able to reverse the increase in basal uptake. Results are the mean ($\pm$ SEM) of one experiment conducted in quadruplicate that was repeated twice with similar results. The difference between the open and hatched bars represents GABA-dependent influx.

Figure 4-8.

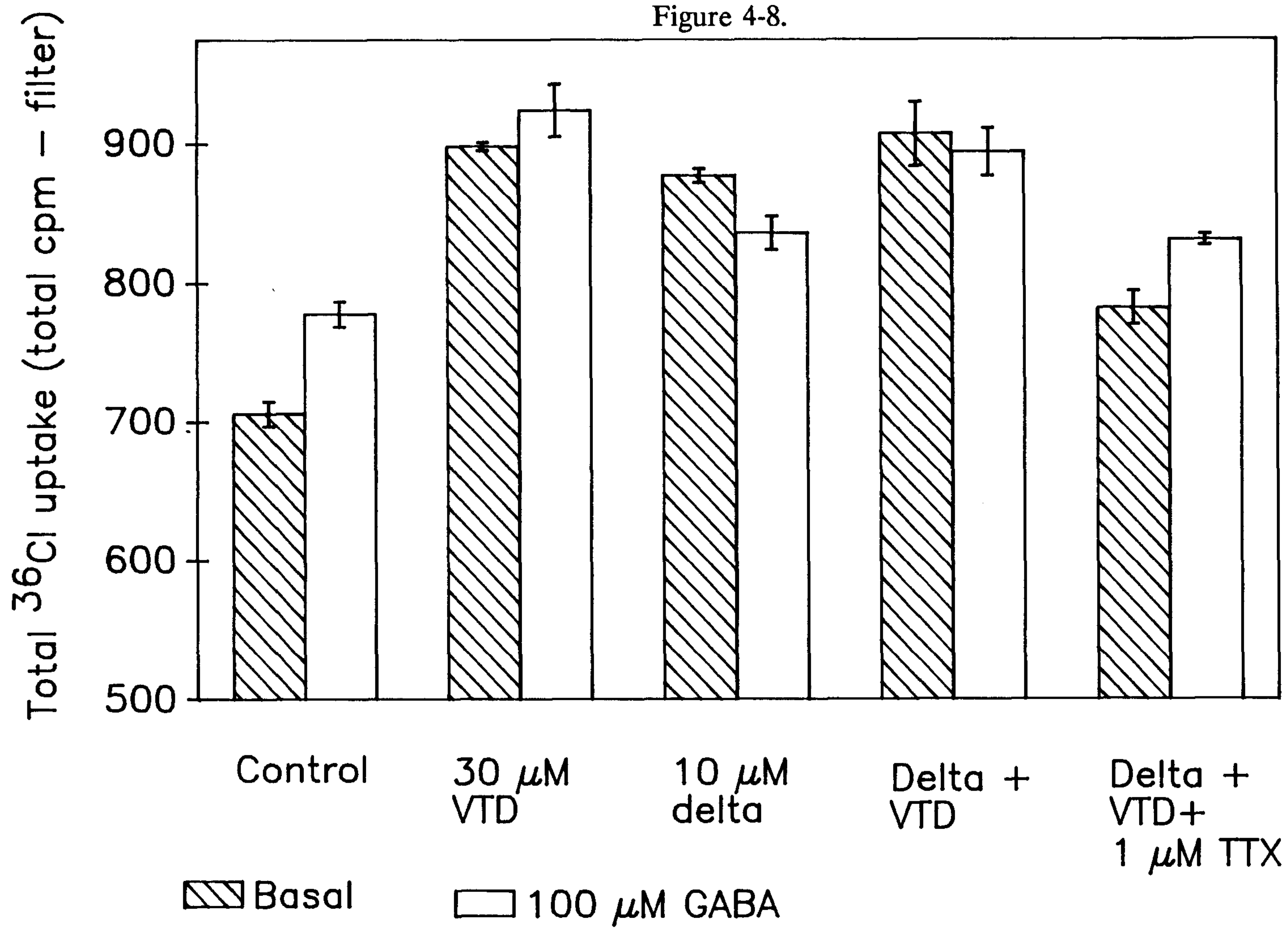


Figure 4-9. Lack of effect of deltamethrin on control, avermectin, (AVA)-, or pentobarbital-induced chloride efflux. $^{36}Cl^-$ efflux produces a larger signal than uptake in trout synaptoneurosomes, making it an attractive alternative to measure perturbations in $^{36}Cl^-$ flux.

A. Avermectin, an anthelmintic, was used as a positive control and significantly increased the rate of efflux. Results presented are the mean ($\pm$ SEM) of a representative experiment conducted in triplicate, repeated twice with similar results. Sensitivity of this assay may be low, as no effect on control efflux could be measured by deltamethrin or GABA (data not shown). B. Pentobarbital (PB, 1 mM) produced a smaller but still detectable increase in $^{36}Cl^-$ efflux. Deltamethrin was without effect on control or PB-stimulated efflux. Data presented are the mean ($\pm$ SEM) of a representative experiment conducted in triplicate, repeated with similar results.

Figure 4-9.

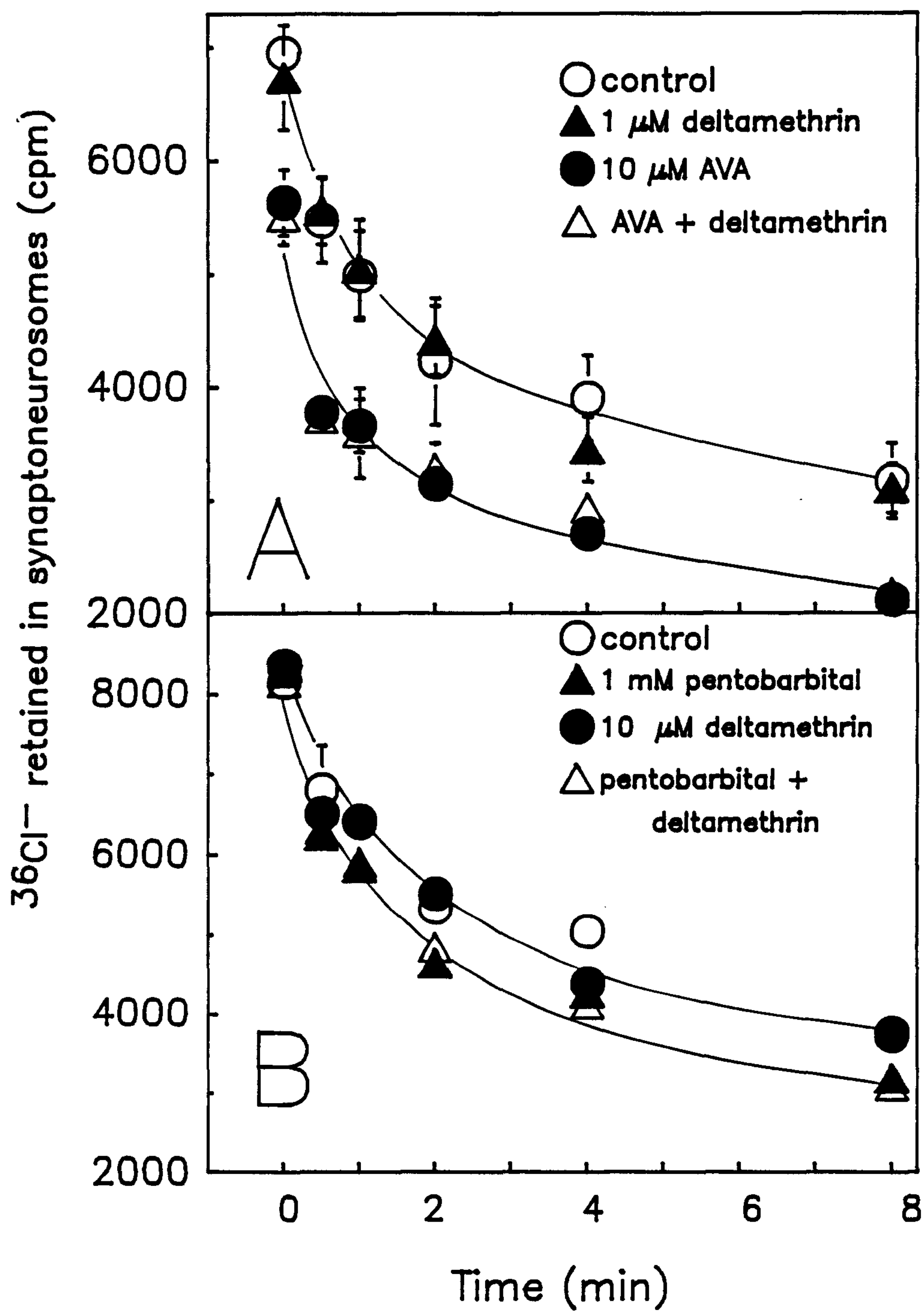


Table 4-1. Pyrethroid inhibition of GABA-dependent $^{36}Cl^-$ influx.

Pyrethroid	IC_{50} (nM)	% inhibition
Deltamethrin	210 ± 50	80
(1RαS)-*cis*-cypermethrin	151 ± 16	82
1R,cis permethrin	9800 ± 3500	72

Pyrethroids were added 12 minutes prior to the addition of 100 μM GABA. At least five concentrations of each compound were tested. (1SαR)-*cis*-cypermethrin did not inhibit GABA-dependent $^{36}Cl^-$ influx over the concentration range tested. Parameters were derived from analysis using the iterative curve-fitting program FITFUN on the PROPHET computer system.

Table 4-2. $^{36}Cl^-$ efflux from trout brain synaptoneurosomes. Effect of avermectin (AVA), veratridine (VTD) and deltamethrin (DEL) on control efflux.

	Parameters derived from two component efflux model			
Treatment	% rapid phase	k_1 min^{-1}	% slow phase	k_2 min^{-1}
Control	33±5.2	1.01±0.27	68±5.3	0.04±0.01
AVA	32±2.9	1.68±0.31	51±2.7	0.05±0.01
VTD	30±8.6	1.03±0.51	71±9.0	0.06±0.02
DEL	32±9.5	0.90±0.41	71±10	0.05±0.02

Data for each experiment was normalized to percentage of control cpm retained at time 0. The means of data from a total of 4, 4, 2, and 3 experiments (Control, AVA, VTD and DEL, respectively), conducted in quadruplicate, were fit simultaneously to the equation given in Methods.

Chapter 5

Convulsant Activity of Deltamethrin in Rainbow Trout

Amy J. Eshleman and Thomas F. Murray

Abstract

The sensitivity of rainbow trout to the neurotoxic activity of pyrethroid insecticides was investigated. Intraaortic dosing of deltamethrin to spinally transected rainbow trout revealed that 3 μg/kg body weight deltamethrin elicited toxic symptoms including seizures. The EC_{50} value for deltamethrin in eliciting seizure activity was 32 μg/kg. PK 11195 had no significant effect on deltamethrin-induced activity over 20 minutes when co-administered. Ro5-4864 at 3 mg/kg body weight elicited severe convulsions which suggests that Ro5-4864 interaction with the $GABA_A$ receptor may be relevant for the epileptogenic properties of this compound. These data confirm and extend previous reports of piscine sensitivity to this type of insecticide.

Introduction

Pyrethroid insecticides are neuroactive, synthetic derivatives of pyrethrins extracted from Chrysanthemum sp. The use of these neurotoxic pesticides is increasing, as they replace insecticides with the negative environmental effects of bioaccumulation and long-term persistence such as the organochlorines (DDT) or insecticides with low margins of safety as measured by lethal doses for insects and humans such as the organophosphates and carbamates. Thus the possibility

of exposure of non-target species to pyrethroids is increasing. Piscine species, which could be exposed by application of pyrethroids to bodies of water, are very sensitive to these insecticides: 96 hr LC_{50} values are less than 10 μg/L (Bradbury and Coats, 1989). Moreover, when administered by intraperitoneal or intravenous injection, permethrin and fenvalerate were 37 and 300 fold more potent in the trout than in the mouse in terms of LD_{50} values (Bradbury and Coats, 1989).

The current research was undertaken to investigate the susceptibility of rainbow trout (*Oncorhynchus mykiss*) to the convulsant actions of pyrethroids at sub-lethal doses. A second goal was to determine if PK 11195, an isoquinoline carboxamide derivative with nanomolar affinity for the peripheral-type benzodiazepine receptor and micromolar affinity for a site on the $GABA_{A3}$ receptor (Costa, 1988), influences pyrethroid convulsant activity. In rodents, PK 11195 inhibited the pro-convulsant activity of pyrethroid insecticides in the pentylenetetrazol seizure threshold model (Devaud et al., 1986). The neurotoxic activity of Ro5-4864 in trout was also assessed.

Methods

Rainbow trout, 1-2 years old, were obtained from the Food Toxicology and Nutrition Laboratory of Oregon State University (Corvallis, OR) and kept in flow-through tanks at ambient lighting. Trout were anaesthetized with MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester, 80 mg/l and $NaHCO_3$, 160 mg/l) and aerated with a recirculating system during surgery. Fish were immobilized with spinal transections, made at the 8th or 9th vertebrae, well behind the medulla oblongata where respiratory and cardiac control centers are

located. Indwelling dorsal aorta cannulas were inserted for dosing. This surgical preparation allowed dosing without the trauma of handling or the confounding effect of anaesthesia on the day of testing. Trout were allowed to recover for 24 hours before testing with pyrethroids.

All compounds were dissolved in a solution of 5% Emulphor, 5% ethanol, and 90% Cortland's saline (g/l: NaCl 7.25, KCl 0.38, $CaCl_2$*$2H_2O$ 0.23, $MgSO_4$*$7H_20$ 0.23, $NaHCO_3$ 1.0, NaH_2PO_4*H_2O 0.41).
The volume of injection was 1 ml/kg body weight.

Seizure severity scoring was based on the following table:

1. opercular flaring, head weaving
2. mild convulsion: single myoclonic head weaving event or wide opercular flaring
3. severe convulsions: continuous myoclonic head weaving with opercular flaring

Fish were observed continuously for a period of 20 minutes, and all activity was recorded. After observations, the total time was segmented into 2 minute epochs and the maximum seizure score during each epoch was tabulated. These maximum scores were summed over the 10 epochs and the totals averaged for at least three fish for each treatment.

The PK 11195 modulation of deltamethrin seizure score was tested using a Latin square design and fish were randomly assigned to treatment groups. Each fish was used for only one treatment. Statistical comparisons of seizure scores were performed with the Student's t-test. The EC_{50} value for deltamethrin was determined using the iterative routine FITFUN on the PROPHET computer

system with the equation $e=(e_{max})/(1+(k/x)^{**}n)$ where e is the seizure score at a given dose of deltamethrin, e_{max} is the highest seizure score possible, k is the EC_{50} value for deltamethrin and n is the slope factor.

Results

The deltamethrin dose-response relationship is shown in Figure 1. This data was obtained during the 20 minute period following dosing. The EC_{50} value for deltamethrin was 32 $\mu g/kg$ body weight (Table 1). The maximum seizure score possible was 30, which would be obtained with a maximum seizure score occurring every 2 minutes for 20 min.. Although a dose of 300 $\mu g/kg$ body weight did not produce the highest score possible, this was the highest dose included in this testing as 1 mg/kg was lethal in the time frame used. At the higher doses, the onset of seizures was rapid, with mild convulsions observed within two minutes and severe convulsions by 4 minutes.

Using a dose of deltamethrin somewhat lower than the EC_{50} value, the effect of PK 11195 was tested. PK 11195 (1 mg/kg) was administered simultaneously with deltamethrin or vehicle and the animals observed for 20 min. Deltamethrin significantly increased the seizure activity score (6 ± 2, $p < 0.05$). PK 11195 attenuated the deltamethrin-induced activity but the effect was not significant. With this protocol, PK 11195 treatment alone was not different from vehicle.

Ro5-4864, administered at 3 mg/kg, produced severe convulsions. The seizure score for 20 minutes of observation was 13.3 ± 2.9, greater than that elicited by 30 $\mu g/kg$ deltamethrin.

Discussion

The results described herein confirm and extend the reports of piscine sensitivity to acute administration of pyrethroid insecticides and indicate that deltamethrin intoxication involves effects on the central nervous system of trout. Toxicity as manifested by convulsions was observed with doses of deltamethrin as low as 3 μg/kg body weight. This convulsant activity was of rapid onset (less than 2 minutes at higher doses).

Reports using various dosing regimens provide data which verify the sensitivity of trout to these compounds. Glickman and Lech (1982) observed toxic signs due to permethrin in rainbow trout at brain concentrations of 1.5 μg/g brain after intraperitoneal injection. Cypermethrin isomers were more potent, having a threshold concentration for toxicity of 0.02-0.07 μg/g brain with a full range of toxic symptoms at 0.25 μg/g and 0.17 μg/g brain for *cis* and *trans* (1RαS) cypermethrin, respectively (Edwards et al., 1987). The higher toxicity of cypermethrin as well as fenvalerate as compared to permethrin has also been observed during aqueous exposures (55, 76 and 135 ppb for 24 hr. LC_{50}, respectively).

During aqueous exposure of fish to pyrethroids, symptom onset can develop much slower- 412 μg/l fenvalerate caused an increased cough rate at 10% survival time, seizures developed at 30% survival time which increased in frequency and intensity up to 70% survival time and death occurred at ~11 hours. The fenvalerate concentration measured in the brain of trout after death was 0.160 μg/g brain, the upper end of our i.v. dosing range with deltamethrin based on body weight (Bradbury et al., 1987).

By comparison, rodents are much less sensitive to these compounds. Intravenous administration of *cis* and *trans* permethrin yielded 13 and > 100 fold differences in 24 hr LD_{50} values in trout and mouse. The oral LD_{50} value for fenvalerate in rat was 450 mg/kg while the i.v. LD_{50} values for *cis*-cypermethrin were 50-100 mg/kg in rats (Vershoyle and Aldridge, 1980). Choreoathetosis appeared in rat at deltamethrin brain concentrations of 0.22-0.55 μg/g (Rickard and Brodie, 1985) while the toxic threshold in mice is at the high end of this range- 0.55 μg/g brain (Ruzo et al., 1979). In contrast, a fenvalerate concentration of 0.160 μg/g brain is lethal in trout (Bradbury et al., 1987). The present results indicate that overt toxic symptoms are elicited at low doses of pyrethroids. At comparable doses but by intraperitoneal injection, pyrethroids were associated with proconvulsant activities but elicited no overt toxic symptoms in rat (Devaud et al., 1986).

A multi-dimensional analysis of several toxicity syndromes in trout has revealed that fenvalerate intoxication produced responses which were significantly different from respiratory uncoupler, narcosis, acetylcholinesterase inhibitor, and respiratory irritant syndromes (Bradbury et al., 1987). The main characteristics which separated the pyrethroid effect were seizures; the other toxicants did not elicit this response. The cough rate increased five fold by half survival time during fenvalerate exposure; this index separated fenvalerate and gill irritant compounds from other response sets. The uniqueness of seizures as a toxic symptom of pyrethroids substantiates the validity of using this symptom to quantify trout susceptibility.

In rodent studies, PK 11195, an isoquinoline carboxamide derivative,

inhibited proconvulsant activity of pyrethroids in the pentylenetetrazol-seizure threshold paradigm. PK 11195 has nanomolar affinity for PTBR and micromolar affinity for a site that interacts with the [^{35}S]TBPS binding site of the $GABA_A$ receptor in both trout and rodents (Le Fur et al., 1983; Devaud, 1988; Eshleman and Murray, 1988; Eshleman and Murray, in press). Because of the similarity of interaction of PK 11195 with binding sites in both species, it was of interest to determine if this compound decreased the convulsant activity of pyrethroids in trout. However, the present results indicate that the deltamethrin-induced convulsant action in trout was resistant to antagonism by PK 11195. Further investigation with higher doses of PK 11195 are necessary to substantiate this inference. The involvement of a PK 11195-insensitive pyrethroid site on the $GABA_A$ receptor can also not be excluded at the present time.

Ro5-4864 produced a strong convulsant activity in rainbow trout. The seizures evoked by Ro5-4864 were qualitatively similar to those evoked by deltamethrin. Ro5-4864 has nanomolar affinity for the PTBR in rodent brain but micromolar affinity for the trout PTBR (Devaud and Murray, 1988; Bolger et al., 1985). In both species, the interaction of Ro5-4864 with the $GABA_A$ receptor as measured by modulation of [^{35}S]TBPS binding is observed at micromolar concentrations of Ro5-4864 (Gee, 1987; Eshleman and Murray, in press). Since there are no high affinity binding sites for Ro5-4864 on trout PTBR, the present results indicate that the lower affinity interaction of Ro5-4864 at the $GABA_A$ receptor may be responsible for its neurotoxic activity.

Figure 5-1. Deltamethrin dose-response curve for seizure activity score in rainbow trout. Trout were observed for 20 minutes post-dosing and all activity was scored. The highest score for each two minute epoch was recorded and these scores totalled. Data presented are the mean and SEM (bars) of 3-6 fish for each dose. The EC_{50} value for deltamethrin was computed to be 32 ± 25 μg/kg. *Significantly different from control at $p<0.05$.

Figure 5-1.

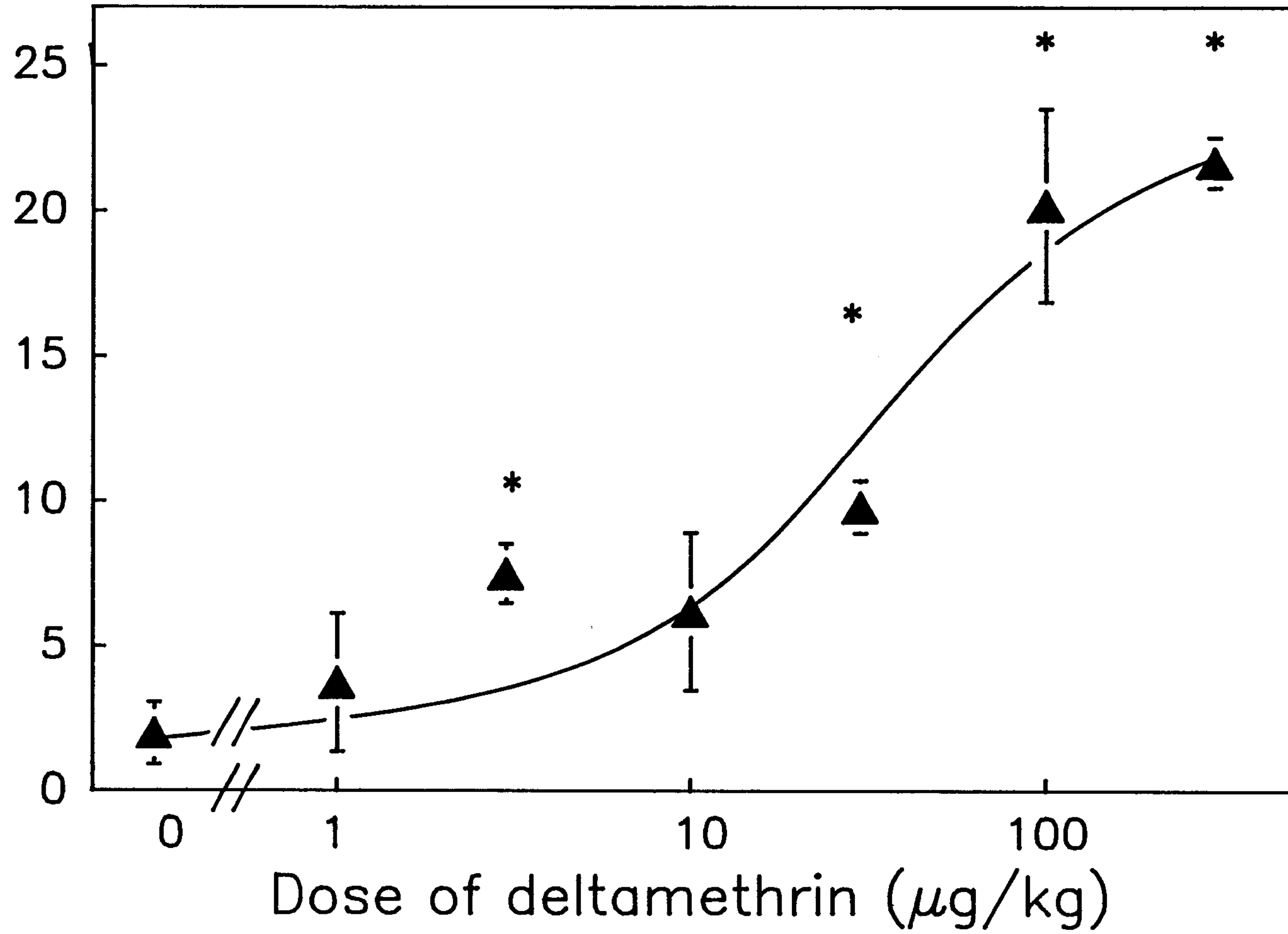
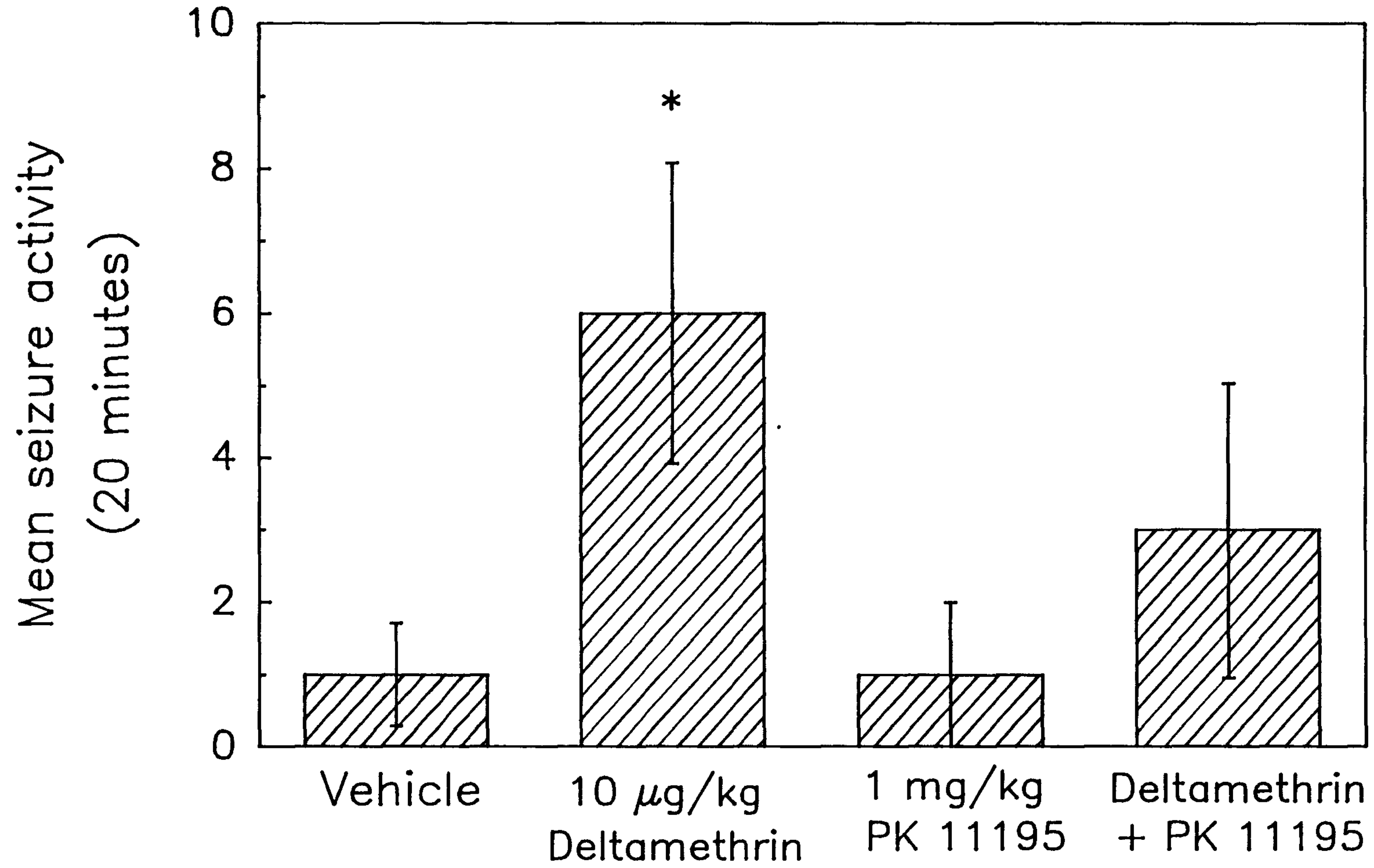


Figure 5-2. PK 11195 modulation of deltamethrin-induced seizure activity following simultaneous administration. Deltamethrin (10 $\mu g/kg$) was administered with or without PK 11195 (1 mg/kg) and all activity recorded for 20 minutes. Data presented are the mean and SEM of 3 or 4 fish per treatment.

*Significantly different from control $p<0.05$.

Figure 5-2.



Chapter 6

Conclusions

Pyrethroid insecticides are widely used in agriculture, animal husbandry and the protection of human health, with possible exposure to non-target species such as humans or fish. Rainbow trout (*Oncorhynchus mykiss)* are exquisitely sensitive to the neurotoxic action(s) of pyrethroids. By intraperitoneal or intravenous exposure they are 1-3 orders of magnitude more susceptible than rodents or quail (Bradbury and Coats, 1989). Due to their susceptibility to these insecticides, trout are a relevant model to explore possible mechanisms of pyrethroid neurotoxicity. Pyrethroid insecticides have been shown to be potent pro-convulsants in rodents as well as convulsants at higher concentrations. By intraarterial administration we have shown that trout are very sensitive to the convulsant activities of pyrethroids at sub-lethal doses (Chapter 5). This thesis investigated two possible sites of action of pyrethroid insecticides in trout brain: the peripheral-type benzodiazepine receptor and the $GABA_A$ receptor.

The PTBR of trout brain was pharmacologically characterized (Chapter 2). Heretofore, the PTBR was thought to have appeared late in evolution, having been detected only in mammalian tissue (Bolger et al., 1985). Our research indicated that the portion of the receptor that binds PK 11195 and the endogenous ligand protoporphyrin IX is abundant in trout brain. However, trout brain membranes are devoid of high affinity binding sites for Ro5-4864. This differential affinity for the two prototypic PTBR ligands is similar to that previously reported for bovine and human profiles (Awad and Gavish, 1987;

Doble et al., 1987), while differing from affinities reported in rodent membranes. The density of these binding sites was not uniform throughout the brain, indicating a region-specific expression of the receptor. Deltamethrin and permethrin had low potency for inhibiting [^{3}H]PK 11195 binding in trout brain.

It is possible that the portion of the rodent PTBR with which pyrethroids have been shown to interact overlaps with the Ro5-4864 binding site. This contention is based on the parallel results obtained in competition profiles for pyrethroids and Ro5-4864 in rodent and trout membranes. In rodents, deltamethrin inhibited [^{3}H]Ro5-4864 and [^{3}H]PK 11195 with nanomolar potency (Devaud and Murray, 1988; Chapter 2). Similarly, Ro5-4864 had nanomolar potency in displacing both of these labelled ligands from rodent brain membranes. In contrast, deltamethrin had low efficacy in trout brain membranes, inhibiting [^{3}H]PK 11195 binding by less than 50% at a concentration of 10 μM. Correspondingly, Ro5-4864 inhibited [^{3}H]PK 11195 binding only at micromolar concentrations in trout. Thus these compounds may share a common binding domain on PTBR for which they have high affinity in rodents but low affinity in trout. Alternatively, the regulation of this binding domain may differ in the two species.

Pyrethroid insecticide interaction with the $GABA_A$ receptor was investigated first by interactions with the [^{35}S]TBPS binding site (Chapter 3). [^{35}S]TBPS labelled a site associated with the anion channel of the $GABA_A$ receptor. GABA acted as an uncompetitive inhibitor of [^{35}S]TBPS binding, decreasing both the number of binding sites and the affinity of TBPS. Deltamethrin inhibited [^{35}S]TBPS binding and this inhibition was modulated by

GABA: both the affinity and the efficacy of this insecticide increased with incremental concentrations of GABA. Deltamethrin also enhanced the potency of GABA as an inhibitor of [^{35}S]TBPS binding. Pyrethroids with and without the α-cyano substituent on the phenoxybenzyl portion of the molecule were tested for efficacy and potency: all pyrethroids tested inhibited [^{35}S]TBPS binding by 55-90% at micromolar concentrations.

Three cypermethrin isomers, with insecticidal potency differing by several orders of magnitude, had IC_{50} values for inhibiting [^{35}S]TBPS binding differing by less than an order of magnitude, indicating a modest stereoselectivity. These results were not in accord with results published by Lawrence and Casida (1983). They reported absolute stereospecificity in displacement of [^{35}S]TBPS by pyrethroids; however, GABA was not included in the assay and the maximum inhibition measured with this protocol was 37%. The results reported herein are similar to those reported by Devaud (1988) in which all pyrethroids tested inhibited [^{35}S]TBPS binding in rodent membranes by 75-98% with IC_{50} values in the micromolar range. Two isomers of cypermethrin and two isomers of permethrin differed in potency by only 4-5 fold. The membrane preparation and GABA concentration employed by Devaud (1988) were similar to that used for the present investigations; hence the results of these studies indicate that the pyrethroid interaction with trout and rodent $GABA_A$ receptors is very similar.

Ro5-4864, the ligand which showed nanomolar affinity for PTBR of rodent but micromolar affinity for the trout PTBR, had a marked effect on [^{35}S]TBPS binding in trout brain, the nature of which was dependent on the concentration of GABA in the incubate. Ro5-4864 exerted this effect at micromolar

concentrations. In the absence of GABA Ro5-4864 inhibited [^{35}S]TBPS binding, whereas in the presence of GABA Ro5-4864 enhanced [^{35}S]TBPS binding, although the absolute amount of binding was identical with either treatment at high concentrations of Ro5-4864. The results reported here are similar to those reported by Devaud (1988) in rodent membranes, both in terms of the potency of Ro5-4864 and modulation by GABA. In summary, the binding studies at the [^{35}S]TBPS recognition site indicated the reciprocal allosteric interactions between a pyrethroid binding site, a Ro5-4864 binding site, the GABA recognition moiety and the TBPS binding site which is common to both trout and rodent brain.

Costa (1988) described a subpopulation of $GABA_A$ receptors, which he termed $GABA_{A3}$, that had affinity for Ro5-4864 and PK 11195 as well as CBR ligands. Recent evidence from expression of specific $GABA_A$ subunit cDNAs in a mammalian cell line indicates that the γ-subunit of the $GABA_A$ receptor is necessary for functional responses mediated by Ro5-4864 as well as CBR ligands (Puia et al., 1989). This was demonstrated by electrophysiological studies; Ro5-4864 inhibition of GABA-evoked current was observed with the expression of α, β and γ-subunits but not with expression of only α and β subunits. A β-carboline (negative modulator at the CBR) also required the expression of the γ-subunit for inhibitory activity. Evidence that these two compounds bind to discrete sites on the $GABA_A$ receptor was presented; flumazenil counteracted the β-carboline, but not the Ro5-4864, inhibitory activity. The TBPS/PTX binding site was shown to be discrete from the Ro5-4864 binding site due to detection of PTX blockade when only α and β subunits were expressed. Thus the $GABA_{A3}$ receptors must have α, β, and γ-subunits. Because PK 11195 was able to inhibit up to 85% of TBPS binding in trout brain, it would appear that a large

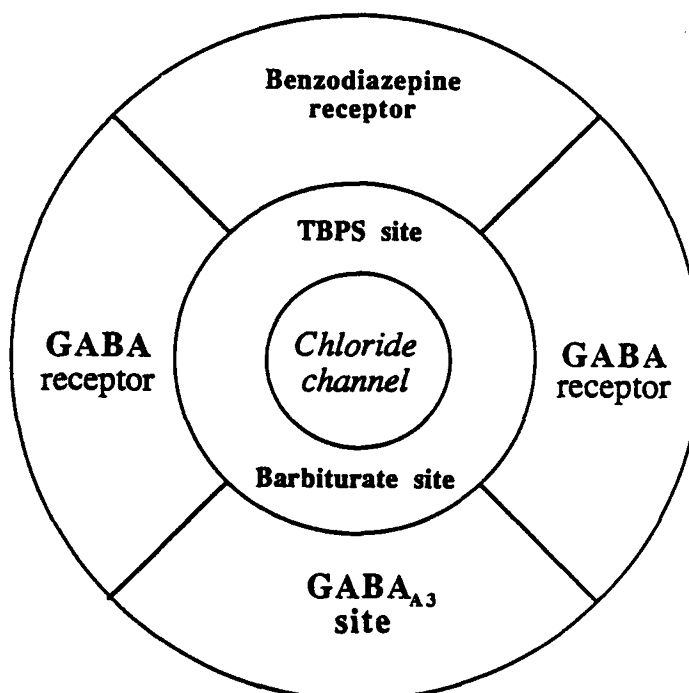
percentage of GABA receptors in trout brain contain all three subunits. Figure 6-1 suggests possible relationships between the different binding sites located on the $GABA_{A3}$ receptor. It would be interesting to determine if the pyrethroid inhibition of [^{35}S]TBPS binding also requires the presence of the γ-subunit. Similar location of binding sites on the $GABA_A$ receptor for pyrethroids and PK 11195 may help to explain the PK 11195 inhibition of the proconvulsant effect of pyrethroids in rodents (Devaud et al., 1986).

The interaction of pyrethroid insecticides with the functioning of the $GABA_A$ receptor was then investigated by measuring the effect of select pyrethroids on GABA-dependent $^{36}Cl^-$ influx into trout brain synaptoneurosomes (Chapter 4). GABA produced a time and concentration-dependent increase in $^{36}Cl^-$ influx that had the pharmacology of an effect mediated by the $GABA_A$ receptor. Pyrethroids produced a large increase in the basal uptake of chloride, thereby compromising the ability of the vesicles to respond to applications of GABA. The EC_{50} values for this effect were in the nanomolar range, the effect was stereospecific, and tetrodotoxin (TTX), a voltage-dependent sodium channel blocker, inhibited a significant portion of this uptake. An increase in basal uptake was also elicited by veratridine (VTD), a voltage-dependent sodium channel activator. These results suggest that the primary effect of pyrethroids, as measured by this assay, is via an interaction with the voltage-dependent sodium channels of rainbow trout brain, increasing sodium flux and thereby increasing a basal uptake of $^{36}Cl^-$ through an anion channel which is voltage sensitive.

Activity of pyrethroids at trout sodium channels would explain some of the

Figure 6-1. A model of the $GABA_A$ receptor-chloride ion channel complex.

$GABA_A$ Receptor - Chloride Ion Channel Complex



Sites of Drug and Toxicant Action

$GABA_A$ receptor	BZ receptor	TBPS site	Barbiturate site
GABA	BZ Agonists	Picrotoxin	Barbiturates
Muscimol	BZ Antagonists	TBPS	
Bicuculline	ß-Carbolines	PTZ	
		Endrin	

$GABA_{A3}$ site

Ro5-4864

PK 11195

Pyrethroids

species-specific sensitivity to these insecticides. Most reports indicate that pyrethroids act at mammalian $GABA_A$ receptors and sodium channels at approximately equal concentrations, ranging from high nanomolar to low micromolar potencies (Devaud, 1988; Lombet et al., 1988). It would thus appear that pyrethroid interaction with both of these systems contributes to mammalian toxicity. Although the [^{35}S]TBPS binding data gives unequivocal evidence that pyrethroids interact allosterically with several sites on the trout $GABA_A$ receptor, this effect was not detected during the biochemical assay assessing modulation of function. In this assay, the increase of basal $^{36}Cl^-$ uptake that was tetrodotoxin-sensitive was the primary effect of pyrethroids. This indicates that, at least with these two assays, the interaction of pyrethroids with the voltage-sensitive sodium channels of rainbow trout brain occurs at a lower concentration.

The convulsant activity of pyrethroids in rainbow trout is qualitatively similar to the convulsant activity of Ro5-4864 (Chapter 5). As stated above, Ro5-4864 interacts with the $GABA_A$ receptor of both trout and rodent as do pyrethroids. Because of the similarity of *in vivo* effects for these two compounds, an interaction of pyrethroids with the $GABA_A$ receptor may be involved in the expression of neurotoxicity in the intact animal, an effect that was not detected in the chloride influx assays.

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