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Thomas F. Murray

Pyrethroid insecticides have come into prominent use in recent years due to their high insecticidal activity and reported low mammalian toxicity. This increased use and the development of newer, more potent pyrethroids has made it even more important to elucidate the specific mechanisms of action by which these compounds exert their neurotoxic effects. It is also important to investigate the effects of subtoxic doses of these insecticides on non-target species. The present study focused on the effects of low doses of pyrethroids utilizing both in vivo and in vitro measures.

Pyrethroid insecticides are potent proconvulsants in the rat. All pyrethroids evincing proconvulsant activity elicited a similar 25-30% maximal reduction of seizure threshold. The Type II pyrethroids were the most potent proconvulsants with 1RαS,cis cypermethrin having an ED_{50} value of 6.3 nmol/kg. The proconvulsant activity of both Type I and Type II pyrethroids was blocked by pretreatment with PK 11195, the peripheral-type benzodiazepine receptor (PTBR) antagonist. In contrast, phenytoin did not

antagonize the proconvulsant activity of either deltamethrin or permethrin.

Pyrethroids displaced the specific binding of [^{3}H]Ro5-4864 to rat brain membranes with a significant correlation between the log EC_{50} values for their activities as proconvulsants and the log IC_{50} values for their inhibition of [^{3}H]Ro5-4864 binding. Both Ro5-4864 and pyrethroid insecticides were found to influence specific [^{35}S]TBPS binding in a GABA-dependent manner. PK 11195 and the Type II pyrethroid, deltamethrin antagonized the Ro5-4864-induced modulation of [^{35}S]TBPS binding.

Pyrethroid insecticides, Ro5-4864 and veratridine influenced GABA-gated ^{36}chloride influx. Moreover, the Type II pyrethroids elicited an increase in ^{36}chloride influx in the absence of GABA-stimulation. Both of these actions were antagonized by PK 11195 and tetrodotoxin.

Considered together, these findings suggest that pyrethroid insecticides exert significant effects on mammals at subtoxic doses. They also share complex interactions with ligands for the PTBR at a site allosterically linked to the $GABA_A$ receptor.

INTERACTIONS OF PYRETHROID INSECTICIDES WITH $GABA_A$ AND PERIPHERAL-TYPE BENZODIAZEPINE RECEPTORS

by

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And in memory of Dorman J. Hyde (December 1, 1925 - December 31, 1987).

CONTRIBUTIONS OF OTHER AUTHORS

Patricia Szot assisted with the first series of pentylenetetrazol seizure threshold determinations.

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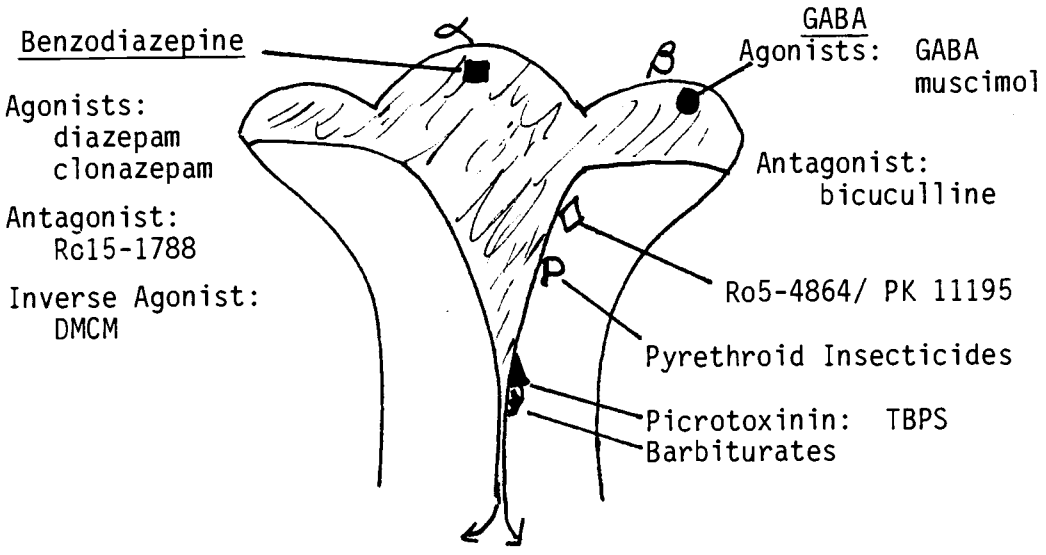
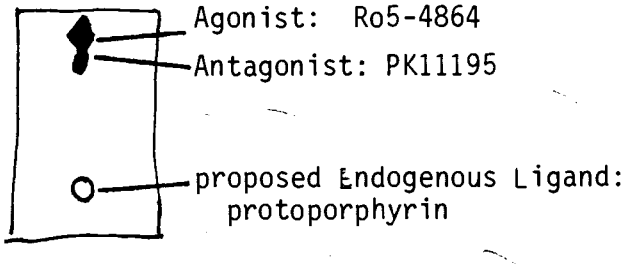


PERIPHERAL-TYPE (MITOCHONDRIAL) BENZODIAZEPINE RECEPTOR



INTERACTIONS OF PYRETHROID INSECTICIDES WITH $GABA_A$ AND PERIPHERAL-TYPE BENZODIAZEPINE RECEPTORS

Chapter 1 INTRODUCTION

Pyrethroid insecticides have seen increasing use in recent years. As one of the four major classes of neurotoxicant insecticides, pyrethroids have been overshadowed by their more potent and persistent counterparts, the organophosphate, methylcarbamate and chlorinated hydrocarbon insecticides. However, with increasing evidence of undue persistance, high mammalian toxicity, the development of pest resistance and other undesirable side effects, these three other classes of insecticides have seen much reduced usage (Davies, 1985). With the focus now shifting to pyrethroids, there is the need for definitive research into the effects of these compounds on non-target organisms.

Pyrethrum is the dried and powdered heads of the flower Chrysanthemum cineriafolium. There are records of this "insect powder" having been used to control pests dating prior to the nineteenth century. Pyrethrum has been in commercial production since the mid 1800's (Casida, 1980).

Extraction of the flower head powder gives a 1-2% yield of a complex series of six closely related insecticidal esters, the pyrethrins. Pyrethrin esters differ only in the terminal substituents on the side chains of the acid and alcohol components of the molecule (Figure 1). The biological activity of pyrethrins depends on the precise structure and stereochemistry of the acid and alcohol moieties (Casida, 1980). The pyrethrins are remarkably effective as insecticides but are photolabile and degrade rapidly upon contact with soil (Elliot, 1978). While this makes them useful for control of pest insects in buildings and for direct application to animals and livestock, they are not stable enough for widespread agricultural use.

Structural modifications of the pyrethrin molecule have led to the synthesis of pyrethroids, highly effective pyrethrum substitutes having increased insecticidal activity and enhanced persistance (Elliot, 1980) (Figure 2). However, the early pyrethroids still lacked long-term stability. Incorporation of αn α-cyano moiety on the alcohol substituent dramatically improved both stability and potency. These newer pyrethroids were categorized as Type II compounds while the earlier analogues were classified as Type I pyrethroids.

The division of pyrethroid insecticides into two classes was supported both by structure-activity relationships (Verschoyle and Aldridge, 1980) and by distinct poisoning symptoms elicited in both insects and mammals by toxic doses of these compounds (Gammon et al, 1981). Type I pyrethroids induce repetitive firing in the

cercal sensory nerves of the cockroach, the giant squid axon and excised frog sensory nerves (Narahashi, 1971; Vijverberg and Van den Bercken, 1982; Lund and Narahashi, 1983). The α-cyano pyrethroids elicit a small but long-lasting depolarizing afterpotential which leads to a gradual depolarization blockade (Vijverberg and Van den Bercken, 1982; Lund and Narahashi, 1983).

The T poisoning syndrome is induced by neurotoxic doses of the Type I compounds and is normally of rapid onset. In both insects and mammals symptomology includes incoordination, an enhanced startle reflex, and aggressive sparring behavior followed by the appearance of mild tremor which progresses to coarse, whole body tremors leading to prostration and death (Verschoyle and Aldridge, 1980; Staatz et al, 1982; Casida et al, 1983).

The CS syndrome, elicited by administration of toxic concentrations of the Type II pyrethroids, has a slower onset of symptoms which begin with increased activity and profuse salivation. This progresses to stereotypical pawing and burrowing behavior, ataxia, clonic/tonic convulsions and choreoathetosis which can become very severe and lead to death of the animal (Staatz et al, 1982; Casida et al, 1983).

Along with the development of pyrethroid insecticides, the 1940's and 1950's saw the advent of many new, highly potent synthetic insecticides. Together these "second generation" compounds rapidly replaced the older, so called "first generation" pesticides which were primarily botanicals (nicotine or rotenoids, for example) or contained toxic metals such as arsenic.

Application of organophosphate, carbamate and organochlorine insecticides provided economical and widespread insect pest control (Elliot, 1980). However, it became apparent that there were serious drawbacks associated with these three classes of insecticides.

Organophosphates (i.e. parathion, malathion and diazanon) exert their insecticidal activity by potently inhibiting acetylcholinesterase (Hayes, 1971). This non-selective interaction makes most of these compounds highly toxic to animals. A major feature of poisoning is the long recovery time following exposure to these compounds due to their irreversible inhibition of acetylcholinesterase. Ingestion of small amounts of certain organophosphates can be rapidly fatal. Furthermore, there can be delayed neurotoxic effects following chronic exposure with only slow and incomplete recovery (Johnson, 1982).

Another class of second generation insecticides, the carbamates, also exert their toxic actions by inhibition of acetylcholinesterase and so evince similar toxicity towards non-target species (O'Brien, 1967). However, the carbamates generally have much lower dermal toxicity (an exception is aldicarb) and most are rapidly reversible inhibitors of acetylcholinesterase. Therefore, while these insecticides are not as potent toxicants nor do they cause as serious a poisoning hazard as organophosphates, carbamates usefulness is still limited because they typically are not broad spectrum insecticides (O'Brien, 1967).

A third class of the second generation insecticides are the

chlorinated hydrocarbons. These compounds exert their insecticidal effects by slowing the inactivation of sodium conductance in nerve membranes and inhibiting the activation of potassium conductance (Vijverberg et al, 1982; Narahashi, 1983). They have gained notoriety due to DDT's long-term persistence and accumulation in the environment which has resulted in a negative impact on wildlife. Other chlorinated hydrocarbons, such as aldrin, dieldrin and lindane have had their use restricted due to their potent convulsant activity.

Thus, these three classes of second generation insecticides, although quite potent and effective, currently have limited usefulness due to their untoward, non-selective effects on non-target organisms, the hazards involved with their manufacture, or the development of insect resistance (Casida, 1980). This has led to a more prominent use of pyrethroid insecticides in recent years which has resulted in continuing investigations into the precise mechanism of action for these compound on both target and non-target species.

Currently several possible sites of action have been suggested for the pyrethroids. Similar to the findings for the chlorinated hydrocarbon class of insecticides, early investigations into a mode of action for the neurotoxic effects of pyrethroids implicated voltage-gated neuronal sodium channels as a major target site (Narahashi, 1971; Narahashi, 1985). When the neuronal membrane has been depolarized these sodium channels briefly become activated and open, shifting rapidly to the closed, inactivated

state. Repolarization shifts the channels back to the resting state. Activation of the sodium channels is seen as an increase in the inward current of sodium ions with the time course of influx following the time course of activation. Channels that are open when the membrane repolarizes rapidly close and return to the resting state, the resultant decay in sodium uptake is called the tail current. Both classes of pyrethroids cause a prolongation of the activation of neuronal sodium channels with quantitative differences on their kinetics of action (Narahashi, 1985; Lund and Narahashi, 1983). Voltage clamp and patch clamp techniques have revealed that while both Type I and Type II pyrethroids slow the sodium current inactivation (prolong the tail current), there is a stronger, prolonged effect of the Type II pyrethroids resulting in a much more slowly decaying tail current (Lund and Narahashi, 1983; Narahashi, 1985). These activities translate to the repetitive firing induced by Type I pyrethroids and the depolarization block elicited by toxic doses of Type II compounds.

Pyrethroids have also been shown to modulate the nicotinic acetylcholine receptor in that they noncompetitively inhibit the binding of [^{3}H]perhydrohistrionicotoxin to sodium channel sites of this receptor (Abassy, 1982; Eldefrawi et al, 1985).

It was suggested early on that pyrethroid insecticides exert significant effects in the central nervous system, based primarily on some of the poisoning signs induced by neurotoxic doses of pyrethroids. Some of the stereotypical symptoms seen following toxic dosing with the Type II pyrethroids, in particular, cannot be

explained solely by a non-selective action on neuronal sodium channels. Investigations showed that both Type I and Type II compounds displayed large increases in potency following intracerebroventricular (icv) administration in mice (Lawrence and Casida, 1982; Staatz et al, 1982). Moreover, both classes of pyrethroids induce the full range of poisoning symptoms following icv dosing. It was also noted that all insecticidally active pyrethroids are more toxic to mice by icv versus intraperitoneal (ip) or oral administration (Lawrence and Casida, 1982). Therefore, centrally mediated activity of pyrethroids is likely.

The search for a specific CNS site of action for pyrethroids was directed towards the $GABA_A$ receptor due to a report of the inhibition of [^{3}H]dihydropictrotoxinin binding by the Type II pyrethroid deltamethrin to the picrotoxinin site on the $GABA_A$ receptor (Leeb-Lundberg and Olsen, 1981). Attempts to gain insight into the possible role of the $GABA_A$ receptor in the toxicity of pyrethroids was first studied with the benzodiazepine, diazepam. A delay in the onset of Type II poisoning signs is observed in both the cockroach and mouse following injection of a low dose of diazepam (Gammon et al,1983). In this study, diazepam treatment did not delay the onset of the T syndrome elicited by the Type I pyrethroids allethrin and permethrin. However, in the frog diazepam was reported to afford more effective protection against the Type II syndrome and also offered some protection against Type I poisoning (Cole and Casida, 1983). More recently, Type II pyrethroids have been reported to inhibit GABA-stimulated

^{36}chloride influx into a synaptoneurosome preparation in a dose-dependent manner (Abalis et al, 1986; Eldefrawi and Eldefrawi, 1987; Bloomquist et al, 1986). Furthermore, Type II pyrethroids were found to act stereospecifically to inhibit the binding of [^{35}S]t-butylbicyclophosphorothionate (TBPS) to the picrotoxinin site of the $GABA_A$ receptor with an excellent correlation between mouse icv toxicity and in vitro inhibition of [^{35}S]TBPS binding (Lawrence and Casida, 1983; Casida and Lawrence, 1985).

Ro5-4864, 4'chlordiazepam, is a ligand used to selectively investigate the peripheral-type benzodiazepine receptor (PTBR), an entity which is pharmacologically distinct from the benzodiazepine binding site on the $GABA_A$ receptor (Marangos et al, 1981; Schoemaker et al, 1983; Richards and Mohler, 1984). Injection of Ro5-4864 caused transient symptoms identical to the early signs seen following administration of the Type II pyrethroid, deltamethrin (Gammon and Sander, 1985). However, pretreatment with a higher dose of Ro5-4864 elicited a delay in both the early and late deltamethrin-induced toxic symptoms in insects (Gammon and Sander, 1985). These results suggested that while Ro5-4864 and diazepam shared some actions with respect to pyrethroid poisoning, Ro5-4864 has multiple, apparently contradictory, effects which depend on the dose employed.

In vitro analysis showed that the Type II pyrethroids deltamethrin and 1RαS,cis cypermethrin inhibited the binding of [^{3}H]Ro5-4864 to rat brain membranes with a 60-fold increase in potency over that observed for [^{35}S]TBPS binding (Lawrence et al,

1985; Gammon and Sander, 1985) further implicating an interaction of pyrethroids with the PTBR.

Thus, prior investigations have implicated multiple receptor interactions for pyrethroid insecticides. However, the specifics of involvement of the $GABA_A$ receptor or the peripheral-type benzodiazepine receptor in the toxicity of pyrethroids has not been established. Moreover, there have been few studies into the effects of sub-toxic doses of pyrethroids. Any low dose effect could have important implications on the commercial use of pyrethroid insecticides.

Therefore, the intent of this thesis project was to investigate the effects of acute, low dose administration of pyrethroid insecticides in concert with biochemical studies in an attempt to better describe the involvement of the PTBR and the $GABA_A$ receptor as sites of action for pyrethroid insecticides.

A study of the effects of low dose administration of pyrethroid insecticides in rats has shown that both Type I and Type II pyrethroids are potent proconvulsants and that this action is blocked by prior administration of PK 11195 (an antagonist of the PTBR) (Chapters 2 and 3). In agreement with these findings, both types of pyrethroids were found to inhibit the specific binding of [^{3}H]Ro5-4864 to rat brain membranes with a rank order of potency significantly correlated with the EC_{50} values obtained from their actions as proconvulsants (Chapter 3). While it is well established that Ro5-4864 is a convulsant and proconvulsant, the precise mechanism by which its interaction with the PTBR elicits

this response has yet to be elucidated (Weissman et al, 1983; Benavides et al, 1984, File et al, 1984). Subcellular localization studies have implicated an association of the PTBR with the outer mitochondrial membrane (Snyder et al, 1987).

Recently, it has been reported that Ro5-4864 interacts with a low affinity site allosterically linked to the $GABA_A$ receptor (Gee, 1987; Gee et al, 1988). This raises the possibility that the proconvulsant activity displayed by both Ro5-4864 and pyrethroids may be mediated via an interaction with the $GABA_A$ receptor.

Investigations into the interaction of pyrethroid insecticides with the $GABA_A$ receptor have shown that both Type I and Type II pyrethroids are able to inhibit GABA-stimulated chloride influx into rat cerebral cortical synaptoneurosomes (Chapter 4). Pyrethroids were also found to inhibit [35S]TBPS binding to rat brain membranes in a GABA-dependent manner, but with a limited potency range and stereospecificity (Chapter 5).

Taken together, these investigations support the involvement of the $GABA_A$ receptor and an allosterically-linked Ro5-4864 binding site in the proconvulsant effects of pyrethroid insecticides

FIGURE 1 Pyrethrins Structures

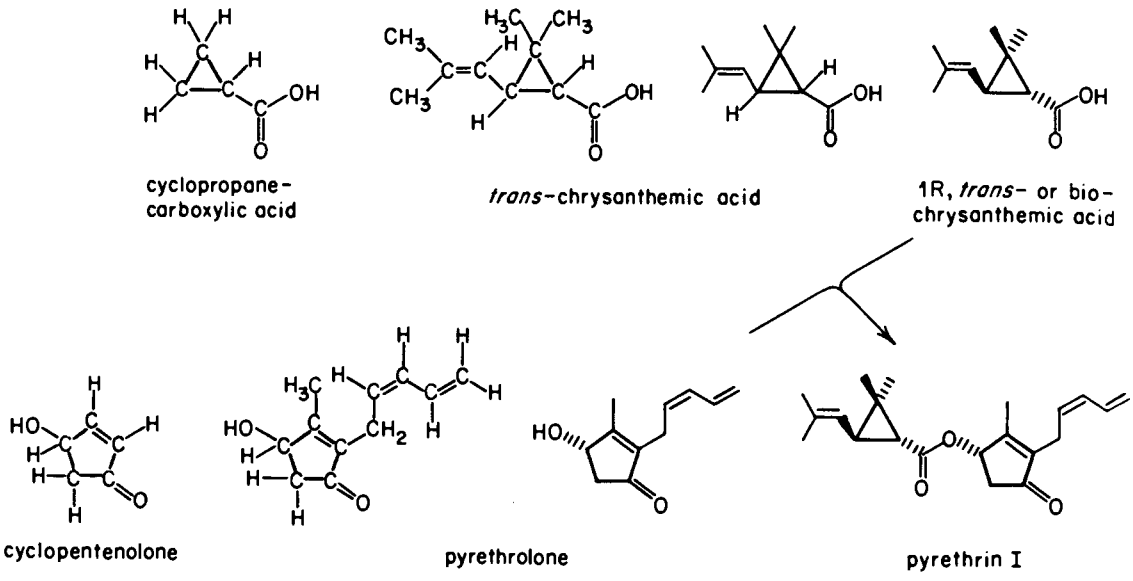
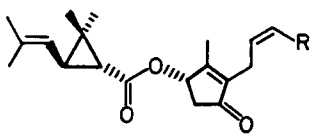
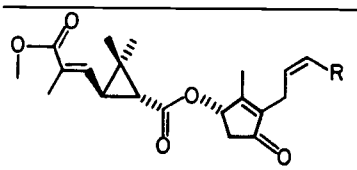
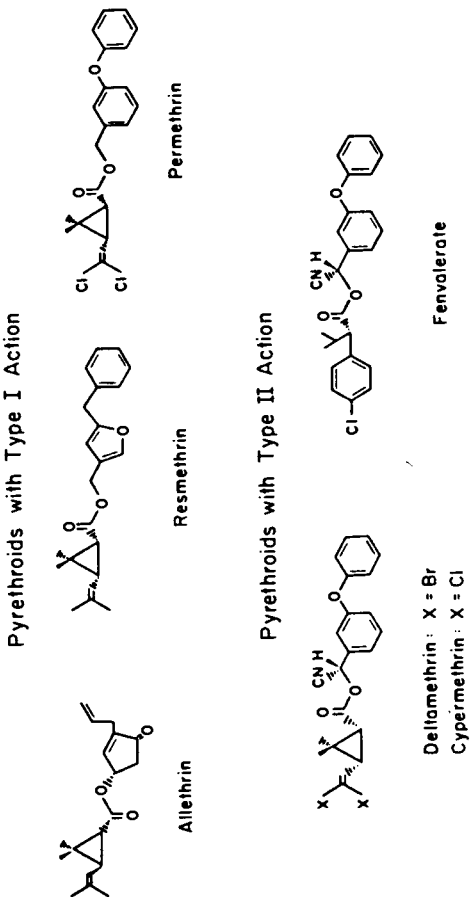


Table 2.

	R	%	Name
		chrysanthemates	
	$CH=CH_2$	35	pyrethrin I
	CH_3	10	cinerin I
	CH_2CH_3	5	jasmolin I
		pyrethrates	
	$CH=CH_2$	32	pyrethrin II
	CH_3	14	cinerin II
	CH_2CH_3	4	jasmolin II

Casida, 1980

FIGURE 2 Pyrethroid Structures



Lawrence et al, 1985

CHAPTER 2 PK 11195 ANTAGONISM OF PYRETHROID-INDUCED PROCONVULSANT ACTIVITY

Leslie L. Devaud, Patricia Szot and Thomas F. Murray

ABSTRACT

The acute administration of 1RαS,cis cypermethrin, deltamethrin, fenvalerate and permethrin produced a dose-dependent lowering of the concentration of pentylenetetrazol required to elicit a seizure in rats. The proconvulsant action of cypermethrin displayed stereospecificity in that the 1RαS,cis isomer was the most potent compound tested, while the non-insecticidal isomer, 1SαR,cis cypermethrin, was devoid of proconvulsant activity. Pretreatment of rats with PK 11195, an antagonist of the peripheral-type benzodiazepine binding site, elicited a complete reversal of the proconvulsant actions of both deltamethrin and permethrin. In contrast, pretreatment with phenytoin did not alter the pyrethroid-induced proconvulsant activity. These results suggest that the effects of pyrethroids on pentylenetetrazol seizure threshold are mediated via an interaction with peripheral-type benzodiazepine binding sites.

Introduction

Synthetic pyrethroids have emerged as a major class of insecticides due to their high potency and selectivity as nerve poisons (Casida et al., 1983). Pyrethroids have been divided into two classes (Type I and Type II) based on their effects on cercal sensory nerves recorded *in vitro* and *in vivo* and on the symptomology they produce in several species following neurotoxic doses (Gammon et al., 1981; Verschoyle and Aldridge, 1980; Lawrence and Casida, 1982). Type I pyrethroids induce a repetitive firing in cercal sensory nerves of the cockroach which is thought to be mediated via a prolongation of the sodium current in nerve axons (Narahashi, 1971). Pyrethroids possessing a Type II action fail to induce repetitive firing in cockroach cercal sensory nerves, and give rise to symptoms including salivation, hyperactivity, sinuous writhing and clonic/tonic convulsions in rats and mice (Gammon et al., 1981; Verschoyle and Aldridge, 1980; Lawrence and Casida, 1982). Pyrethroids with a Type I action are typically those lacking a cyano substituent in the alcohol moiety, whereas the newer and more potent analogs containing a cyano substituent at the α-carbon of the phenoxybenzyl alcohol moiety posses a Type II action (Casida et al., 1983).

There is increasing evidence that the Type II pyrethroid

action involves an interaction with the GABA receptor-ionophore complex. Onset of Type II, but not of Type I, pyrethroid poisoning signs have been reported to be delayed by diazepam pretreatment in both insects and mammals (Gammon et al., 1982). Moreover, Type II pyrethroids, including cypermethrin, deltamethrin and fenvalerate act stereospecifically to inhibit the specific binding of [^{35}S]TBPS to the picrotoxinin binding site of rat brain membranes (Lawrence and Casida, 1983). More recent investigations have shown that Type II pyrethroids also inhibit the specific binding of [^{3}H]Ro5-4864, which is a selective ligand for the peripheral-type benzodiazepine binding site (Gammon and Sander, 1985; Lawrence et al., 1985). It is noteworthy in this regard that cypermethrin was shown to be 60 times more potent as an inhibitor of [^{3}H]Ro5-4864 than for [^{35}S]TBPS binding (Lawrence et al., 1985).

Although the neurotoxic effects of pyrethroids have been well characterized, no studies have examined the interaction between low dose pyrethroid exposure and seizure susceptibility. The purpose of the present investigation was to determine the effects of selected pyrethroids on pentylenetetrazol (PTZ) seizure threshold in rats, and to evaluate the involvement of peripheral-type benzodiazepine binding sites in this action of pyrethroids.

Material and Methods

Male Sprague-Dawley rats (Simonsen Labs, Gilroy, CA) weighing 140-180 g were used in all experiments. The rats were housed at an ambient temperature of 22^{o}C with food and water provided ad

libitum. A standard light/dark cycle of 12 hrs was maintained and all testing was conducted within the first 6 h of the light cycle. Each rat was used only once for any determination.

Seizure thresholds for PTZ in rats was determined using a timed infusion technique as previously described (Murray et al., 1985). Briefly, the PTZ solution (2 mg/ml) was infused via a 25 gauge butterfly needle inserted into the lateral tail vein of rats. The solution was infused at a constant rate of 1.6 ml/min, and the endpoint of the infusion was taken as the first myoclonic twitch of the head and neck. The time to the first myoclonic twitch was recorded to the nearest 0.1 s and the dose of PTZ required to elicit the seizure was calculated from this time, the concentration of the PTZ solution, and the body weight of the rat.

Pyrethroids and drugs were dissolved in a vehicle consisting of 5% Emulphor (EL-620, GAF, New York, N.Y.), 5% ethanol and 90% isotonic saline. Solutions of PK 11195, Ro5-4864 and the higher doses of pyrethroids were sonicated until time of injection. All injections were intraperitoneal with the volume of injection being 1 ml/kg for pyrethroids and Ro5-4864, and 2 ml/kg for PK 11195 and phenytoin. In pilot experiments it was determined that the time to peak effect for the pyrethroids employed was 30 min. In the drug interaction experiments, the test compounds were injected 30 min prior to challenge with pyrethroids and seizure thresholds were determined 30 min later.

Modified Hill plots were used to compare quantitatively the dose-response curves for pyrethroids and the ED_{50} values and 95%

confidence limits were calculated as previously described (Murray et al., 1985). Levels of significance between PTZ seizure threshold group means were evaluated using a two-tailed Students t-test. Deltamethrin was obtained from Roussel-Uclaf (Paris, France), technical-grade fenvalerate from Shell Development Company (Modesto, CA), and technical-grade permethrin and cypermethrin isomers were from FMC Corporation (Princeton, N.J.). The PK 11195 was a gift of Dr. G. LeFur, Pharmuka Laboratories (Gennevilliers, France) and the Ro5-4864 was obtained from Hoffman-LaRoche (Nutley, N.J.).

Results

The acute administration of 1RαS,cis cypermethrin, deltamethrin, fenvalerate and permethrin all resulted in dose-dependent reductions in the dose of PTZ required to elicit a seizure. As shown in Figure 3, all four of these pyrethroids displayed conventional log dose-response curves with maximum reductions of PTZ seizure threshold of 30-35%. The 1RαS,cis isomer of cypermethrin was the most potent pyrethroid tested with a dose as small as 3 μg/kg (i.p.) producing an 18.7% ($P<0.05$) decrease in the seizure threshold for PTZ. The effects of 1RαS,cis cypermethrin plateaued at a dose of 100 μg/kg. Rats injected with the 3 μg/kg dose of 1RαS,cis cypermethrin did not evince any overt behavioral signs of stimulation or excitement. The proconvulsant

action of cypermethrin displayed stereospecificity in that the non-insecticidal isomer, 1SαR,cis cypermethrin, was essentially devoid of activity (Figure 3). The ED_{50} values (and the 95% confidence limits) for the pyrethroids tested were: 1Rασ,cis cypermethrin =2.6 (1.3-5.3) μg/kg, deltamethrin= 43 (15-123) μg/kg, fenvalerate = 342 (169-692) μg/kg and permethrin = 593 (164-2128) μg/kg.

To evaluate the role of peripheral-type benzodiazepine receptors in the proconvulsant actions of pyrethroids, the effect of pretreatment with PK 11195 on the response to deltamethrin and permethrin was determined. PK 11195 in doses of 10 and 30 mg/kg, which by themselves had no significant effect on PTZ seizure threshold, produced a significant antagonism of both the deltamethrin- and permethrin-induced proconvulsant effects (Table 1, Figure 4). Moreover, in accordance with previous reports in mice (Benavides et al., 1984), PK 11195 antagonized the proconvulsant effects of Ro5-4864 in rats (Figure 5). In contrast to the results obtained with PK 11195, pretreatment with phenytoin (30 mg/kg) did not reverse the proconvulsant activity of deltamethrin, permethrin or Ro5-4864(Table 1, Figures 6 and 7).

Deltamethrin induced poisoning signs consistent with the Type II (CS) syndrome with an ED_{50} value of 5.3 ± 0.43 mg/kg. The severity of symptoms elicited by a 5 mg/kg dose of deltamethrin were significantly alleviated by pretreatment with 10 mg/kg PK 11195 (Figure 8).

Discussion

Results of present investigations have demonstrated that selected pyrethroid insecticides are potent proconvulsants in rats. A relationship between the observed proconvulsant action and the insecticidal activity of pyrethroids was suggested by the results obtained with the isomers of cypermethrin. The non-insecticidal 1SαR,cis isomer of cypermethrin was devoid of proconvulsant activity, while the insecticidal 1RαS,cis isomer of cypermethrin was the most potent pyrethroid tested. These results are in agreement with the previously reported stereoselectivity of cypermethrin isomers as inhibitors of both [^{35}S]TBPS binding (Lawrence and Casida, 1983) and [^{3}H]Ro5-4864 binding (Lawrence et al., 1985) to rat brain synaptic membranes.

In addition to the proconvulsant effects of the Type II pyrethroids (1RαS,cis cypermethrin, deltamethrin and fenvalerate), the Type I compound permethrin also elicited a dose-related decrease in PTZ seizure threshold. In contrast to these results, permethrin has been reported to be inactive at [^{35}S]TPBS sites (Lawrence and Casida, 1983) and a relatively weak inhibitor of [^{3}H]Ro5-4864 binding (Lawrence et al., 1985) However, in the latter study, the Type II pyrethroid, fenvalerate, was also a relatively weak inhibitor of [^{3}H]Ro5-4864 specific binding to rat brain membranes. It is of interest to note that permethrin resembles Type II pyrethroids such as fenvalerate and cypermethrin,

but not Type I compounds, in its potency and speed of action as a displacer of carbamylcholine-stimulated [^{3}H]perhydrohistrionicotoxin binding in Torpedo electroplax membranes (Eldefrawi et al., 1985). Further investigations with additional Type I pyrethroids are needed to clarify the relative efficacies of Type I vs Type II pyrethroids as proconvulsants.

Given the recent evidence implicating the peripheral-type benzodiazepine binding site as a target for the neurotoxic actions of pyrethroids, we assessed the ability of PK 11195 to reverse the proconvulsant effects of pyrethroids. PK 11195 has been reported to be an antagonist of the peripheral-type benzodiazaepine binding site (LeFur et al., 1983). PK 11195 administration, in doses which by themselves did not alter PTZ seizure threshold, elicited a virtually complete antagonism of the proconvulsant activity of deltamethrin and permethrin. Moreover, PK 11195 also blocked the reduction in PTZ seizure threshold produced by the administration of Ro5-4864. The ability of PK 11195 to antagonize the proconvulsant effects of pyrethroids suggest the involvement of the peripheral-type benzodiazepine receptor. However, the complete pharmacological profile of PK 11195 remains to be established, thereby precluding more definitive statements regarding the role of this recognition site in the observed actions of pyrethroids. In contrast to the effects of PK 11195, pretreatment with phenytoin, an anticonvulsant which binds to activation gates of sodium channels to slow recovery from inactivation, failed to antagonize either the permethrin or deltamethrin proconvulsant action.

The possibility that pyrethroids, Ro5-4864 and PK 11195 are exerting their effects via an interaction with the picrotoxinin site of the GABA receptor-ionophore complex cannot be excluded by the present experiments. Ro5-4864 has been shown to be a competitive inhibitor of [^{35}S]TBPS binding (picrotoxinin site) in rat brain membranes (Ticku and Ramanjaneyulu, 1984). Moreover, Ro5-4864 has been reported to possess proconvulsant activity when administered in combination with pictrotoxin (File et al., 1984). Therefore, a further understanding of the involvement of GABAergic transmission in the observed effects of pyrethroids must await a delineation of the molecular mechanisms by which Ro5-4864 interacts with the picrotoxinin site of the GABA receptor-ionophore complex.

Considered together, these results provide additional support for the contention that at least some of the neuropharmacologic effects of pyrethroids are mediated through an interaction with the peripheral-type benzodiazepine binding site. The potent effects of pyrethroids to increase seizure susceptibility and the antagonism of this effect by PK 11195 may have significant clinical implications. Further studies of the precise mechanisms by which pyrethroids interact with the peripheral-type benzodiazepine binding site are clearly warranted.

Acknowledgements

This work was supported by a grant for the Oregon State University Environmental Health Sciences Center. The excellent secretarial assistance of Elaine Luttrull is gratefully acknowledged.

TABLE 1 Effect of PK 11195 on the proconvulsant action of deltamethrin, permethrin and Ro5-4864[a]

Treatment	PTZ seizure threshold(mg/kg)	%of control
Experiment 1		
Control (vehicle only)	24.32 ± 0.61	--
PK 11195 (10 mg/kg)	23.37 ± 0.80	96.1
PK 11195 (30 mg/kg)	24.10 ± 0.59	99.1
Deltamethrin (0.3 mg/kg)	18.42 ± 0.81*	75.7
Deltamethrin (0.3 mg/kg)+ PK 11195 (10 mg/kg)	22.32 ± 1.22	91.8
Deltamethrin (0.3 mg/kg)+ PK 11195 (30 mg/kg)	23.34 ± 0.51	96.0
Permethrin (10 mg/kg)	17.37 ± 1.41*	71.4
Permethrin (10 mg/kg)+ PK 11195 (10 mg/kg)	24.94 ± 0.95	102.5
Permethrin (10 mg/kg)+ PK 11195 (30 mg/kg)	22.52 ± 0.99	92.6
Experiment II		
Control (vehicle only)	22.72 ± 1.48	--
Ro5-4864 (3 mg/kg)	15.40 ± 0.99*	67.8
Ro5-4864 (3 mg/kg)+ PK 11195 (10 mg/kg)	20.40 ± 1.00	90.1
Ro5-4864 (3 mg/kg)+ PK 11195 (30 mg/kg)	22.89 ± 0.42	100.7
Experiment III		
Control (vehicle only)	22.72 ± 0.54	--
Phenytoin (30 mg/kg)	21.69 ± 0.38	95.5
Deltamethrin (0.3 mg/kg)+ Phenytoin (30 mg/kg)	17.01 ± 0.76*	74.9
Permethrin (10 mg/kg)+ Phenytoin (30 mg/kg)	17.27 ± 0.78*	76.0

[a] Values represent the mean S.E.M for at least 6 animals for each treatment, * $p < 0.01$ (two-tailed t-test).

Figure 3. Dose-response curves for the effects of 1RαS,cis cypermethrin (▲), deltamethrin (●), fenvalerate (⬣), permethrin (■), and 1SαR,cis cypermethrin ( ), on PTZ seizure thresholds. All doses were administered intraperitoneally. Values are expressed as the mean % decrease in seizure thresholds as compared to vehicle-injected controls. Each value represents the mean of 6-12 rats.

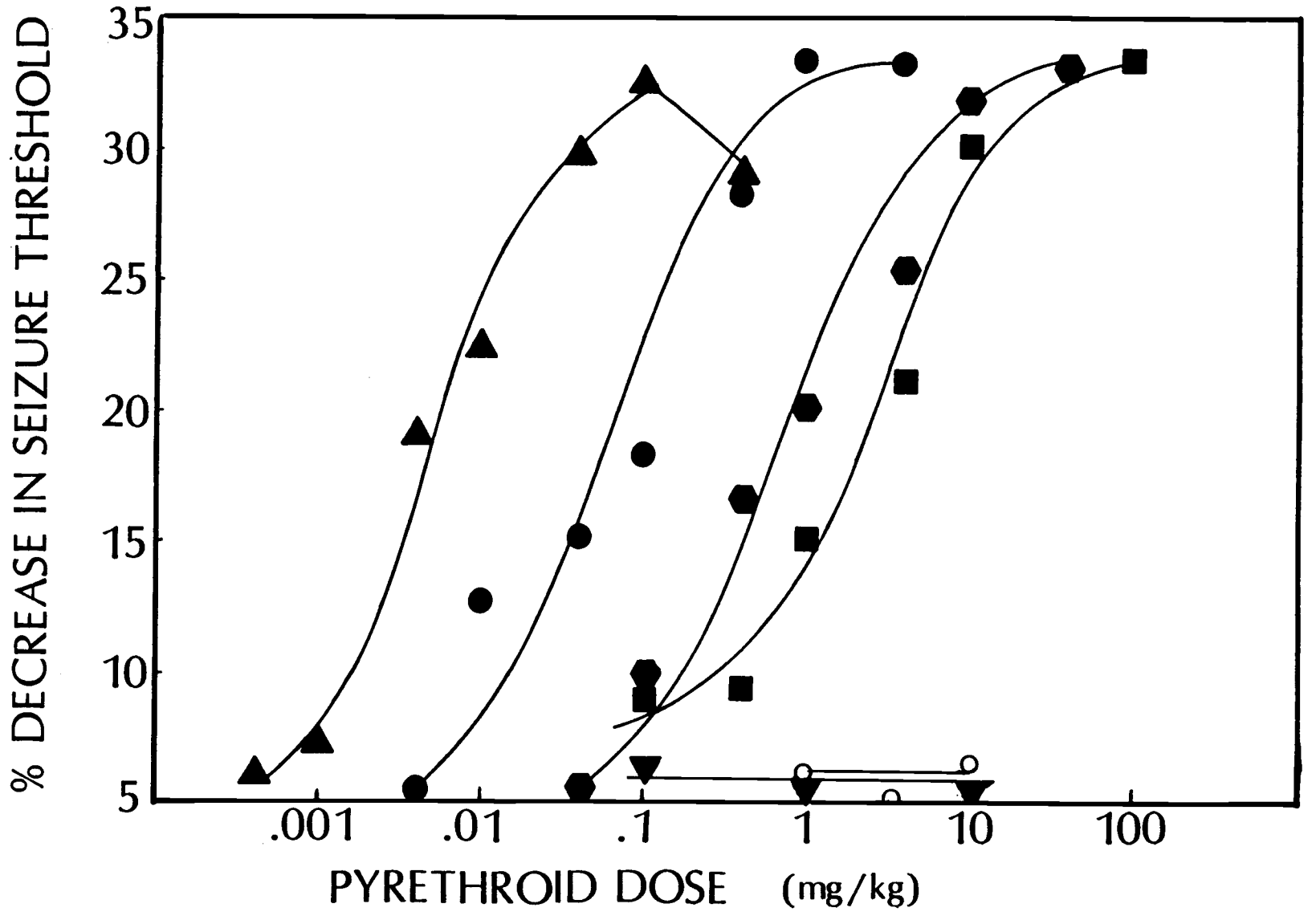

% DECREASE IN SEIZURE THRESHOLD
35
30
25
20
15
10
5
.001
.01
.1
1
10
100
PYRETHROID DOSE (mg/kg)

FIGURE 3

Figure 4. PK 11195 antagonism of the proconvulsant effects of deltamethrin and permethrin. Groups of rats (n=8-12) received an ip injection of vehickle or PK 11195 (either 10 or 30 mg/kg) 30 minutes prior to an ip injection of vehichle, deltamethrin (0.3 mg/kg) or permethrin (10 mg/kg). PTZ seizure thresholds were determined 30 minutes after pyrethroid administration. Pyrethroid treatment elicited a significant reduction in the PTZ seizure threshold as compared to vehicle-only treatment ($P < 0.05$). Administration of PK 11195 and either pyrethroid did not significantly alter PTZ seizure threshold as compared to controls.

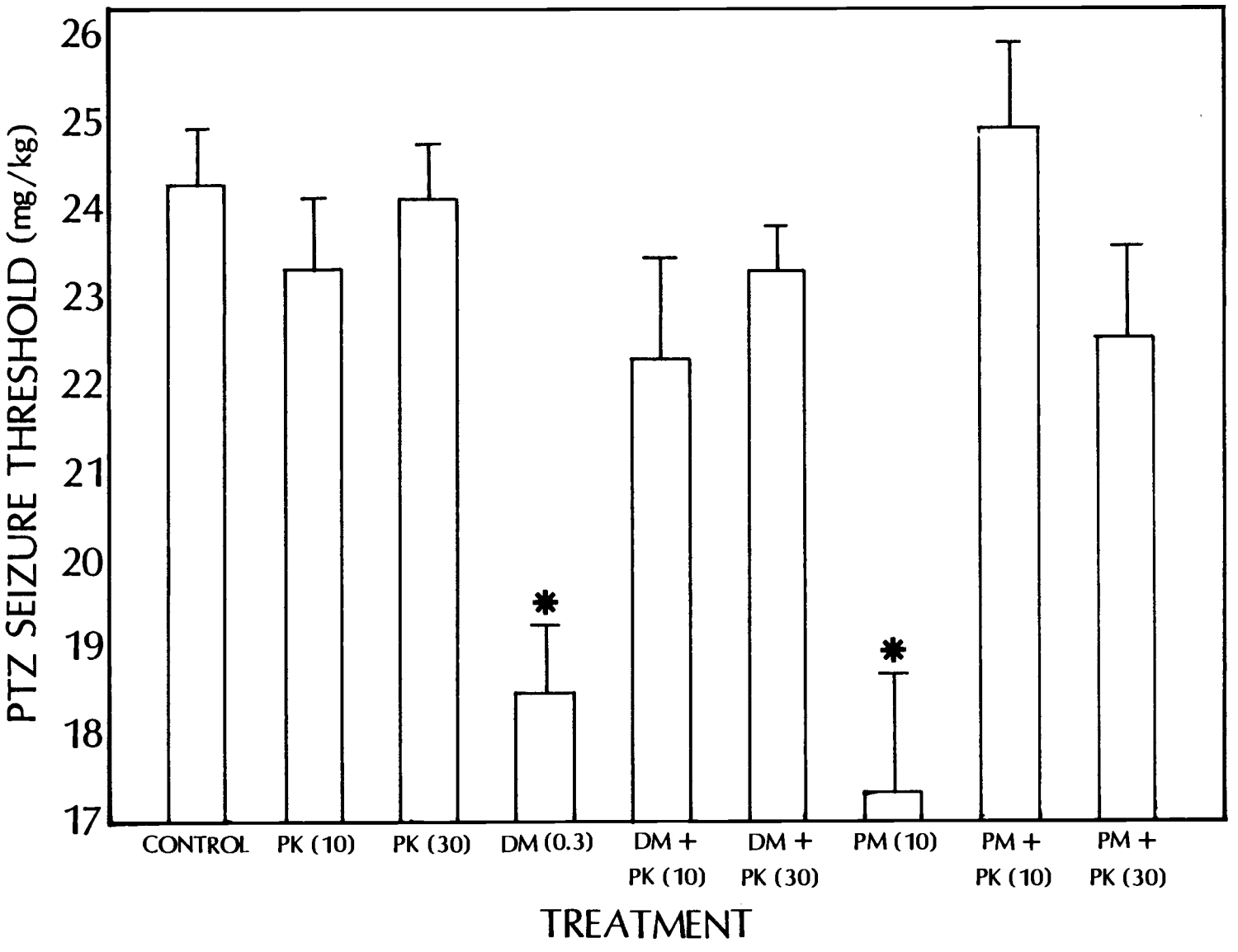

PTZ SEIZURE THRESHOLD (mg/kg)
26
25
24
23
22
21
20
19
18
17
*
*
CONTROL
PK (10)
PK (30)
DM (0.3)
DM + PK (10)
DM + PK (30)
PM (10)
PM + PK (10)
PM + PK (30)
TREATMENT

FIGURE 4

Figure 5. PK 11195 antagonism of the proconvulsant effects of Ro5-4864. Groups of rats (n=8-12) received an ip injection of vehicle or PK 11195 (10 or 30 mg/kg) 30 minutes prior to an ip injection of vehickle or Ro5-4864 (3 mg/kg). PTZ seizure thresholds were determined 30 minutes after Ro5-4864 administration. Ro504864 treatment elicited a significant reduction in the PTZ seizure threshold as compared to control ($P < 0.01$). Administration of PK 11195 and Ro5-4864 did not significantly alter PTZ seizure threshold as compared to controls.

FIGURE 5

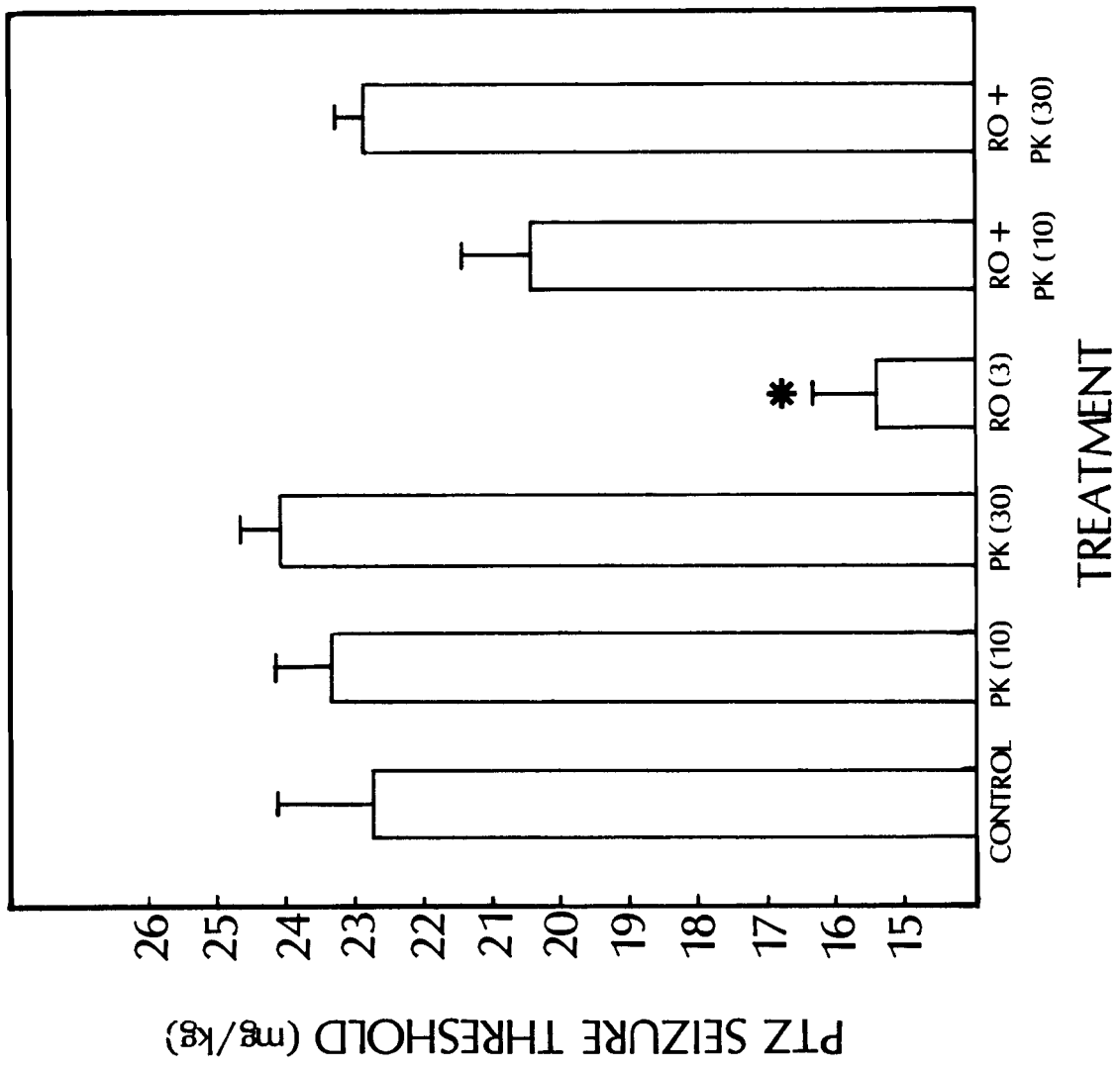

PTZ SEIZURE THRESHOLD (mg/kg)
26
25
24
23
22
21
20
19
18
17
16
15
CONTROL
PK (10)
PK (30)
RO (3)
RO + PK (10)
RO + PK (30)
TREATMENT
*

Figure 6. Phenytoin effects on the proconvulsant actions of deltamethrin and permethrin. Groups of rats (6-10) received an ip injection of phenytoin (30 mg/kg) or vehicle 30 minutes prior to an ip injection of pyrethroid or vehicle. Pyrethroid treatment elicited a significant reduction in PTZ seizure threshold as compared to vehicle-only treatment ($P < 0.05$) Administration of phenytoin failed to antagonize the pyrethroid-induced reduction in PTZ seizure threshold for either pyrethroid with phenytoin and pyrethroid treatment groups not being significantly different.

FIGURE 6

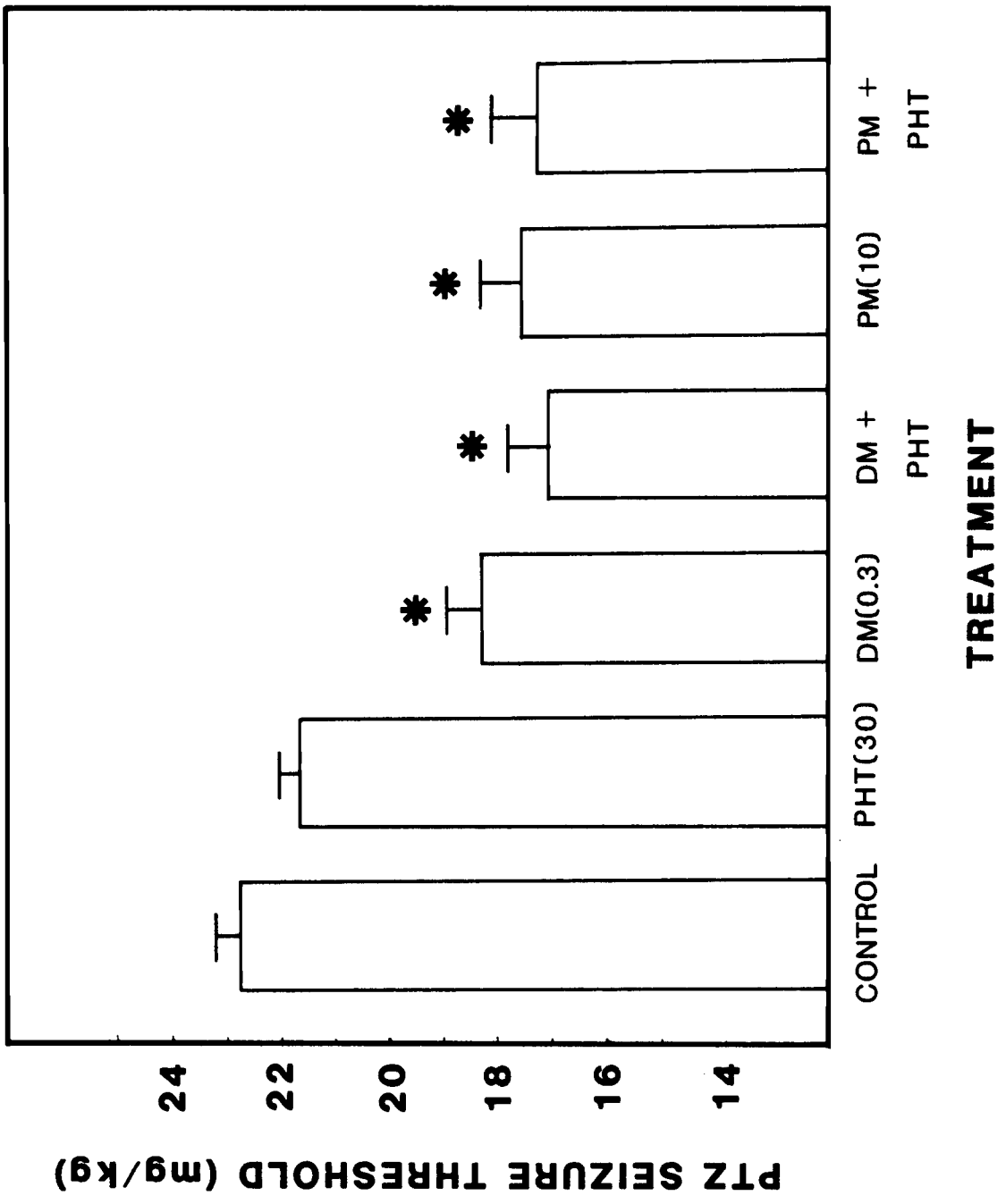

PTZ SEIZURE THRESHOLD (mg/kg)
24
22
20
18
16
14
CONTROL
PHT(30)
DM(0.3)
DM + PHT
PM(10)
PM + PHT
TREATMENT

Figure 7. Phenytoin effects on the proconvulsant actions of Ro5-4864. Groups of rats (6-10) received an ip injection of phenytoin (30 mg/kg) or vehicle 30 minutes prior to an ip injection of Ro5-4864 (3 mg/kg). Ro5-4864 treamtent elicited a significant reduction in PTZ seizure threshold as compared to vehicle-only treated treatment ($P < 0.005$). Administration of phenytoin failed to antagonize the Ro5-4864-induced reduction in PTZ seizure threshold.

FIGURE 7

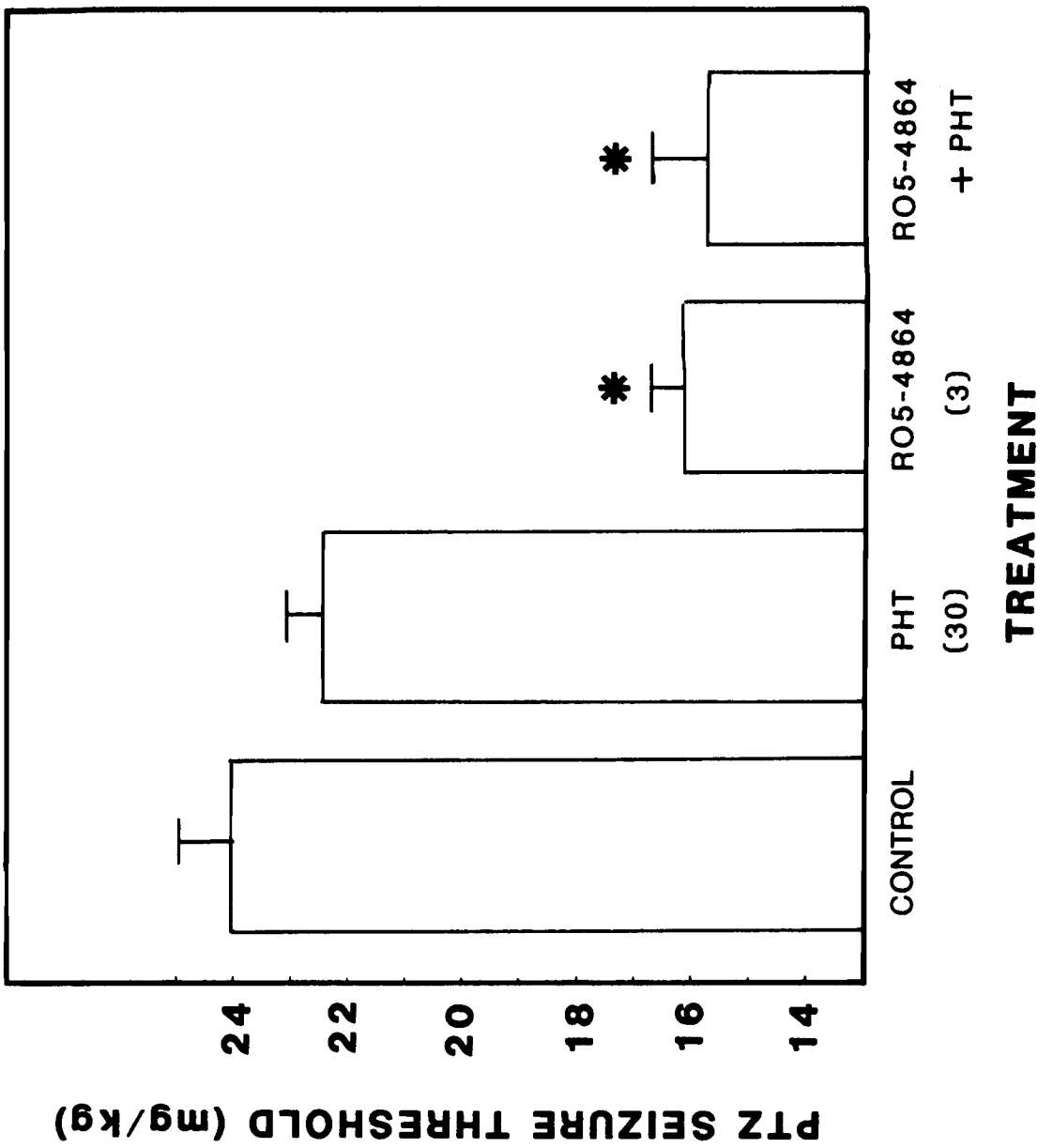

PTZ SEIZURE THRESHOLD (mg/kg)
24
22
20
18
16
14
CONTROL
PHT (30)
RO5-4864 (3)
RO5-4864 + PHT
TREATMENT
*
*

Figure 8. Antagonism of the neurotoxic effects of deltamethrin by PK 11195. Vehicle or PK 11195 (10 mg/kg) were injected ip 30 minutes prior to an ip injection of deltamethrin (5 mg/kg). Animals were observed at 10 minute intervals for toxic symptoms. Scoring of CS syndrome: 1: increae in spontaneous motor activity, i.e. grooming behavior, head rearing, general movement; 2: profuse salivation and/or piloerections; 3: Stereotypical pawing and burrowing behavior; 4: Ataxia and/ or forepaw clonus; and 5: choreoathetosis.

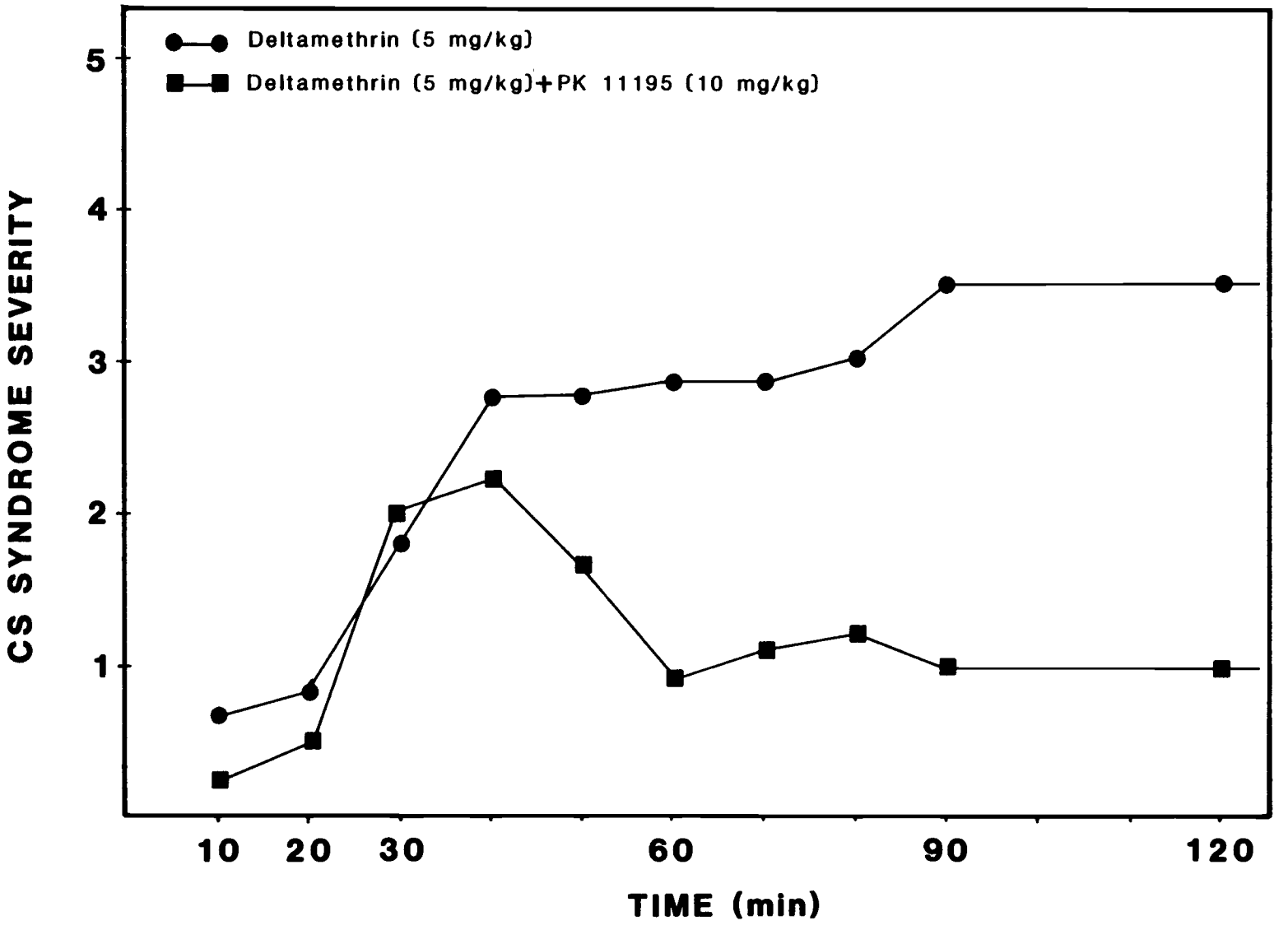

Deltamethrin (5 mg/kg)
Deltamethrin (5 mg/kg)+PK 11195 (10 mg/kg)
CS SYNDROME SEVERITY
5
4
3
2
1
10
20
30
60
90
120
TIME (min)

FIGURE 8

CHAPTER 3 INVOLVEMENT OF PERIPHERAL-TYPE BENZODIAZEPINE RECEPTORS IN THE PROCONVULSANT ACTIONS OF PYRETHROID INSECTICIDES

Leslie L. Devaud and T.F. Murray

ABSTRACT

It has previously been demonstrated that select Type II pyrethroids are potent proconvulsants in the rat and that the proconvulsant actions of deltamethrin are blocked by administration of PK 11195, an antagonist of the peripheral-type benzodiazepine receptor (PTBR). The present investigation has extended these findings to include various Type I pyrethroids as proconvulsants. Additionally, the proconvulsant activity of cismethrin was reversed by administration of PK 11195. Pyrethroid displacement of specific [^{3}H]Ro5-4864 binding to rat brain membranes was investigated to further define the interaction of pyrethroids with the PTBR. Both Type I and Type II pyrethroids potently inhibited [^{3}H]Ro5-4864 binding with affinities ranging from nanomolar to micromolar. The ED_{50} values for the proconvulsant effects of both Type I and Type II pyrethroids were significantly correlated with their respective IC_{50} values as inhibitors of [^{3}H]Ro5-4864 binding. [^{3}H]Ro5-4864

saturation isotherms performed in the presence of fixed concentrations of deltamethrin or cismethrin showed that these pyrethroids increased the observed Kd values for [^{3}H]Ro5-4864 with no change in the maximum number of binding sites. However, Schild plot analysis of the effect of deltamethrin on [^{3}H]Ro5-4864 affinity was nonlinear with the Kd shift approaching a limiting value. Considered together these results suggest an allosteric effect of pyrethroids on [^{3}H]Ro5-4864 binding, and provide additional support for the involvement of the PTBR in the proconvulsant actions of pyrethroids.

INTRODUCTION

Following the labeling of specific brain benzodiazepine receptors with [^{3}H]diazepam, it was shown that this ligand also labeled binding sites in various peripheral tissues (Braestrup and Squires, 1977; Mohler and Okada, 1977). Characterization of the peripheral-type benzodiazepine binding site was advanced with the introduction of a tritiated diazepam analog, [^{3}H]Ro5-4864 (4-chlorodiazepam), having high selectivity for this site. Use of this radioligand in conjunction with selective central-type benzodiazepine receptor ligands has allowed classification of the peripheral-type benzodiazepine receptor (PTBR) as pharmacologically distinct from the $GABA_A$ receptor complex-coupled, central-type benzodiazepine receptor (Marangos et al, 1981; Schoemaker et al,

1983; Richards and Mohler, 1984).

The PTBR is ubiquitously distributed in a variety of tissues throughout the body (Braestrup and Squires, 1977; Marangos et al, 1982). In the mammalian brain, the site labeled by [^{3}H]Ro5-4864 is present in a diffuse distribution of generally low density with the exception of the olfactory bulb where these sites are relatively enriched (Bolger et al, 1984; Richards and Mohler, 1984). Development of PK 11195, an isoquinoline derivative and purported antagonist of the PTBR, has further aided characterization of this site (LeFur et al, 1983).

Investigations to determine the physiological relevance of the PTBR have indicated that among its various actions Ro5-4864 is a potent convulsant and proconvulsant compound (Benavides et al, 1983; Weissman et al, 1984; File et al, 1984; Devaud et al, 1986). Administration of PK 11195 blocks the epileptogenic effects of Ro5-4864 (Benavides et al, 1983; Weissman et al, 1984; Devaud et al, 1986). Although several biochemical and physiological actions of Ro5-4864 have now been well documented, the precise mechanism for seizure induction has yet to be elucidated.

Pyrethroid insecticides are selective nerve poisons which act to prolong the activation of neuronal sodium channels (Narahashi, 1971; Lund and Narahashi, 1983). Pyrethroids have been divided into two classes based on structure-activity relationships and on the symptomology they produce following neurotoxic doses (Verschoyle and Adlridge, 1980; Gammon et al, 1981; Staatz et al, 1982; Lawrence and Casida, 1982). Type I pyrethroids lack an α-

cyano substituent on the phenoxybenzyl moiety while Type II compounds possess this substituent. The poisoning syndrome observed in both insects and mammalian species elicited by Type I pyrethroids is characterized by a rapid onset of restlessness, incoordination and whole body tremors (Staatz et al, 1982; Verschoyle and Aldridge, 1980). The symptomology induced by Type II compounds has a more delayed onset and includes hyperactivity, clonic/tonic convulsions and choreoathetosis (Lawrence and Casida, 1982; Staatz et al, 1982).

Increasing evidence suggests an interaction of Type II pyrethroids with the benzodiazepine/$GABA_A$ receptor/Cl ionophore complex. The onset of Type II poisoning symptoms have been reported to be delayed by diazepam pretreatment in both insects and mammals (Gammon et al, 1982). Moreover, select Type II compounds stereospecifically inhibit the binding of [^{35}S]t-butylbicyclophosphorothionate (TBPS) to the picrotoxin site of the $GABA_A$ receptor complex in rat brain membranes (Lawrence and Casida, 1983; Seifert and Casida, 1985; Crofton et al, 1987).

Recent investigations have also pointed to an involvement of the PTBR as a site for pyrethroid interaction. The Type II pyrethroids deltamethrin and 1RαS,<u>cis</u> cypermethrin inhibited the specific binding of [^{3}H]Ro5-4864 with a 60-fold increase in potency over that reported for inhibition of [^{35}S]TBPS binding (Gammon and Sander, 1985; Lawrence et al, 1985). Our own <u>in vivo</u> studies, utilizing a sensitive measure of seizure susceptibility, have demonstrated a potent proconvulsant action for the Type II

pyrethroids 1RaS,*cis* cypermethrin, deltamethrin and fenvalerate, and the atypical Type I pyrethroid permethrin (Devaud et al, 1986). Furthermore, the proconvulsant activity produced by both deltamethrin and permethrin was effectively reversed by pretreatment with PK 11195 (Devaud et al, 1986).

Thus, the purpose of the present investigation was twofold: first to determine whether the potent proconvulsant actions of Type II pyrethroids are shared by Type I compounds, and second to better define the interactions of pyrethroid insecticides with the PTBR using [^{3}H]Ro5-4864 as a radioligand probe for this site.

METHODS

Seizure Threshold Measurements: Male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing 140 to 180 g were used in all experiments. Pentylenetetrazol (PTZ) seizure thresholds were determined using the timed infusion technique as previously described (Murray et al, 1985; Devaud et al, 1986). Briefly, the PTZ solution (2 mg/ml) was infused via a 25 gauge butterfly needle inserted into the lateral tail vein. The solution was infused at a constant rate of 1.6 ml/min and the endpoint was taken as the first myoclonic twitch of the face or neck. The time to the first myoclonic twitch was recorded and the dose of PTZ required (mg/kg) to elicit the seizure was calculated from this time, the concentration of the PTZ solution and the body weight of the animal. The endpoint for this method, a myoclonic twitch of the

face or neck, occurs synchronously with the first EEG discharge and is a reliable and accurate method for determining seizure susceptibility (Nutt et al, 1980; Nutt et al, 1981).

Pyrethroids and PK 11195 were dissolved in a vehicle consisting of 5% Emulphor (El-620, GAF, New York, NY), 5% ethanol and 90% isotonic saline and injected intraperitoneally (ip) in a volume of 1 ml/kg (pyrethroids) or 2 ml/kg (PK 11195). Pyrethroids were administered 30 minutes prior to PTZ seizure threshold determination and PK 11195 was injected 30 minutes prior to pyrethroid treatment.

[^{3}H]Ro5-4864 Equilibrium Binding Assay: Membrane preparation: Male Sprague-Dawley rats were decapitated and the olfactory bulbs and/or cerebral cortices quickly dissected. The tissue was gently homogenized using a Teflon-glass homogenizer in 100 volumes of 0.32 M sucrose containing 10 mM Hepes, pH 7.4. The homogenate was centrifuged at 1000 x g for 10 minutes. The supernatant was decanted and centrifuged at 9000 x g for 20 minutes. The resulting P2 pellet was resuspended in 100 volumes of ice cold assay buffer (50 mM sodium phosphate in normal saline, pH 7.2). Final protein content was 0.04-0.06 mg/tube for the olfactory bulb membrane preparation and 0.30-0.40 mg/tube for the cerebral cortical membrane preparation as determined by the method of Lowry following solubilization with 0.5N NaOH (Lowry et al, 1951). Bovine serum albumin was used as the standard for protein assays.

Equilibrium competition assays: Assays were performed in a final volume of 1 ml containing aliquots of the membrane preparation, varying concentrations of pyrethroids in 5 μl 100% DMSO or DMSO only, and 50 μl of [^{3}H]Ro5-4864 at a final concentration of approximately 1 nM. Non-specific binding was determined in the presence of 1 μM PK 11195 and was less than 10% of total in the olfactory bulb preparation and 20-30% of the total in the cerebral cortical membrane preparation. The reaction was initiated by addition of the radioligand. Following incubation at 4°C for 120 minutes samples were rapidly filtered through Whatman GF/B filters using a Brandel Cell Harvester (Gaithersburg, MD) and washed four times with 4 mls each of ice cold assay buffer. After overnight elution, bound radioactivity was measured by liquid scintillation spectroscopy. IC_{50} values and slope factors were determined using the nonlinear least-squares curve fitting program LIGAND (Munson and Rodbard, 1980).

[^{3}H]Ro5-4864 equilibrium saturation binding assays: Using an identical protocol for the radioligand binding assay as described above, the specific binding of 12 concentrations of [^{3}H]Ro5-4864 ranging from 0.2-24 nM was determined. The final concentration of DMSO was 0.5% in all assays. Individual saturation isotherms were analyzed by nonlinear regression analysis assuming either ligand binding to a single receptor site or binding to two independent species of receptor. Parameter estimates were derived using the Lundon-1 iterative curve-fitting program (Lundeen and Gordon, 1985). Data are described as a better fit by one model of ligand

binding than another when a partial F-test comparing the two models indicated significant improvement in residual sum of squares ($P < 0.05$) as described by Hoyer et al. (1984). Data showing the effect of deltamethrin on [^{3}H]Ro5-4864 affinity were plotted according to the Schild equation:

$$\log (x-1) = \log [I] - \log K_i$$

where [I] is the concentration of deltamethrin, K_i is the equilibrium dissociation constant of deltamethrin, and x is the affinity shift induced by deltamethrin (Arunlakshana and Schild, 1959).

Materials: [^{3}H]Ro5-4864 (specific activity 78.9 Ci/mmol) was purchased from DuPont-NEN, Boston, MA. Unlabeled Ro5-4864 was generously supplied by Dr. Peter Sorter, Hoffman-LaRoche, Inc., Nutley, N.J. PK 11195 was a generous gift of Dr. G. LeFur, Pharmuka Laboratories, Gennevilliers, France. Pyrethroids: 1RαS,cis cypermethrin, 1SαR,cis cypermethrin, 1R,cis permethrin, 1S,cis permethrin and allethrin were gifts from Dr. Arthur Ramsey, FMC Corporation, Princeton, New Jersey. Tetramethrin was obtained from the EPA, Research Triangle Park, N.C. Kadethrin, cismethrin and deltamethrin were gifts from Dr. P. Foulhoux, Roussel Uclaf, Romainville, France. PK 11195 and all pyrethroids were dissolved in 100% DMSO for use in the binding assays.

RESULTS

The acute administration of the Type I pyrethroids

cismethrin, kadethrin, resmethrin, tetramethrin and 1R,*cis* permethrin all resulted in dose-dependent lowering of chemoconvulsant seizure threshold. Examination of the dose-response curves depicted in Figure 9 shows that all five of these pyrethroids produced maximal reductions in PTZ seizure threshold of approximately 25-35%. Kadethrin was the most potent pyrethroid tested with a dose as low as 100 μg/kg ip reducing PTZ seizure threshold from a control value of 25.2 ± 0.82 mg/kg to 21.5 ± 0.82 mg/kg ($P < 0.001$). As shown in Table 2 the rank order potency of the Type I pyrethroids tested as proconvulsants was kadethrin > 1R,*cis* permethrin > resmethrin > cismethrin > tetramethrin. In contrast, allethrin, a prototypic Type I pyrethroid, and 1S,*cis* permethrin, the insecticidally inactive isomer of permethrin, were completely devoid of proconvulsant activity in doses as high as 10 mg/kg. A dose of 30 mg/kg allethrin produced generalized tremors and prostration which led to death in all animals within 20 minutes of injection. The strong tremorogenic effects of allethrin even at low doses may have rendered detection of the myoclonic twitch induced by infusion of PTZ difficult. The maximum dose of kadethrin which could be used in the seizure threshold determinations was also limited by the tremors induced following its administration.

The involvement of peripheral-type benzodiazepine receptors in the proconvulsant actions of Type I pyrethroids was assessed by determining the effect of pretreatment with PK 11195 on the response to cismethrin. The 10 mg/kg dose of PK 11195 had no

effect on PTZ seizure threshold when administered alone (Figure 10). However, as shown in Figure 10, this dose of PK 11195 significantly antagonized the proconvulsant effects of the 3 mg/kg dose of cismethrin. The 3 mg/kg dose of cismethrin reduced seizure threshold by 24.7% ($P < 0.001$) in vehicle-injected control animals, while the same dose of cismethrin produced a non-significant reduction of 7.3% in PK 11195 pretreated rats. These findings are in accordance with our previous demonstration of PK 11195 antagonism of the proconvulsant activity of deltamethrin and permethrin (Devaud et al, 1986). These results suggest that an activation of peripheral-type benzodiazepine receptors may be operative in the proconvulsant actions of both Type I and Type II pyrethroids.

The pyrethroid-induced inhibition of [^{3}H]Ro5-4864 binding to rat olfactory bulb membranes was next studied to further characterize the interaction of pyrethroids with peripheral-type benzodiazepine receptors. The initial experiments utilized Ro5-4864, PK 11195, diazepam and clonazepam as inhibitors of specific [^{3}H]Ro5-4864 binding to establish the pharmacological profile of this binding site. Both Ro5-4864 and PK 11195, two ligands selective for the PTBR, were able to potently displace specific [^{3}H]Ro5-4864 binding (Table 3). PK 11195 and Ro5-4864 were equipotent at this binding site. Diazepam has been reported to bind with high affinity to both the central- and peripheral-type benzodiazepine receptor, having a low nanomolar K_i for the central-type and mid nanomolar K_i for the peripheral-type receptor

(Marangos, 1982). Consistent with previous reports, a mean IC_{50} value of 224 ± 52 nM for diazepam was observed in olfactory bulb membranes in the present study. Clonazepam, a benzodiazepine with high selectivity for the central-type benzodiazepine receptor and low (micromolar) affinity for the peripheral-type receptor (Weissman et al, 1984) inhibited specific [^{3}H]Ro5-4864 binding with an IC_{50} of 12.0 ± 3.8 μM. Thus, the pharmacological profile of the site labeled by [^{3}H]Ro5-4864 was that expected for the PTBR.

A series of both Type I and Type II pyrethroids were examined for their ability to inhibit specific binding of [^{3}H]Ro5-4864 to rat olfactory bulb membranes. As depicted in Figure 11, selected Type I and Type II pyrethroids elicited a concentration-dependent inhibition of the specific binding of [^{3}H]Ro5-4864. The IC_{50} values for deltamethrin and 1RαS,cis cypermethrin were 40.3 and 43.1 nM, respectively, indicating that these compounds have a high affinity for [^{3}H]Ro5-4864 binding sites in olfactory bulb membranes. In contrast, the insecticidally inactive 1SαR,cis isomer of cypermethrin displayed a 306-fold lower potency as an inhibitor of [^{3}H]Ro5-4864 binding (Table 3). The interaction between pyrethroids and [^{3}H]Ro5-4864 did not appear to be competitive since the maximum inhibition reached a limiting value which varied for the various compounds employed (Figure 11, Table 3). Consistent with a non-competitive mechanism for the inhibition of [^{3}H]Ro5-4864 binding, the Hill slopes for the majority of the pyrethroids tested were significantly lower than unity (Table 3). The observed efficacies for pyrethroid inhibition of specific

[^{3}H]Ro5-4864 binding ranged from greater than 90% for the most potent pyrethroids to less than 50% for those compounds with lower affinity for the [^{3}H]Ro5-4864 binding site. The maximum concentration of pyrethroids employed was 10 μM due to the limited aqueous solubility of these compounds.

Of the five insecticidally active Type I pyrethroids tested, kadethrin, an atypical Type I compound, was found to be equipotent with deltamethrin and 1RαS,cis cypermethrin in its ability to inhibit specific [^{3}H]Ro5-4864 binding (Figure 11). The rank order of potencies of Type I pyrethroids as inhibitors of specific [^{3}H]Ro5-4864 binding to olfactory bulb membranes was kadethrin > (1R)cis permethrin > allethrin > tetramethrin > cismethrin (Figure 10 and Table 3). 1S,cis permethrin, the insecticidally inactive isomer of permethrin, did not inhibit [^{3}H]Ro5-4864 binding over the concentration range employed.

Although routine equilibrium competition binding experiments were performed with olfactory bulb membranes due to the high signal to noise ratio afforded by the enrichment of the PTBR in this brain area, further experiments were undertaken to compare the inhibitory potencies of pyrethroids at the PTBR using rat cerebral cortical membranes. As shown in Figure 12, the three most potent pyrethroids in the olfactory bulb membrane preparation, deltamethrin, 1RαS,cis cypermethrin and kadethrin were also the most potent at inhibiting specific [^{3}H]Ro5-4864 binding to cerebral cortical membranes. The results obtained with deltamethrin and 1RαS,cis cypermethrin are in excellent agreement with the

previously reported findings of Lawrence and coworkers (Lawrence et al, 1985). Compounds such as deltamethrin, 1RαS,*cis* cypermethrin and kadethrin tended to be somewhat less potent as inhibitors of specific [^{3}H]Ro5-4864 binding in the cerebral cortex as compared with the olfactory bulb. However, the [^{3}H]Ro5-4864 binding assay in cerebral cortical membranes required substantially more protein than that used with olfactory bulb preparations. Additional competition experiments demonstrated that the IC_{50} value for deltamethrin increased from 53.6 nM to 158.3 nM when olfactory bulb membrane protein content of the assay tubes was increased from 46 mg to 220 mg (data not shown). Thus, the differences in pyrethroid potencies in the two brain areas appears to be related to the higher tissue concentrations used in assays with cerebral cortical membranes, presumably due to depletion of pyrethroids to nonspecific binding sites.

The relationship between the affinities of pyrethroids for the [^{3}H]Ro5-4864 binding site and the proconvulsant potencies of both the Type I compounds reported herein and the Type II compounds reported earlier (Devaud et al, 1986) was explored. A significant correlation was found between the logs of the ED_{50} values for the Type I and Type II pyrethroids examined *in vivo* as proconvulsants and their respective IC_{50} values as inhibitors of [^{3}H]Ro5-4864 binding, $P < 0.01$ by Kendall's coefficient of rank correlation (Sokol and Rohlf, 1969) (Figure 13).

To further assess the mechanism of pyrethroid inhibition of [^{3}H]Ro5-4864 binding to the PTBR, [^{3}H]Ro5-4864 saturation binding

isotherms were performed in the presence or absence of fixed concentrations of either cismethrin or deltamethrin. The presence of either deltamethrin or cismethrin in the incubation was associated with a decrease in [^{3}H]Ro5-4864 affinity (i.e., increased Kd) with no significant alteration in the density of binding sites (Table 4). Representative saturation isotherms and corresponding Scatchard replots of these saturation data for [^{3}H]Ro5-4864 binding in the presence and absence of deltamethrin and cismethrin are depicted in Figure 14 and 15, respectively. Computer-modeling of these saturation data revealed that the isotherms were adequately described by a one-site fit in 52 of 52 individual experiments. Thus, the two-site model did not provide a significant improvement in the fit in any of the [^{3}H]Ro5-4864 saturation isotherms either in the presence or absence of pyrethroids. A Schild plot of the log (affinity shift-1) versus log (deltamethrin concentration) as shown in Figure 16 revealed that the shift in [^{3}H]Ro5-4864 affinity approached a limiting value of approximately 3 at deltamethrin concentrations of 50 to 150 nM. The maximum Kd shift varied from 2.66 to 3.14 over this concentration range of deltamethrin and these mean values did not differ from one another statistically. These results further suggest that the interaction of deltamethrin with the [^{3}H]Ro5-4864 binding site is noncompetitive in nature.

DISCUSSION

Pyrethroid insecticides have come into prominent use in recent years due to their high insecticidal activity and reported low animal toxicity. Research into mechanisms of action of pyrethroids has shown that they interact with neuronal sodium channels to produce prolongation of the sodium current (Narahashi, 1971; Lund and Narahashi, 1983). The Type II pyrethroids, however, appear to have additional actions at or near the TBPS site on the $GABA_A$ receptor/Cl ionophore (Lawrence and Casida, 1983; Seifert and Casida, 1985; Crofton et al, 1987). Recent evidence also implicates the peripheral-type benzodiazepine receptor as a site of action for Type II pyrethroids (Gammon and Sander, 1985; Lawrence et al, 1985).

The administration of low doses of select Type II pyrethroids has shown that these compounds are potent proconvulsants and that this effect can be blocked by pretreatment with PK 11195, an antagonist of the PTBR (Devaud et al, 1986). These previous results were extended in the present investigation to show that selected Type I compounds shared this proconvulsant activity. Although generally less potent than Type II pyrethroids, the Type I compounds were found to be equally as efficacious as proconvulsants suggesting a common mechanism of action for both classes of pyrethroids in eliciting this response. Moreover, the stereospecificity of action previously noted for the isomers of the Type II pyrethroid cypermethrin was also observed in the present study for the isomers of the Type I pyrethroid permethrin.

The previously demonstrated antagonism of the proconvulsant effects of deltamethrin and permethrin by PK 11195 was extended to include the Type I compound, cismethrin. The PK 11195 sensitivity of pyrethroid-induced lowering of seizure threshold provides evidence for the involvement of the PTBR in this action shared by both Type I and Type II compounds. These results support the toxicological relevance of pyrethroid interaction with the PTBR.

To further assess the role of the PTBR in pyrethroid-induced lowering of seizure threshold, Type I and Type II pyrethroids were evaluated as displacers of specific [^{3}H]Ro5-4864 binding to rat olfactory bulb and cerebral cortical membranes. All insecticidally active pyrethroids studied were capable of inhibiting specific [^{3}H]Ro5-4864 binding with a similar rank order of potency in both membrane preparations. Moreover, the pyrethroid inhibition of [^{3}H]Ro5-4864 binding was found to be stereoselective. The insecticidally active 1RαS,cis isomer of cypermethrin was observed to be 306 times more potent than its respective 1SαR,cis isomer as an inhibitor of [^{3}H]Ro5-4864 binding. A similar stereoisomeric preference was seen with permethrin in that the insecticidally inactive 1S,cis isomer was unable to displace [^{3}H]Ro5-4864 binding. The stereoselectivity exhibited by the 1RαS,cis isomer of cypermethrin and the 1R,cis isomer of permethrin extended to their in vivo potencies where the respective 1S isomers of these two pyrethroids were essentially inactive as proconvulsants. Moreover, a significant correlation was found between the affinities of pyrethroids as inhibitors of [^{3}H]Ro5-4864 binding and their

respective proconvulsant potencies. Thus, the stereoselectivity and pharmacological specificity of these actions of pyrethroids provide evidence in support of the involvement of the PTBR in the modulation of seizure threshold elicited by these compounds.

The pyrethroid inhibition of [^{3}H]Ro5-4864 binding described herein cannot be accounted for by a competitive interaction of pyrethroids with a single population of [^{3}H]Ro5-4864 binding sites. The observation that in rat olfactory bulb membranes the maximum inhibition of [^{3}H]Ro5-4864 specific binding produced by pyrethroids plateaued at values ranging from 45 to 98% inhibition suggested a noncompetitive interaction of pyrethroids with the site labeled by [^{3}H]Ro5-4864. The noncompetitive nature of pyrethroid interaction with the [^{3}H]Ro5-4864 site was further supported by a Schild plot analysis of deltamethrin's effect on [^{3}H]Ro5-4864 affinity (Figure 15). The deltamethrin-induced shift in [^{3}H]Ro5-4864 affinity was not linear and approached a limiting value of approximately 3 at deltamethrin concentrations of 50-150 nM. From these data it may be inferred that the inhibitory influences of pyrethroids on [^{3}H]Ro5-4864 binding occur via an allosteric mechanism, conceivably with pyrethroids binding at a site adjacent to the [^{3}H]Ro5-4864 recognition site.

In addition to their interactions with the PTBR, a recent report has confirmed the ability of Type II pyrethroids to inhibit [^{35}S]TBPS binding by demonstrating that deltamethrin produced a significant decrease in the affinity of [^{35}S]TBPS for the chloride channel in rat brain membranes (Lummis et al, 1987). Moreover,

Seifert and Casida (1985) have recently shown that the presence of micromolar concentrations of GABA enhance the ability of 1RαS,cis cypermethrin to displace [^{35}S]TBPS in rat brain. This demonstration of a GABA-dependent mechanism for α-cyano pyrethroid displacement of [^{35}S]TBPS suggests that the pyrethroid binding domain and the chloride channel of the $GABA_A$ receptor complex may be allosterically coupled. Ro5-4864 shares with pyrethroids the ability to inhibit the specific binding of [^{35}S]TBPS (Ticku and Ramanjaneyalu, 1984). The IC_{50} for Ro5-4864 as an inhibitor of [^{35}S]TBPS binding was reported to be 20 μM. Additional modulatory influences of Ro5-4864 on [^{35}S]TBPS binding may be observed in the presence of GABA. Ro5-4864 (1-5 μM) was recently shown to partially reverse the inhibitory effect of GABA on [^{35}S]TBPS binding in one report (Squires and Saederup, 1987), and to stimulate [^{35}S]TBPS binding to the chloride channel of rat brain in the submicromolar to micromolar concentration range in a second report (Gee, 1987). Thus, in addition to labeling the PTBR, Ro5-4864 appears to recognize a site on or near the chloride channel associated with $GABA_A$ receptors. The interactions of both Ro5-4864 and α-cyano pyrethroids with the [^{35}S]TBPS recognition site may indicate that the binding domains for these compounds on the $GABA_A$ receptor-chloride channel complex are adjacent and allosterically coupled. Additional studies will be necessary to assess this hypothesis and to further explore the potential heterogeneity of Ro5-4864 binding sites.

Acknowledgements

This work was supported by a grant from the Oregon State University Environmental Health Sciences Center. The excellent word processing assistance of Elaine Luttrull is gratefully acknowledged.

Table 2. Relative potencies of Type I pyrethroids on seizure threshold for PTZ in rats

Compound	ED_{50} (95% confidence limits) mg/kg	mmol/kg	Maximum[a] Effect
kadethrin	153 (42-560)	0.39 (0.11-1.40)	26.7
IR cis permethrin	183 (2-1340)	0.47 (0.01-34.3)	25.4
resmethrin	234 (72-762)	0.69 (0.21-2.25)	30.1
cismethrin	374 (174-802)	1.10 (0.52-2.40)	26.7
tetramethrin	409 (326-513)	1.24 (0.99-1.55)	32.5
IS cis permethrin	inactive		--
allethrin	inactive		--

[a]Maximum percentage reduction in dose of PTZ required to elicit a seizure

Table 3. Relative potencies of pyrethroids and ligands for peripheral- and central-type benzodiazepine receptors as inhibitors of specific [^{3}H]Ro5-4864 binding in rat brain membranes

Benzodiazepine receptor ligands	Olfactory Bulb			Cerebral Cortex		
	IC_{50}[a]	n_H[b]	max[c] inhibition %	IC_{50}[a]	n_H[b]	max[c] inhibition %
Ro5-4864	2.9 ± 0.33	0.87 ± 0.07	100	2.95 ± 1.18	0.94 ± 0.01	100
PK 11195	2.8 ± 0.28	1.13 ± 0.21	100	1.05 ± 0.16	0.94 ± 0.06	100
diazepam	224 ± 52	0.79 ± 0.08	94.9	n.d.[d]	n.d.	n.d.
clonazepam	11900 ± 3800	1.05 ± 0.06	84.2	n.d.	n.d.	n.d.
Pyrethroids						
deltamethrin	40.3 ± 8.7	0.49 ± 0.04	93.3	258 ± 22.9	0.75 ± 0.1	97.6
1RαS,*cis* cypermethrin	43.1 ± 6.1	0.61 ± 0.06	94.9	142 ± 18.8	0.81 ± 0.03	100
kadethrin	44.9 ± 15.3	0.51 ± 0.08	89.6	178 ± 39	0.60 ± 0.04	93.4
1R,*cis* permethrin	1550 ± 140	0.48 ± 0.01	68.9	3590 ± 650	0.83 ± 0.09	69.4
allethrin	2250 ± 260	1.13 ± 0.10	98.1	1730 ± 130	0.94 ± 0.1	89.9
tetramethrin	4100 ± 130	1.08 ± 0.04	78.1	3680 ± 600	1.30 ± 0.08	77.8
cismethrin	10100 ± 3500	0.74 ± 0.30	46.4	12400 ± 4100	0.78 ± 0.05	63.4
1SαR,*cis* cypermethrin	13200 ± 6500	0.33 ± 0.02	52.7	34300 ± 29680	0.51 ± 0.05	59.7
resmethrin	15100 ± 5200	0.43 ± 0.02	45.0	16600 ± 1660	0.59 ± 0.09	42.7
1S,*cis* permethrin	inactive			n.d.		

[a]IC_{50}'s and [b]slope factors were calculated by computer-assisted analysis as described in Methods section. Values presented are means ± standard errors of the mean of three to seven competition experiments.

[c]Maximal % inhibition is the inhibition observed at concentrations of 1-10 μM. [^{3}H]Ro5-4864 was present at a final concentration of 0.6-1.2 nM.

[d]n.d. indicates not determined.

Table 4. Effects of the Type II pyrethroid (A) deltamethrin and the Type I pyrethroid (B) cismethrin on [^{3}H]Ro5-4864 saturation isotherm binding parameters in rat olfactory bulb membranes

Experimental Condition		N	Kd[a] (nM)	Bmax[a] (fmol/mg protein)	Kd Shift[b]
A.	control	3	2.19 ± 0.30	1229 ± 232	
	deltamethrin (25 nM)	3	4.08 ± 0.71	1400 ± 272	1.97
	control	4	3.02 ± 0.45	1243 ± 115	
	deltamethrin (50 nM)	4	8.72 ± 2.0*	1530 ± 335	3.14
	control	4	2.44 ± 0.77	1040 ± 85	
	deltamethrin (75 nM)	4	7.41 ± 2.70	1680 ± 268	3.11
	control	4	4.83 ± 1.60	1483 ± 263	
	deltamethrin (100 nM)	4	13.28 ± 4.40	1630 ± 120	2.66
	control	3	4.92 ± 1.40	1227 ± 13	
	deltamethrin (150 nM)	3	12.53 ± 1.89*	1930 ± 262	2.80
B.	control	3	3.22 ± 0.20	1373 ± 12	
	cismethrin (5 mM)	3	8.72 ± 1.20*	1683 ± 213	2.62
	control	3	2.98 ± 0.52	1477 ± 84	
	cismethrin (10 mM)	3	9.27 ± 1.10*	1907 ± 197	3.22

[a] Values presented are means ± standard error of the mean

[b] The affinity shift values for each pyrethroid concentration represent the means of the K_d shift derived from individual equilibrium saturation experiments with control and deltamethrin- or cismethrin-treated samples assayed in parallel.

*$P < 0.05$ by Student's t test

Figure 9. Dose response curves for the effects of (1R)cis permethrin (■), tetramethrin (▼), resmethrin (⬢), cismethrin (●), kadethrin (△), (1S)cis permethrin (□), and allethrin (○), on PTZ seizure threshold. Values are expressed as the mean percentage decrease in seizure threshold as compared to vehicle injected controls. Each point represents the mean of 6-12 animals. Kadethrin ($P < 0.001$), cismethrin ($P < 0.05$) and (1R)cis permethrin ($P < 0.01$) elicited a statistically significant decreases in PTZ seizure threshold at doses equal to or greater than 0.1 mg/kg. Resmethrin ($P < 0.05$) and tetramethrin ($P < 0.01$)-induced decreases in PTZ seizure threshold were significant at doses equal to or greater than 0.3 mg/kg using the Student's t-test.

FIGURE 9

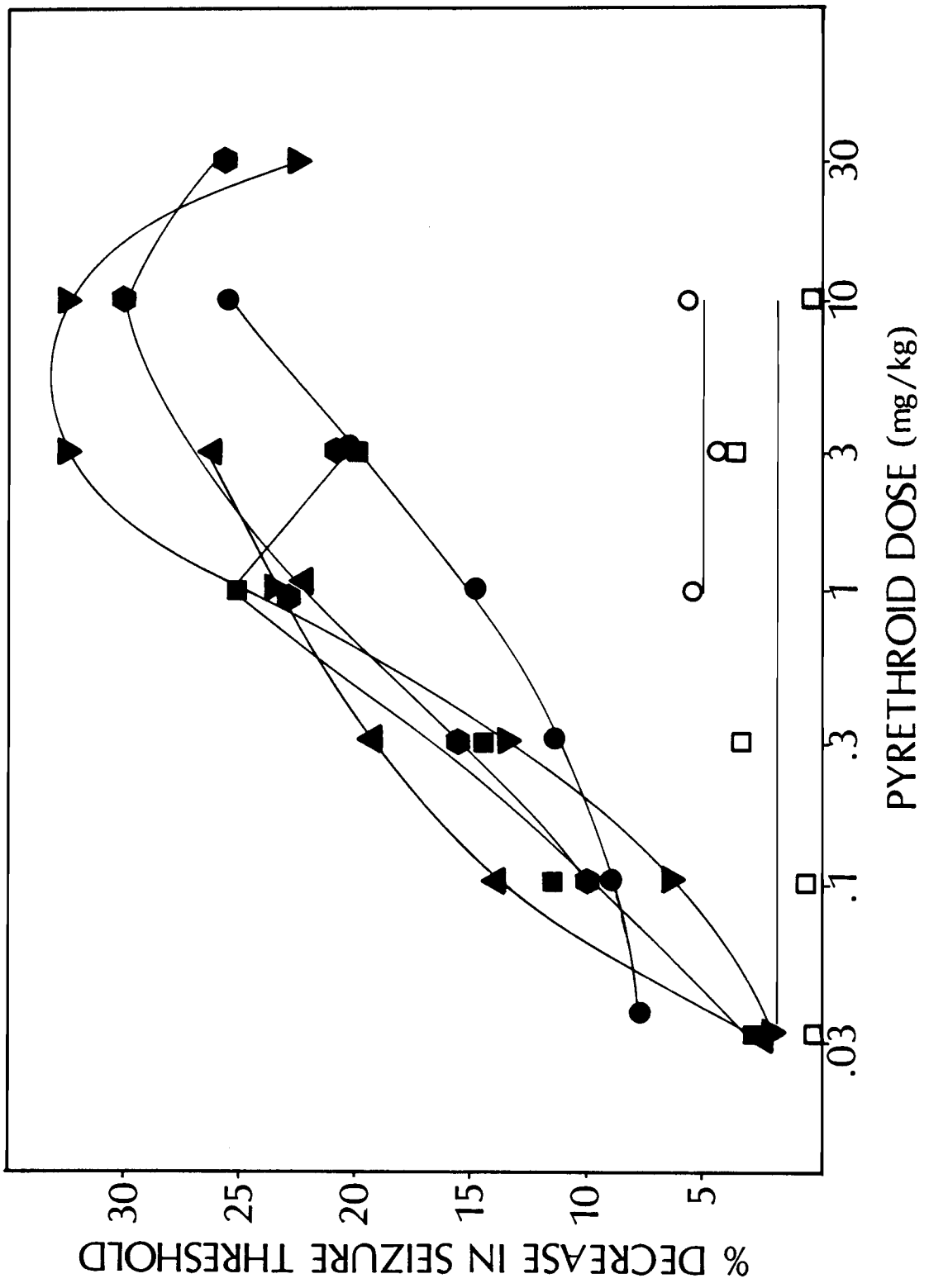

% DECREASE IN SEIZURE THRESHOLD
30
25
20
15
10
5
.03
.1
.3
1
3
10
30
PYRETHROID DOSE (mg/kg)

Figure 10. PK 11195 antagonism of the proconvulsant effects of cismethrin. Groups of rats (n = 8-10) received an ip injection of vehicle or PK 11195 (10 mg/kg) 30 minutes prior to an ip injection of vehicle or cismethrin (3 mg/kg). PTZ seizure thresholds were determined 30 minutes after cismethrin administration. Cismethrin treatment elicited a significant reduction in the PTZ seizure threshold as compared to vehicle-only treatment ($P < 0.001$). Administration of PK 11195 and cismethrin did not significantly alter PTZ seizure threshold as compared to controls but did produce a statistically significant increase versus cismethrin-only treated animals ($P < 0.001$).

FIGURE 10

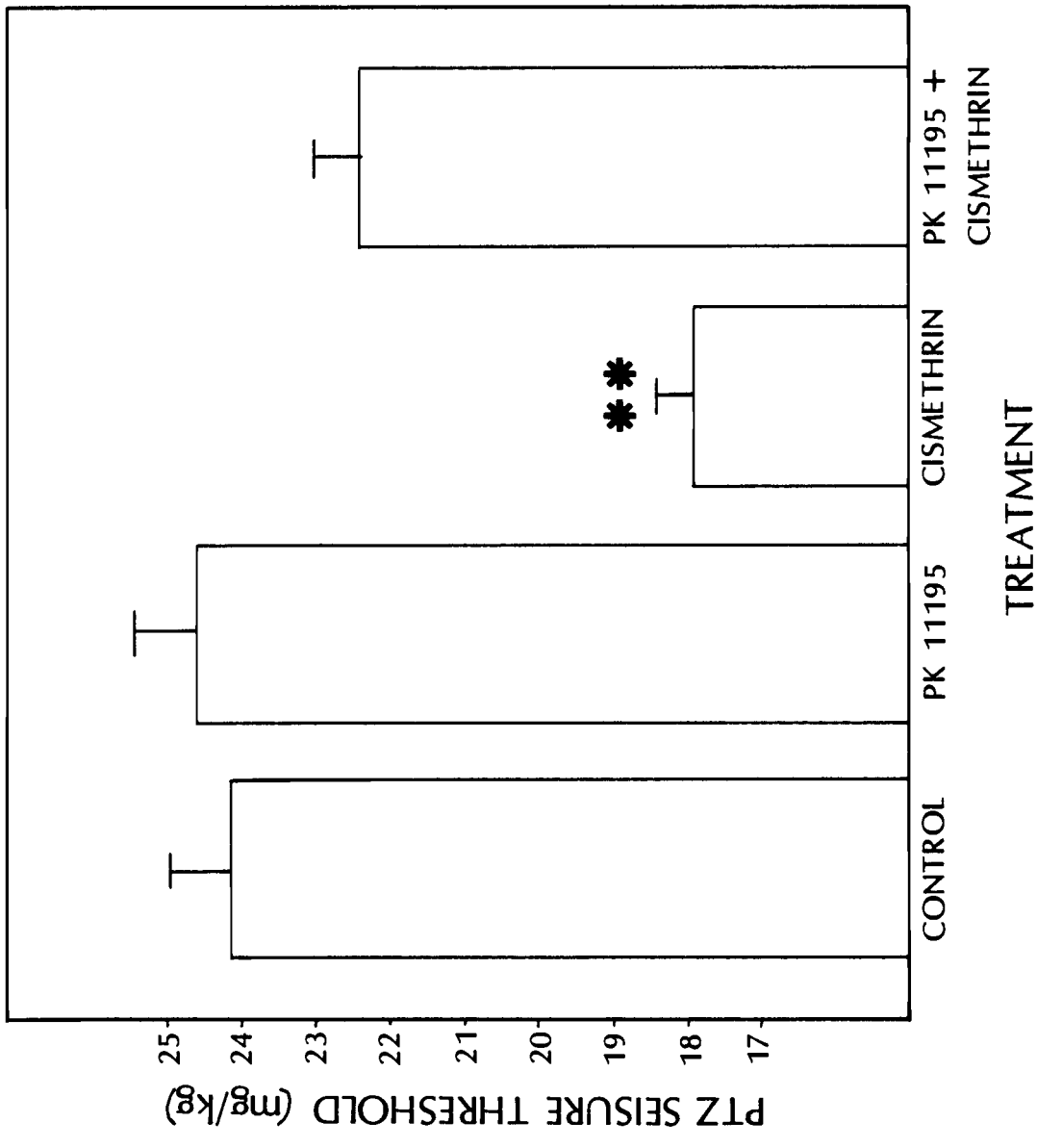

PTZ SEISURE THRESHOLD (mg/kg)
25
24
23
22
21
20
19
18
17
**
CONTROL
PK 11195
CISMETHRIN
PK 11195 + CISMETHRIN
TREATMENT

Figure 11. Pyrethroid dose-dependent inhibition of specific [^{3}H]Ro5-4864 binding to rat olfactory bulb membranes. The ordinate represents the % inhibition of control specific [^{3}H]Ro5-4864 binding. Ro5-4864 (○); pyrethroids: deltamethrin (●), (1R≪S)cis cypermethrin (▲), kadethrin (△), (1R)cis permethrin (■), cismethrin (□), allethrin (⬢), resmethrin (◇), tetramethrin (⬡), (1S≪R)cis cypermethrin (◆), and (1S)cis permethrin (▽).

FIGURE 11

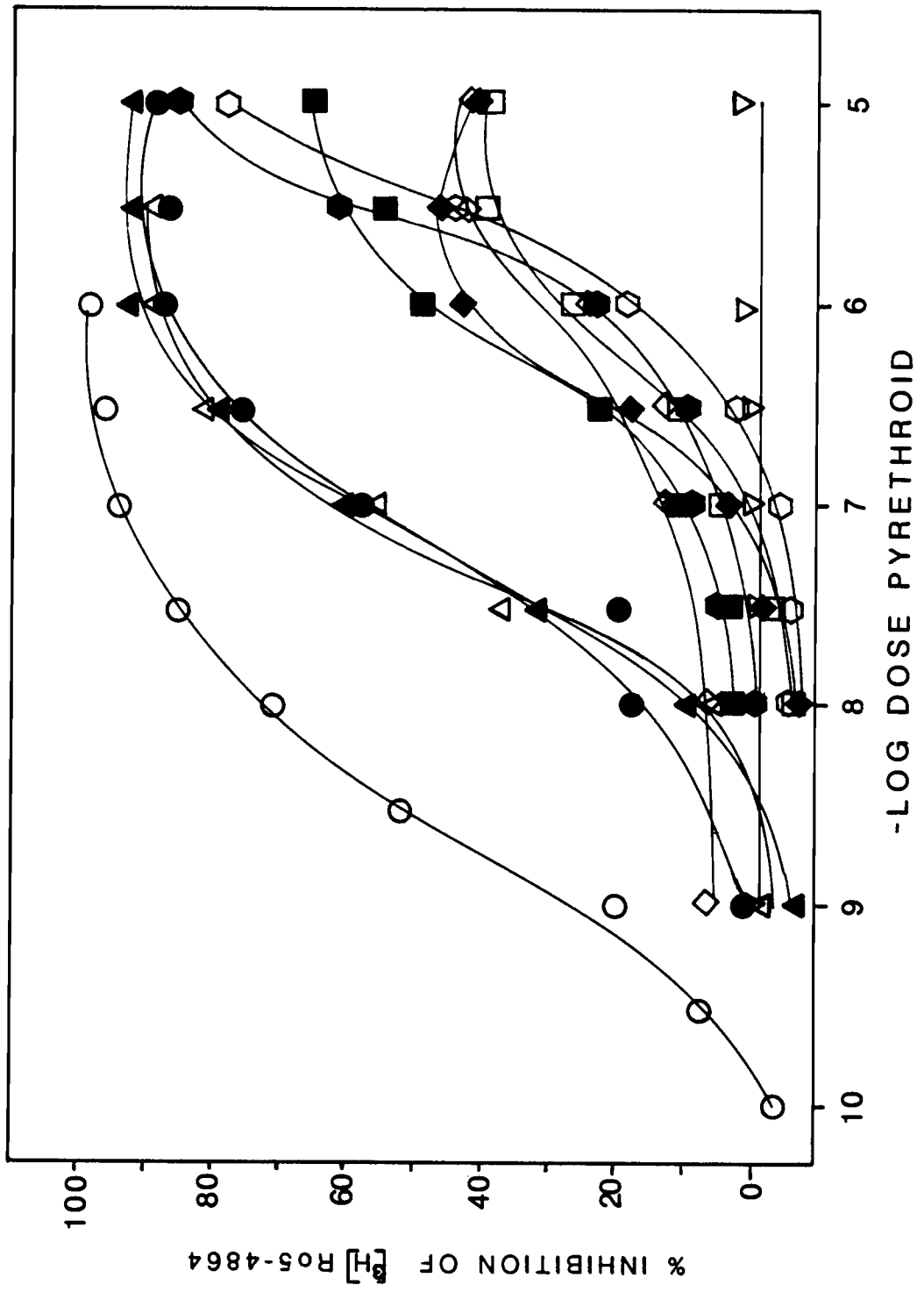

% INHIBITION OF [3H] Ro5-4864
0
20
40
60
80
100
5
6
7
8
9
10
-LOG DOSE PYRETHROID

Figure 12. Pyrethroid dose-dependent inhibition of specific [^{3}H]Ro-4864 binding to rat cerebral cortical membranes. The ordinate represents the % inhibition of control specific [^{3}H]Ro5-4864 binding. Ro5-4864 (○); pyrethroids: deltamethrin (●), (1R≪S)cis cypermethrin (▲), kadethrin (Δ), (1R)cis permethrin (■), cismethrin (□), allethrin (⬣), resmethrin (◇), tetramethrin (⬡), and (1S≪R)cis cypermethrin (◆). (1S)cis permethrin was not included in this set of experiments as it was inactive in the olfactory bulb membrane preparation.

FIGURE 12

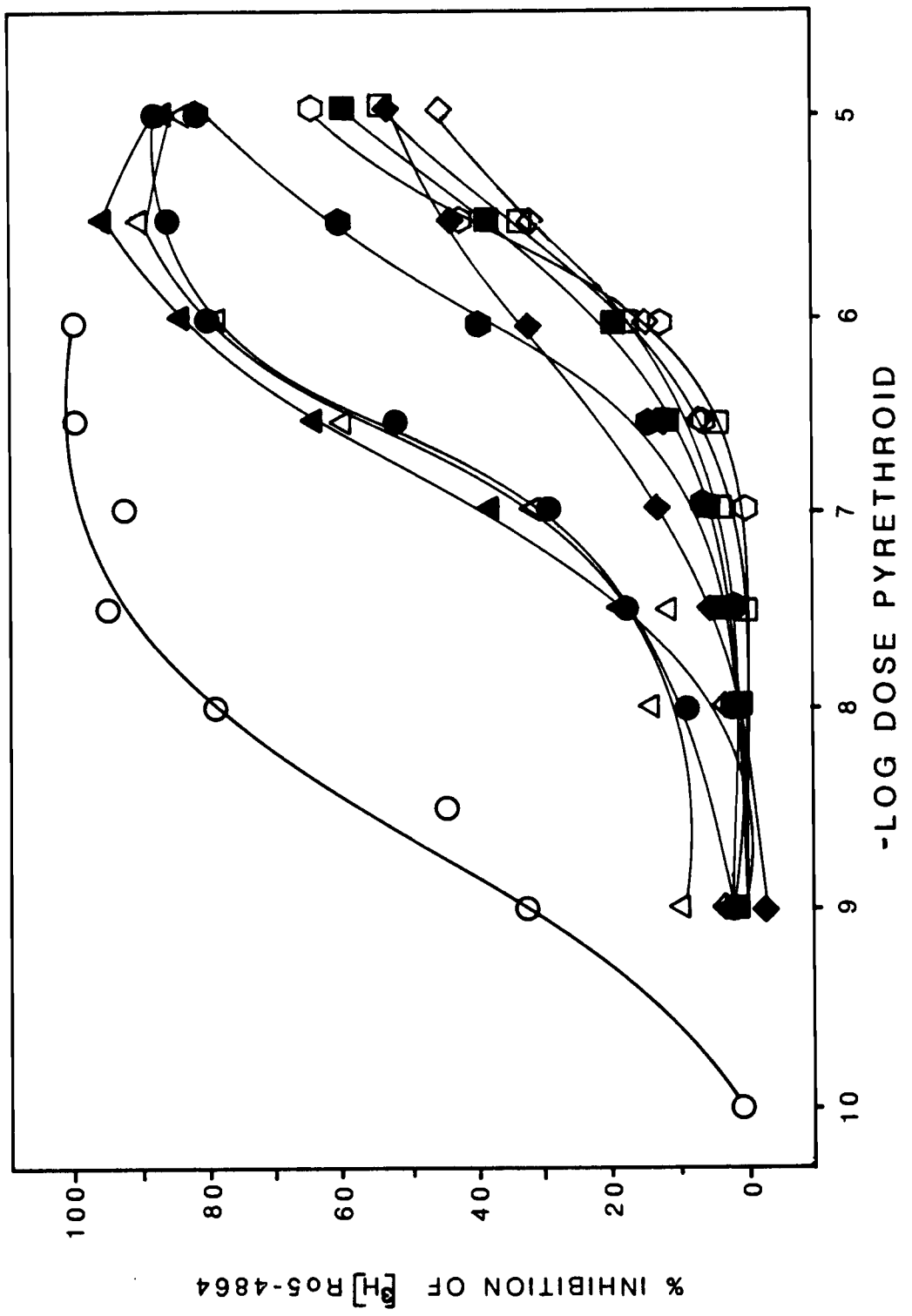

% INHIBITION OF [3H] RO5-4864
0
20
40
60
80
100
5
6
7
8
9
10
-LOG DOSE PYRETHROID

Figure 13. Correlation between pyrethroid proconvulsant activity and potency as inhibitors of specific [^{3}H]Ro5-4864 binding in olfactory bulb membranes. For correlational analysis the pyrethroid ED_{50} values as proconvulsants and respective IC_{50} values as inhibitors of [^{3}H]Ro5-4864 binding were ranked. The ED_{50} values for the inactive pyrethroids allethrin, 1SαR,<u>cis</u> cypermethrin and 1S,<u>cis</u> permethrin were arbitrarily set at a value of 100 μmol/kg, while the IC_{50} value for 1S,<u>cis</u> permethrin was similarily set at 100 μmol/kg. $P < 0.01$ by Kendall's coefficient of rank correlation, $\tau = 0.689$ (Sokol and Rolf, 1969). Pyrethroids: 1RαS,<u>cis</u> cypermethrin (1), deltamethrin (2), kadethrin (3), 1R,<u>cis</u> permethrin (4), resmethrin (5), cismethrin (6), tetramethrin (7), allethrin (8), 1SαR,<u>cis</u> cypermethrin (9) and 1S,<u>cis</u> permethrin (10).

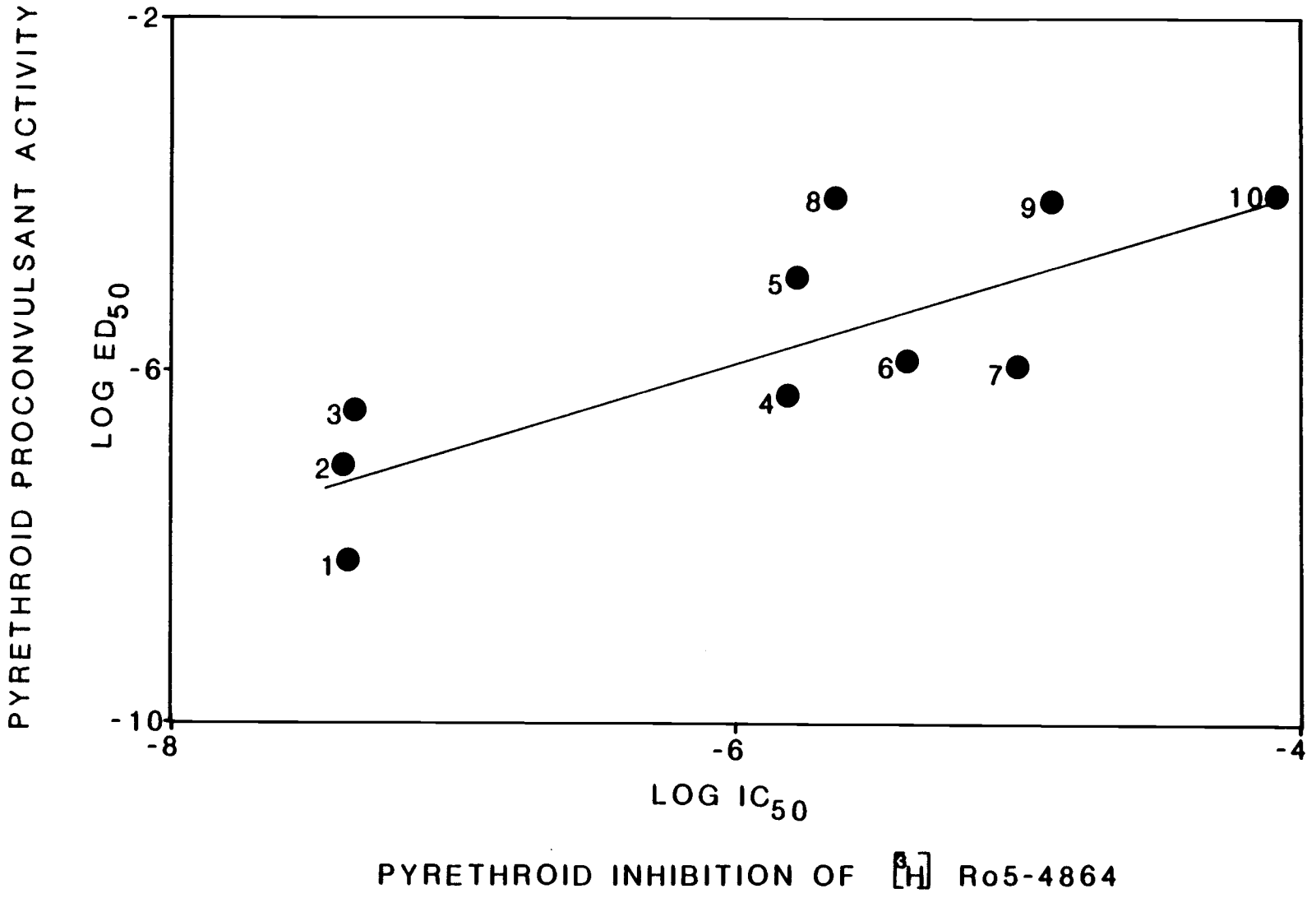

PYRETHROID PROCONVULSANT ACTIVITY
LOG ED_{50}
-2
-6
-10
-8
-6
-4
LOG IC_{50}
PYRETHROID INHIBITION OF [^{3}H] Ro5-4864
1
2
3
4
5
6
7
8
9
10

FIGURE 13

Figure 14. Saturation isotherm (A) and Scatchard replot (B) of [^{3}H]Ro5-4864 binding to rat olfactory bulb membranes in the absence (▲) or presence (Δ) of 50 nM deltamethrin. Data shown are from a representative experiment which was replicated three times. The curves drawn are for a one-site model which adequately described the data in these and three additional experiments. The presence of deltamethrin in the incubation did not influence non-specific binding (■). The K_d and B_{max} values for the control curve shown are 1.86 nM and 1230 fmol/mg protein, respectively. In the presence of 50 nM deltamethrin, the K_d value for [^{3}H]Ro5-4864 increased to 9.99 nM while the B_{max} value was 1870 fmol/mg protein.

FIGURE 14

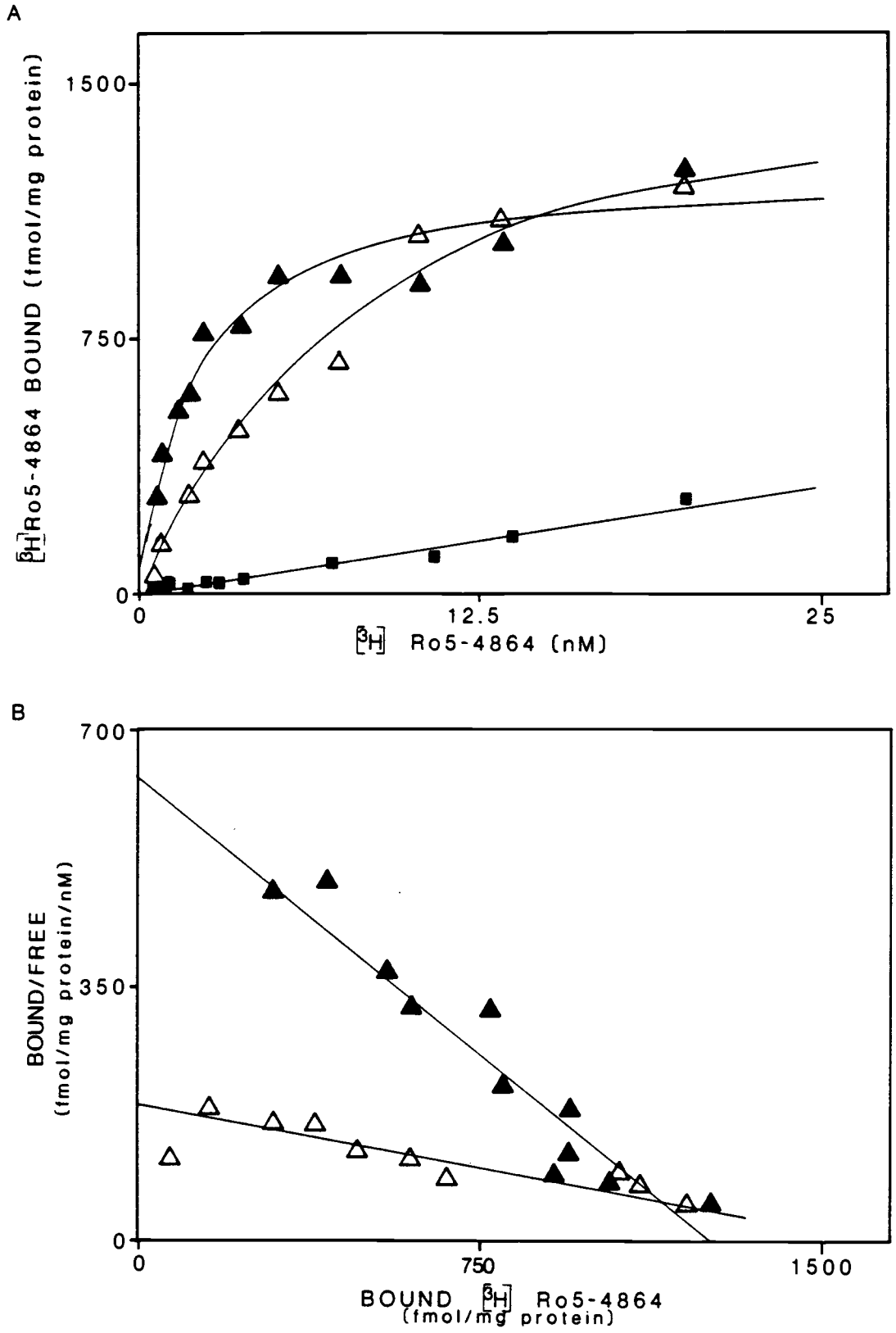

A
1500
750
0
[3H]Ro5-4864 BOUND (fmol/mg protein)
0
12.5
25
[3H] Ro5-4864 (nM)
B
700
350
0
BOUND/FREE
(fmol/mg protein/nM)
0
750
1500
BOUND [3H] Ro5-4864
(fmol/mg protein)

Figure 15. Saturation isotherm (A) and Scatchard replot (B) of [^{3}H]Ro5-4864 binding to rat olfactory bulb membranes in the absence (●) or presence (○) of 10 mM cismethrin. Data shown are from a representative experiment which was replicated twice. The curves drawn are for a one-site model which adequately described the data in these and three additional experiments. The presence of cismethrin in the incubation did not influence non-specific binding (■). The K_d and B_{max} values for the control curve shown are 2.38 nM and 1360 fmol/mg protein, respectively. In the presence of 10 mM cismethrin, the K_d value for [^{3}H]Ro5-4864 was 7.10 nM and the B_{max} value was 1690 fmol/mg protein.

FIGURE 15

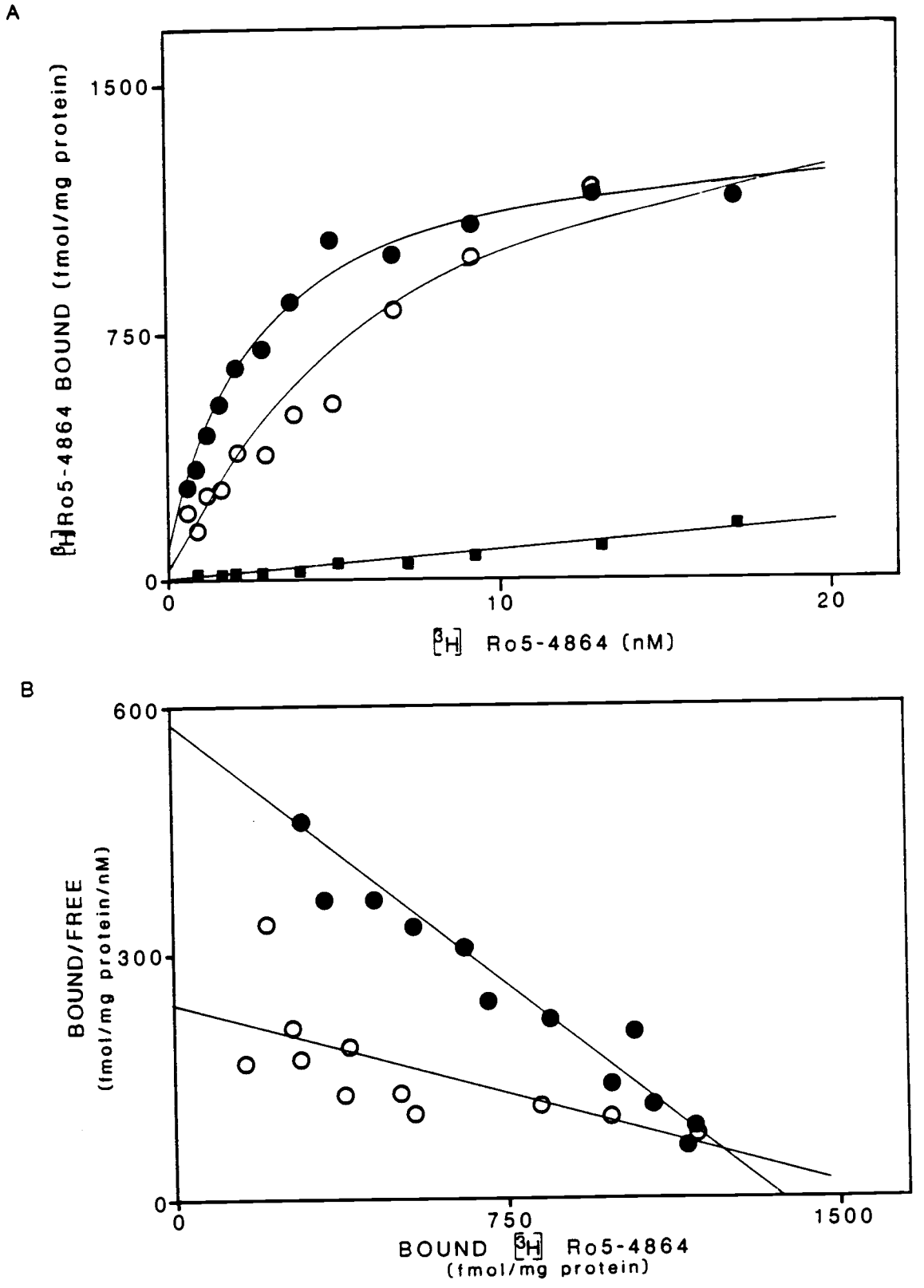

A
1500
750
0
[3H]Ro5-4864 BOUND (fmol/mg protein)
0
10
20
[3H] Ro5-4864 (nM)
B
600
300
0
BOUND/FREE
(fmol/mg protein/nM)
0
750
1500
BOUND [3H] Ro5-4864
(fmol/mg protein)

Figure 16. Schild plot of deltamethrin-induced [^{3}H]Ro5-4864 affinity shift in rat olfactory bulb membranes. The affinity shifts were determined from [^{3}H]Ro5-4864 saturation experiments with control and deltamethrin samples assayed in parallel. The mean affinity shift values from 3-4 experiments for each deltamethrin concentration were used to construct the Schild plot.

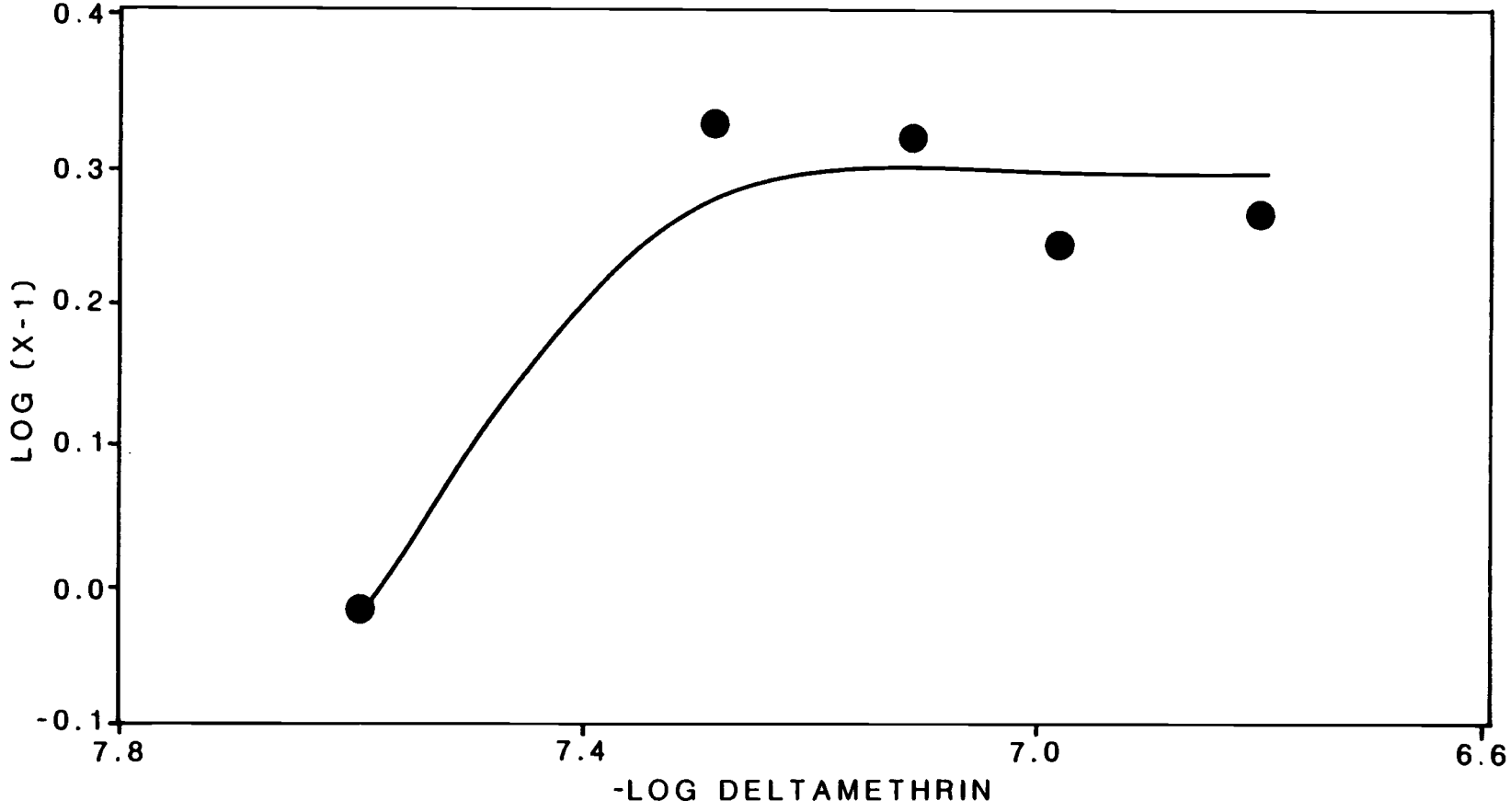


FIGURE 16

CHAPTER 4 PYRETHROID INSECTICIDE AND VERATRIDINE INHIBITION OF GABA-GATED ^{36}CHLORIDE INFLUX IN RAT BRAIN SYNAPTONEUROSOMES

Leslie L. Devaud and Thomas F. Murray

ABSTRACT

Pyrethroid insecticides inhibited 50 μM GABA-gated ^{36}chloride influx with a rank order of potency being 1RαS,cis cypermethrin > deltamethrin > kadethrin > 1R,cis permethrin > cismethrin. Resmethrin and allethrin and the insecticidally inactive isomers of cypermethrin and permethrin did not inhibit chloride influx over the dose range employed. The potent sodium channel activator, veratridine, also inhibited $^{36}Cl^-$ influx with an EC_{50} value similar to that for deltamethrin. Moreover, the Type II pyrethroids and veratridine enhanced chloride influx independent of GABA-stimulation, an effect blocked by tetrodotoxin. Tetrodotoxin also prevented the GABA-dependent inhibition of chloride influx elicited by deltamethrin and veratridine. Ro5-4864 and PK 11195 elicited a modest inhibition of GABA-gated chloride influx, and PK 11195 partially antagonized both the GABA-dependent and GABA-independent activity of deltamethrin and veratridine. Taken together these results indicate complex interactions of pyrethroids, Ro5-4864 and PK

11195 with the $GABA_A$ receptor.

INTRODUCTION

Pyrethroid insecticides are synthetic analogs of the naturally occuring pyrethrins. They have come into prominent use in recent years due to their high insecticidal activity with minimal toxicity towards mammalian species. Pyrethroids have been divided into two classes, Type I and Type II, based on structure-activity relationships, in vitro effects on nerve preparations, and the symptomology they produce following acutely toxic doses (Verschoyle and Aldridge, 1980; Gammon et al., 1981; Casida et al, 1983). In addition to their neurotoxic activities, both Type I and Type II pyrethroids are potent proconvulsants in the rat (Devaud et al, 1986; Devaud and Murray, 1988).

Pyrethroid insecticides have been shown to interact with multiple sites in the nervous systems of insects and vertebrates. A major target of these compounds is neuronal sodium channels, where they prolong the activation of voltage-dependent sodium channels and increase sodium influx (Narahashi, 1971; Lund and Narahashi, 1983; Ghiasuddin and Soderland, 1985). Pyrethroids have also been shown to modulate the nicotinic acetycholine receptor in that they noncompetitively inhibit the binding of [^{3}H]perhydrohistrionicotoxin to channel sites on this receptor (Abbassy et al, 1982). There is increasing evidence for an interaction of pyrethroids with the $GABA_A$

receptor. Poisoning symptoms induced by toxic doses of Type II pyrethroids are delayed by diazepam pretreatment (Gammon et al, 1982; Cole and Casida, 1983). Higher doses of diazepam raise the intracerebral LD_{50} in mice for both the Type II pyrethroid, deltamethrin and the Type I compound, permethrin (Gammon et al, 1982). Pyrethroids stereospecifically inhibit the binding of [^{35}S]t-butylbicyclophosphorothionate (TBPS) to the picrotoxinin site of the $GABA_A$ receptor in a GABA-dependent manner (Lawrence and Casida, 1983; Seifert and Casida, 1985).

The Type II pyrethroids deltamethrin and 1RαS,<u>cis</u> cypermethrin also inhibit the specific binding of [^{3}H]Ro5-4864, a ligand selective for the peripheral-type benzodiazepine receptor, with a 60-fold increase in potency over that observed for inhibition of [^{35}S]TBPS binding (Gammon and Sander, 1985; Lawrence et al, 1985). Both Type I and Type II pyrethroids stereospecifically inhibit the specific binding of [^{3}H]Ro5-4864 to rat brain membranes (Devaud and Murray, 1988). As additional evidence for the involvement of the peripheral-type benzodiazepine receptor as a site of action for pyrethroids, pretreatment with PK 11195, a peripheral-type benzodiazepine receptor antagonist, protects against the proconvulsant activity of pyrethroids (Devaud et al, 1986; Devaud and Murray, 1988). However, both Ro5-4864 and PK 11195 also modulate [^{35}S]TBPS binding, the nature of the interaction depending on the level of GABA present (Ticku and Ramanjayala, 1984; Squires and Saederup, 1987; Gee, 1987). Thus, it is possible that the interaction of pyrethroids with an Ro5-4864 binding site may be one that is allosterically linked to the $GABA_A$ receptor.

Studies of inhibition of binding to sites associated with the $GABA_A$ receptor do not identify the mechanism of the displacer's interaction. Development of the GABA-gated ^{36}chloride flux assay has allowed for the determination of the functional coupling of $GABA_A$ receptors to the chloride ionophore (Harris and Allen, 1985). This methodology distinguishes between GABA-mimetic versus GABA-opposing activity (Allan and Harris, 1986). While it has been shown that pyrethroids inhibit GABA-stimulated chloride influx (Abalis et al, 1986; Bloomquist et al, 1986), the pharmacological profile of pyrethroid inhibition of GABA-gated chloride influx, their effects on GABA-independent chloride influx and their interactions with the purported $GABA_A$ receptor-coupled Ro5-4864 site have not been reported.

Thus, one aim of the present investigation was to characterize the effects of both Type I and Type II pyrethroids on ^{36}chloride influx. A second goal of this research was to study the interactions of Ro5-4864, PK 11195 and pyrethroids in their modulation of GABA-gated chloride influx in an attempt to gain a better understanding of the possible mechanism by which pyrethroids exert their proconvulsant actions.

METHODS

Materials: $^{36}Cl^-$ (15.59 mCi/g) was purchased from DuPont-NEN, Boston, MA. Ro5-4864 was generously supplied by Dr. Peter Sorter, Hoffman-LaRoche, Inc., Nutley, N.J. PK 11195 was a gift of Dr. G.

LeFur, Pharmuka Laboratories, Gennevilliers, France. Pyrethroids: The isomers of cypermethrin, 1Rcis permethrin, 1Scis permethrin and allethrin were gifts from Dr. Arthur Ramsey, FMC Corporation, Princeton, N.J. Tetramethrin and resmethrin were obtained from the EPA, Research Triangle Park, N.C. Kadethrin, cismethrin and deltamethrin were gifts from Dr. P. Foulhoux, Roussel Uclaf, Romainville, France.

Preparation of synaptoneurosomes: Male Sprague-Dawley rats, 140-180 g (Simonsen Labs, Gilroy, CA) were decapitated and their cerebral cortices rapidly dissected over ice. The tissue was gently homogenized in 10 volumes of ice cold assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose and 10 mM Hepes, adjusted to pH 7.4 with Tris base) by 5 strokes with a loose fitting (B) glass/glass Dounce. The homogenate was filtered through nylon mesh (160 μm) and centrifuged at 1000 x g for 15 minutes. The pellet was gently resuspended in 10 volumes ice cold assay buffer and washed twice more. The final pellet was resuspended in 3.6 volumes ice cold buffer for a final protein concentration of 6-7 mg/ml as determined by the method of Lowry following solubilization with 0.5 N NaOH (Lowry et al., 1951).

^{36}Chloride influx measurements: Aliquots of 200 μl of the synaptoneurosome preparation were preincubated at 30^{o}C for 10 minutes with test compounds dissolved in 2 μl 100% DMSO or DMSO only for a final concentration of 0.5% DMSO. The presence of 2 μl DMSO in the preparation did not alter ^{36}chloride influx levels. The addition of 4 μl DMSO in the interaction experiments did decrease the GABA-dependent ^{36}chloride influx approximately 10% and was included in all

incubations. ^{36}chloride influx was initiated by addition of a 200 μl solution containing 0.12 μCi of $^{36}Cl^-$ and appropriate concentrations of GABA or buffer and incubated for 4 seconds. The incubation was terminated by the rapid addition of 4 mls of ice cold assay buffer followed by vacuum filtration through Schleicher &Schuell 32 glass fiber filters. The filters were washed twice more with 4 mls each of ice cold assay buffer. After elution, bound radioactivity was measured by liquid scintillation spectroscopy.

Data Analysis: GABA-independent values are the $^{36}Cl^-$ cpm observed at basal conditions, i.e. with no addition of GABA to the incubation (minus binding to filters in the absence of tissue). GABA-dependent values were obtained by subtracting GABA-independent cpm from total cpm in the presence of GABA. Following normalization of the data to percent of control stimulation, E_{max} and EC_{50} values were determined using the non-linear MK Model Plus curve-fitting routine (Holford, 1983).

RESULTS

The protocol employed in this series of experiments utilized a protein concentration of approximately 1.0-1.2 mg/400 μl total assay volume. Increasing the amount of protein to approximately 2.0 mg/tube decreased the magnitude of effect noted for both Ro5-4864 and deltamethrin. High levels of protein could disperse these compounds to non-specific sites and so limit the amount of free modifier available to interact at the $GABA_A$ receptor. Employing 1.2-1.4 mg

protein aliquots and 0.12 μCi $^{36}Cl^-$ resulted in approximately 200 cpm/ 4 sec for the tissue blanks, 100 cpm/4sec for GABA-independent levels and GABA-dependent counts of 250 cpm/4 sec.

GABA stimulated chloride influx in a concentration-dependent manner over the range of 3-300 μM. The EC_{50} value for this effect was 30.6± 2.6 μM, in good agreement with previously published reports (Harris and Allen, 1985).

The addition of either Ro5-4864 or deltamethrin reduced the maximal stimulation of chloride influx elicited by GABA without significantly altering the potency of GABA. The presence of 100 nM Ro5-4864 reduced the percent stimulation induced by GABA by 12.3 ±2.8% (Figure 17). The EC_{50} value was 28.8±3.2 μM, no change from that determined for the control. Inclusion of a higher concentration of Ro5-4864 (1 μM) in the assay did not further reduce the maximal effect elicited by GABA (E_{max}= 91.5±3.3 μM) nor did it significantly alter the EC_{50} value (36.1±3.2 μM).

Deltamethrin also elicited a decrease in the maximal stimulation of chloride influx induced by GABA with little effect on the EC_{50} value (Figure 18). The presence of 100 nM deltamethrin reduced the maximal effect by 13.2 ±1.4% with an EC_{50} value of 32.1± 5.3 μM. However, in contrast to the effect observed with Ro5-4864, the addition of 1 μM deltamethrin further reduced the stimulation of chloride influx by GABA by 37.4 ±2.0% (EC_{50}= 19.9±5.4 μM). These results show that while both Ro5-4864 and deltamethrin alter GABA-gated chloride influx in a non-competitive manner, deltamethrin is more efficacious than Ro5-4864 in this regard.

To further investigate the mechanism of interaction of

pyrethroid insecticides, Ro5-4864 and PK 11195 with the $GABA_A$ receptor, their ability to inhibit 50 μM GABA-gated chloride influx in a concentration-dependent fashion was assessed. Ro5-4864 (30 nM to 10 μM) induced a maximal reduction of chloride influx of 22.0 ± 3.6 % below control levels from 23.65 ± 2.11 to 16.16 ± 2.60 nmol/mg protein with an EC_{50} value of 1.15 ± 0.29 μM (Table 5, Figure 19). Ro5-4864 did not effect GABA-independent levels at any concentration tested. PK 11195 induced a similar response with a maximal inhibition of 18.2% from control levels elicited by a dose of 10 μM PK 11195 (data not shown).

The Type II pyrethroids deltamethrin and 1RαS,<u>cis</u> cypermethrin induced a marked, dose-dependent inhibition of 50 μM GABA-gated chloride influx. 1RαS,<u>cis</u> cypermethrin almost completely prevented GABA-dependent chloride influx, inducing a 95.1 ± 6.6% inhibition from 28.02 ± 1.64 to 3.64 ± 0.89 nmol/mg protein with an EC_{50} value of 657.3 ± 146.0 nM (Figure 20, Table 5). Similarly, deltamethrin reduced GABA-gated chloride influx from a level of 32.30 ± 1.70 to 4.25 ± 1.27 nmol/mg protein, a 94.1 ± 5.7% reduction with an EC_{50} of 899.4 ± 168 nM (Figure 21 and Table 5). Moreover, both 1RαS,<u>cis</u> cypermethrin and deltamethrin had significant effects on GABA-independent chloride flux. In a concentration-dependent manner, these Type II pyrethroids induced a nearly two fold enhancement of basal chloride influx, from a basal level of 9.67 ± 1.38 to 15.55 ± 1.24 nmol/mg protein for 1RαS,<u>cis</u> cypermethrin and from 8.04 ± 2.8 to 16.75 ± 1.90 nmol/mg protein in the presence of deltamethrin (Figures 20 and 21). This pyrethroid influence on GABA-independent chloride influx indicates complex interactions of Type II pyrethroids in the

chloride influx assay in that they enhance chloride influx without addition of GABA but elicit a marked inhibition of chloride influx in the presence of GABA.

Type I pyrethroids also inhibit GABA-gated chloride influx but with reduced efficacy and potency as compared to deltamethrin and 1RαS,cis cypermethrin. Kadethrin, an atypical Type I pyrethroid which was very potent both as a proconvulsant and as an inhibitor of [^{3}H]Ro5-4864 binding (Devaud and Murray, 1988), decreased GABA-gated chloride influx by 64.4±3.8%, from 26.33 ± 0.91 to 10.68 ± 0.62 nmol/mg protein with an EC_{50} value of 2.60±0.31 μM (Figure 22). 1R,cis permethrin modulated chloride influx in a fashion similar to kadethrin: 59.8± 10.2% maximal inhibition with an EC_{50} value of 6.03±2.50 μM reducing influx from 30.20 ± 2.34 to 12.34 ± 1.29 nmol/mg protein (Figure 23). The Type I pyrethroid cismethrin inhibited chloride influx by 48.0% at a concentration of 30 μM but there was little inhibition observed at lower doses so parameter estimates could not be obtained. Higher concentrations of pyrethroids were not investigated due to the low aqueous solubility of these compounds.

In addition, as seen in Figures 22 and 23, the Type I pyrethroids tested did not exert significant effects on GABA-independent chloride influx. The Type I pyrethroids resmethrin and allethrin did not inhibit GABA-gated chloride influx over the concentration range employed. Taken together, these results suggest that while both Type I and Type II pyrethroids are able to inhibit $GABA_A$-coupled chloride channel activity, they differ not only in potency but also with respect to their actions on GABA-independent

chloride influx.

The stereoselectivity of effect previously noted for pyrethroids as insecticides (Casida et al, 1983) and in their proconvulsant activity (Devaud et al, 1986; Devaud et Murray, 1988) was also observed in their modulation of GABA-gated and GABA-independent chloride influx (Figures 20 and 23). Of the series of pyrethroids tested, the 1RαS,cis isomer of cypermethrin was the most potent inhibitor of chloride influx while its stereoisomer, 1SαR,cis was unable to alter either GABA-dependent or GABA-independent influx. The insecticidally inactive isomer of the Type I pyrethroid permethrin was also devoid of activity in the chloride influx assay.

An interaction of pyrethroids with PK 11195 has been established by the ability of this peripheral-type benzodiazepine receptor ligand to block the proconvulsant effects of pyrethroids. Thus, it was of interest to test for an interaction of PK 11195 with deltamethrin in the chloride flux assay. A 300 nM dose of PK 11195 reduced the maximal inhibition of GABA-gated chloride influx elicited by deltamethrin from 81.0 ± 5.8 % to only 44.7 ± 3.4 % over a concentration range of 30 nM to 10 μM deltamethrin (Figure 24). There was a non-significant shift in the EC_{50} from 882.3 ± 199 nM for deltamethrin alone to 617 ± 129 nM with the addition of PK 11195. Furthermore, the 300 nM dose of PK 11195 also antagonized the effects of deltamethrin on GABA-independent chloride influx, reducing the per cent enhancement from 59.3 ± 7.6 for the control to 34.1 ± 5.0 % in the presence of PK 11195. Again, there was no shift in the EC_{50} value (from 1.02 ± 0.39 μM to 1.04 ± 0.42 μM). This apparent non-competitive antagonism of the deltamethrin-induced effects on

chloride influx by PK 11195 supports the involvement of the $GABA_A$ receptor as a site of action for both pyrethroid insecticides and ligands for the PTBR.

It has been well documented that pyrethroids have significant effects on voltage-gated neuronal sodium channels (Narahashi, 1985). In an attempt to separate their voltage-gated sodium flux enhancement effects from their interactions with $GABA_A$ receptor chloride influx, 1 μM tetrodotoxin was added to the ten minute preincubation with deltamethrin. The presence of tetrodotoxin prevented both the GABA-independent enhancement and GABA-dependent inhibition of chloride influx elicited by deltamethrin (Figue 25). Tetrodotoxin also blocked the GABA-gated inhibition of chloride influx elicited by the Type I pyrethroid, kadethrin. These findings implicate some form of interaction between the effects of pyrethroids on voltage-gated sodium flux with their actions on ^{36}chloride influx.

To better ascertain the involvement of voltage-gated sodium channel activation with pyrethroid interaction with the $GABA_A$ receptor mediated chloride flux, we investigated the effects on chloride influx elicited by a potent voltage-gated sodium channel activator, veratridine (Tamkun and Catterall, 1980). As seen with the Type II pyrethroids, veratridine inhibited GABA-gated chloride influx in a dose-dependent manner, reducing chloride uptake by 81.3 ±3.5% from 20.5 ± 0.9 to 4.5 ± 2.0 nmol/mg protein with an EC_{50} value of 863.7 ± 102.0 nM (Figure 26). Veratridine also induced about a 50% enhancement of GABA-independent chloride influx. As seen previously with deltamethrin, both these actions of veratridine were eliminated by inclusion of 1 μM tetrodotoxin in the preincubation

(Figure 26). In addition, preincubation with 300 nM PK 11195 also antagonized the modulation of chloride influx elicited by veratridine by increasing the EC_{50} from 774.8 ± 91.3 nM to 2.58 ± 1.3 μM (Figure 27). The E_{max} value shifted from 78.3 ± 3.3 % inhibition in the presence of GABA to 62.1 ± 14.5 % inhibition with the addition of PK 11195. The GABA-independent EC_{50} value for veratridine-induced enhancement of chloride uptake was increased from 1.07 ± 0.05 μM to 3.8 ± 0.68 μM with the addition of 300 nM PK 11195 and the E_{max} value reduced from 60.8 ± 1.4 % enhancement to 35.4 ± 2.7 %.Taken together, these results suggest a commonality of activity for veratridine and the Type II pyrethroids deltamethrin and 1RαS,cis cypermethrin as modifiers of both GABA-gated and GABA-independent chloride influx.

DISCUSSION

The present investigation has demonstrated that both Type I and Type II pyrethroids display a functional interaction with the $GABA_A$ receptor by sterospecifically inhibiting GABA-gated chloride influx in a concentration-dependent fashion. There is a similar rank order of effectiveness for the pyrethroids in this assay as compared with their insecticidal potency (Casida et al, 1983;), proconvulsant activity (Devaud et al, 1986; Devaud and Murray, 1988), and ability to inhibit [^{35}S]TBPS or [^{3}H]Ro5-4864 binding (Lawrence and Casida, 1983; Gammon and Sander; 1985; Lawrence et al, 1985; Devaud and Murray, 1988). However, the present investigation also discerned a

marked, dose-dependent enhancement of GABA-independent chloride uptake specific to the Type II pyrethroids 1RαS,cis cypermethrin and deltamethrin.

Pyrethroids have also been shown to have potent interactions with Ro5-4864 binding sites (Lawrence et al, 1985; Gammon and Sander, 1985; Devaud and Murray, 1988). In the present investigation, both Ro5-4864 and deltamethrin inhibited the maximal stimulation of chloride influx elicited by GABA with little effect on the potency of GABA, suggesting that both compounds interact non-competitively to interfere with chloride influx and further supporting the interaction of both pyrethroids and Ro5-4864 with sites associated with the $GABA_A$ receptor. PK 11195, at a concentration which did not affect GABA-gated chloride influx, non-competititevly attentuated the degree of chloride uptake inhibition induced by deltamethrin. In vivo experiments have shown that PK 11195 pretreatment blocks the proconvulsant effect of pyrethroids (Devaud et al, 1986; Devaud and Murray, 1988). Gee has reported that PK 11195 enhances the potency of GABA as an inhibitor of [^{35}S]TBPS binding to the picrotoxinin site in the chloride channel (Gee, 1987) and Ro5-4864 has been shown to modulate specific [^{35}S]TBPS binding in a GABA-dependent fashion (Squires and Saederup, 1987; Gee, 1987). Additional radioligand binding studies indicate that pyrethroids inhibit both [^{3}H]Ro5-4864 and [^{35}S]TBPS binding to rat brain membranes (Lawrence and Casida, 1983; Gammon and Sander, 1985; Seifert and Casida, 1985; Devaud and Murray, 1988). These results, considered together with those from the current investigation, signify complex interactions between pyrethroids, Ro5-4864, PK 11195, and the $GABA_A$ receptor and suggest

the possible involvement of a Ro5-4864/PK 11195 site allosterically coupled to the $GABA_A$ receptor in the proconvulsant activity of pyrethroid insecticides.

The voltage-gated sodium channel activator, veratridine was also found to potently modulate chloride influx in a similar, tetrodotoxin-sensitive manner to deltamethrin. Schwartz and coworkers have recently reported the ability of various inhibitors of nicotinic receptor-gated sodium influx to inhibit GABA-gated chloride uptake (Schwartz and Mindlin, 1988). These findings, together with those of the current investigation, make evident that many compounds are able to exert significant effects on both sodium and chloride fluxes.

There are several plausible explanations for these multiple activities. One possibility is that these compounds which are known to enhance sodium influx induce a partial depolarization of the membrane. This change in membrane potential may effect either the affinity of GABA for its receptor or the ability of pyrethroids to interact with this site (Creveling et al, 1980; Akaike et al, 1986). It has been reported that both GABA and glycine coupled-chloride channels display multiple conductance states (Hamill et al, 1983). A shift in the membrane potential could alter gating properites and be observed as a shift in the chloride-conductance induced by GABA.

Another explanation can be put forth with the recent cloning and determination of the molecular structure of the $GABA_A$ receptor (Schofield et al, 1987; Barnard et al, 1987). This work has shown that there appears to exist a "super family" of receptor-gated ion channels, including $GABA_A$, glycine and nicotinic, with many

similarities among their membrane-spanning regions. The homology may be found to extend to the voltage-gated sodium channel as well. This suggest that compounds interacting with a specific site in sodium channels may also recognize and interact with a similar site in the $GABA_A$ receptor-coupled chloride channel. It would be very interesting to determine the effects of pyrethroids and nicotinic or voltage-gated sodium channel activators on glycine-mediated chloride influx or the sodium influx gated by some excitatory amino acid receptors to determine the extent of these compounds interactions with other voltage-gated or receptor-gated ion channels.

The antagonism by PK 11195 of both the veratridine- and deltamethrin-induced inhibition of chloride influx should also be further investigated for a possible interaction with other ion-channel modifiers. GABA-gated chloride influx is inhibited by a broad range of convulsant or proconvulsant compounds, from the convulsant barbiturates (Allen and Harris, 1985), to non-competitive inhibitors of nicotinic cholinergic receptors (Schwartz and Mindlin, 1988) to pyrethroids.

In conclusion, the results of the present investigation have extended previous findings to include both Type I and Type II pyrethroids as well as the sodium channel activator, veratridine as inhibitors of GABA-gated chloride influx. This work ahs also provided additional evidence for interactions of ligands for the PTBR with the $GABA_A$ receptor.

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TABLE 5 Inhibition of 50 uM GABA-gated chloride influx by pyrethroids and peripheral-type benzodiazepine receptor ligands

Treatment	E_{max} (% inhibition)	EC_{50} (μM)	N
Ro5-4864	22.0 ± 3.6	1.15 ± 0.29	1.43 ± 0.23
PK 11195	18.8	n.d.[a]	n.d.
1RαS,*cis* cypermethrin	95.1 ± 6.6	0.675 ± 0.146	1.01 ± 0.16
deltamethrin	94.1 ± 5.7	0.899 ± 0.168	1.00 ± 0.12
kadethrin	64.4 ± 3.8	2.60 ± 0.31	1.51 ± 0.18
1R,*cis* permethrin	59.8 ± 10.2	6.03 ± 2.50	1.20 ± 0.35
cismethrin	48.0	n.d.	n.d.
resmethrin	inactive		
allethrin	inactive		
1SαR,*cis* cypermethrin	inactive		
1S,*cis* permethrin	inactive		

[a]not determined

For each compound n=6-9, from 2-3 experiments consisting of three runs each.

Figure 17. Modulation of GABA-stimulated ^{36}chloride influx by Ro5-4864. GABA stimulated chloride influx with an EC_{50} value of 31.8 ± 2.6 μM. The addition of either 100 nM or 1 μM Ro5-4864 elicited a significant reduction in the maximal stimulation induced by 300 μM GABA ($P < 0.05$). N=3

FIGURE 17

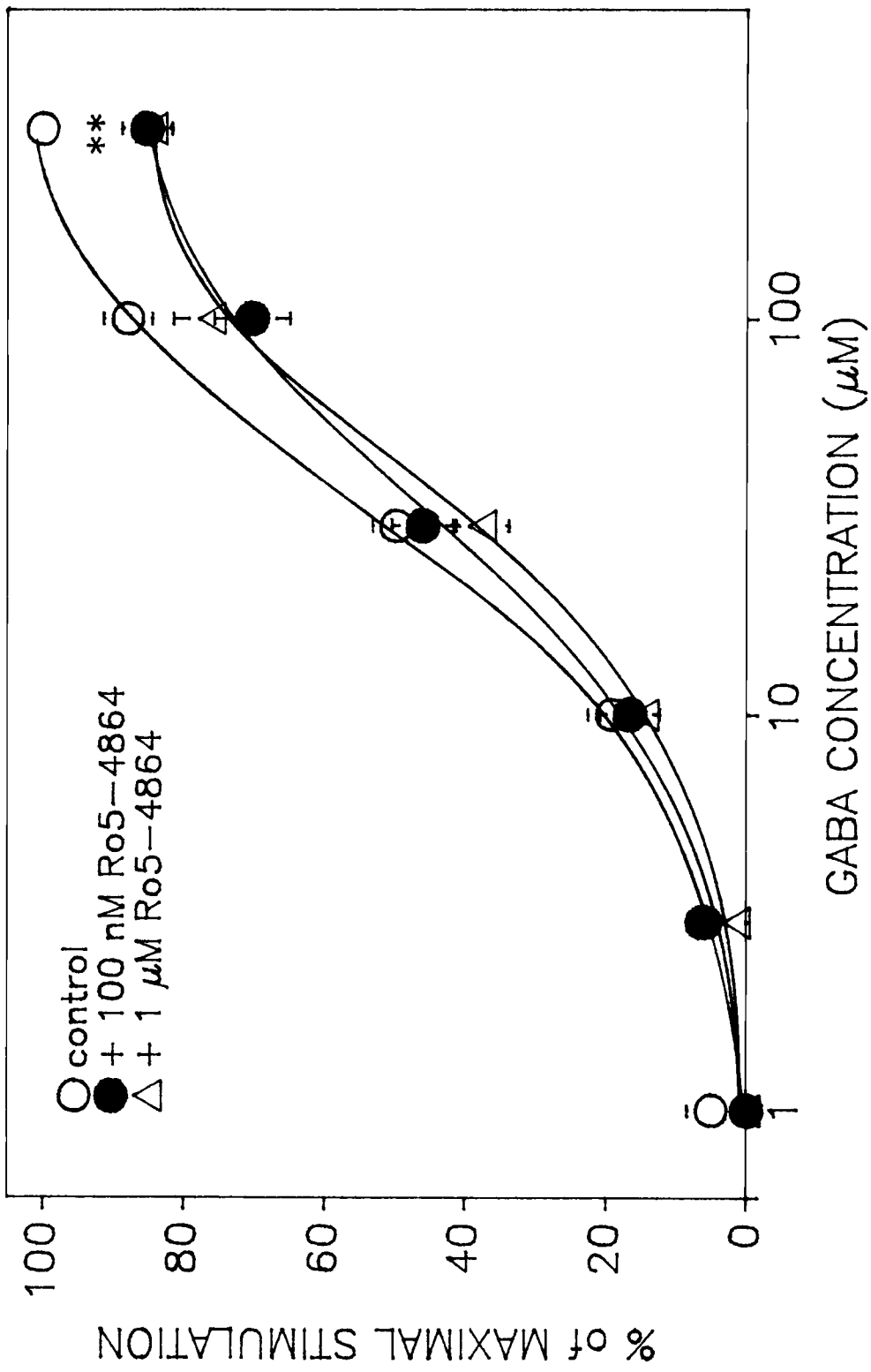

% of MAXIMAL STIMULATION
0
20
40
60
80
100
1
10
100
GABA CONCENTRATION (μM)
control
+ 100 nM Ro5-4864
+ 1 μM Ro5-4864
**

Figure 18. Modulation of GABA-stimulated ^{36}chloride influx by the Type II pyrethroid, deltamethrin. GABA stimultaed chloride influx with an EC_{50} value of 28.9 ± 3.0 μM. Both 100 nM and 1 μM deltamethrin significantly antagonized the maximal GABA-induced stimulation ($P < 0.01$) while 1 μM deltamethrin significantly antagonized the stimulation elicited by 100 μM GABA ($p < 0.05$). N=3-4.

FIGURE 18

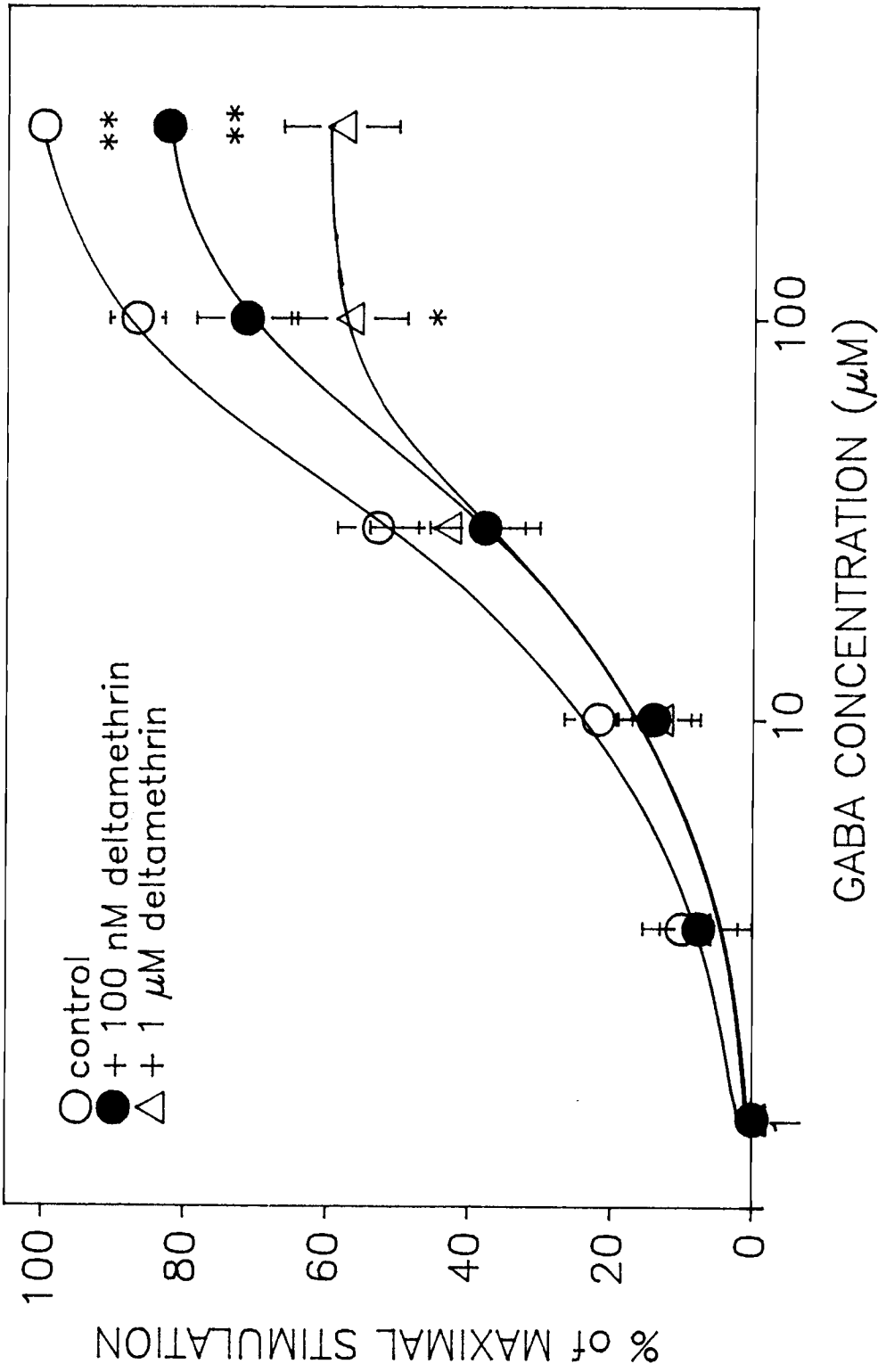

% of MAXIMAL STIMULATION
100
80
60
40
20
0
1
10
100
GABA CONCENTRATION (μM)
control
+ 100 nM deltamethrin
+ 1 μM deltamethrin
**
**
*

Figure 19. Ro5-4864 inhibition of 50 μM GABA-gated chloride influx. N=2 separate experiments with 3 runs each. The maximal inhibition was 22.0 ± 3.6 % with an EC_{50} value of 1.15 ± 0.29 μM.

FIGURE 19

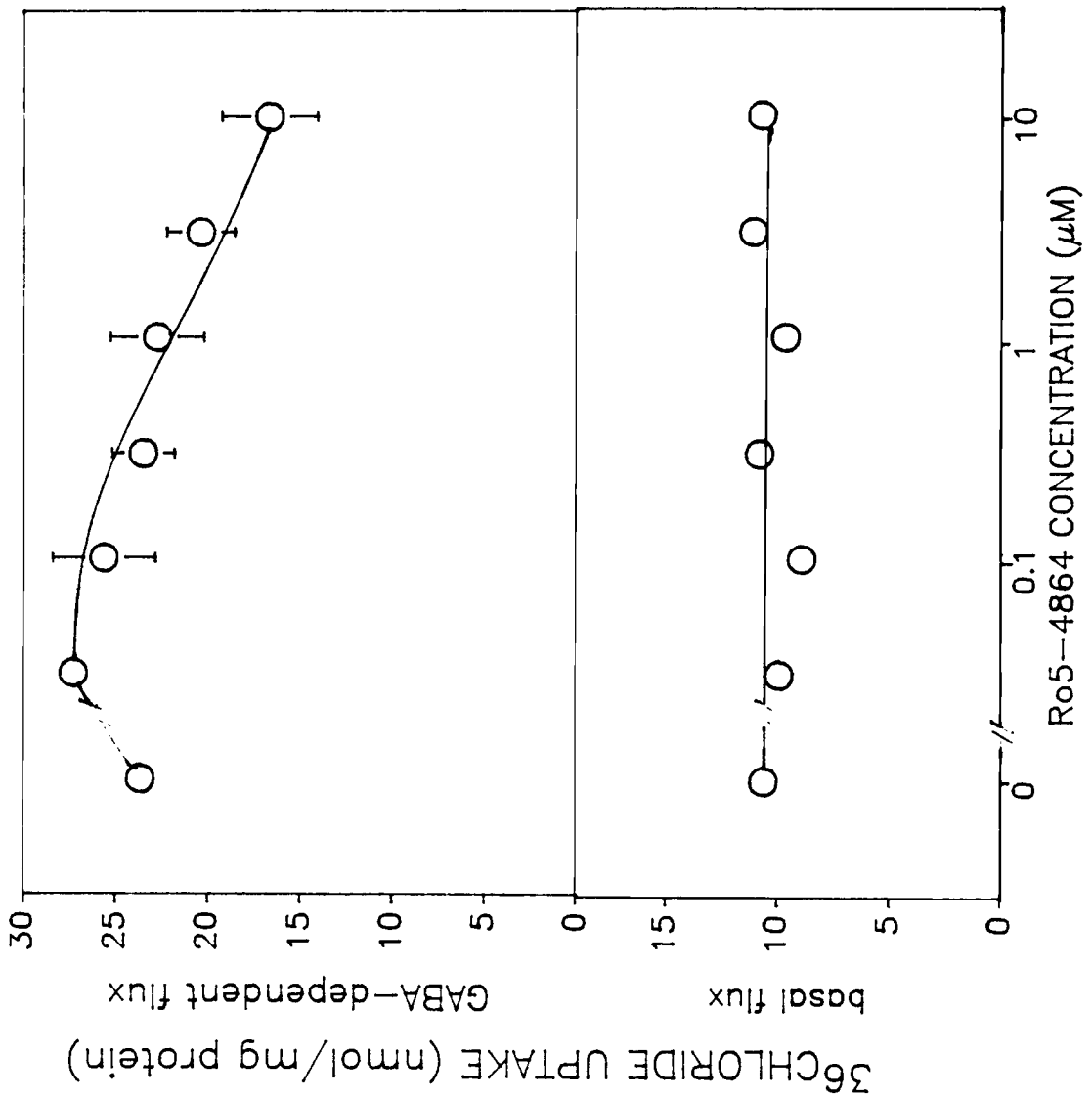

^{36}CHLORIDE UPTAKE (nmol/mg protein)
GABA–dependent flux
basal flux
30
25
20
15
10
5
0
15
10
5
0
0
0.1
1
10
Ro5–4864 CONCENTRATION (μM)

Figure 20. Inhibition of 50 μM GABA-gated chloride influx by the Type II pyrethroid, cypermethrin. 1RαS,<u>cis</u> cypermethrin is the insecticidally active isomer with the 1SαR,<u>cis</u> isomer being devoid of insecticidal activity. N=3 experiments with three runs each.

FIGURE 20

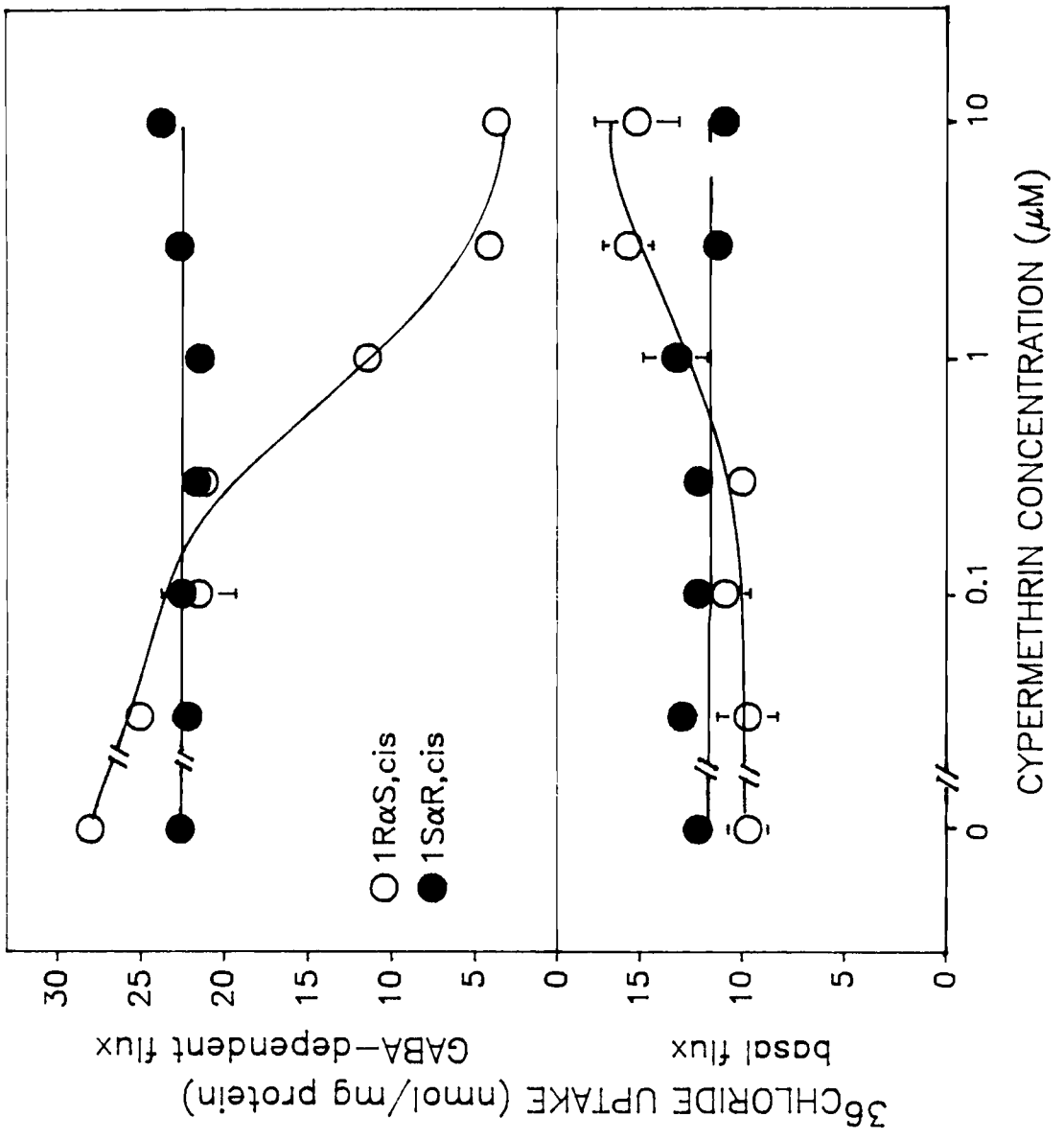

^{36}CHLORIDE UPTAKE (nmol/mg protein)
GABA–dependent flux
basal flux
1RαS,cis
1SαR,cis
30
25
20
15
10
5
0
15
10
5
0
0
0.1
1
10
CYPERMETHRIN CONCENTRATION (μM)

Figure 21. Inhibition of 50 μM GABA-gated chloride influx by the Type II pyrethroid deltamethrin. N=3 separate experiments with three runs each.

FIGURE 21

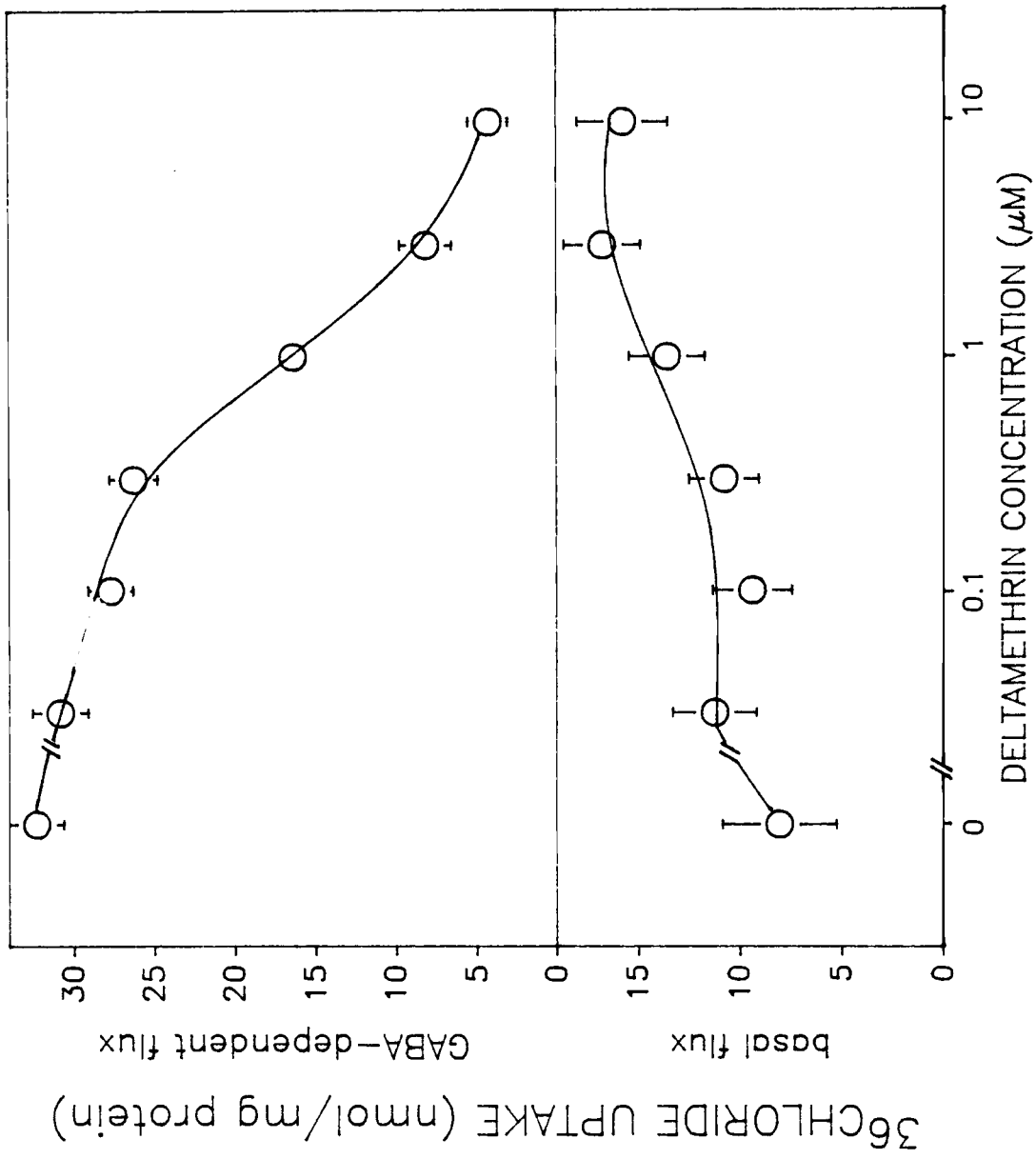

^{36}CHLORIDE UPTAKE (nmol/mg protein)
GABA-dependent flux
basal flux
0
5
10
15
20
25
30
0
0.1
1
10
DELTAMETHRIN CONCENTRATION (μM)

Figure 22. Inhibition of 50 μM GABA-gated chloride influx by the atypical Type I pyrethroid, kadethrin. N= 3 separate experiments of three runs each.

FIGURE 22

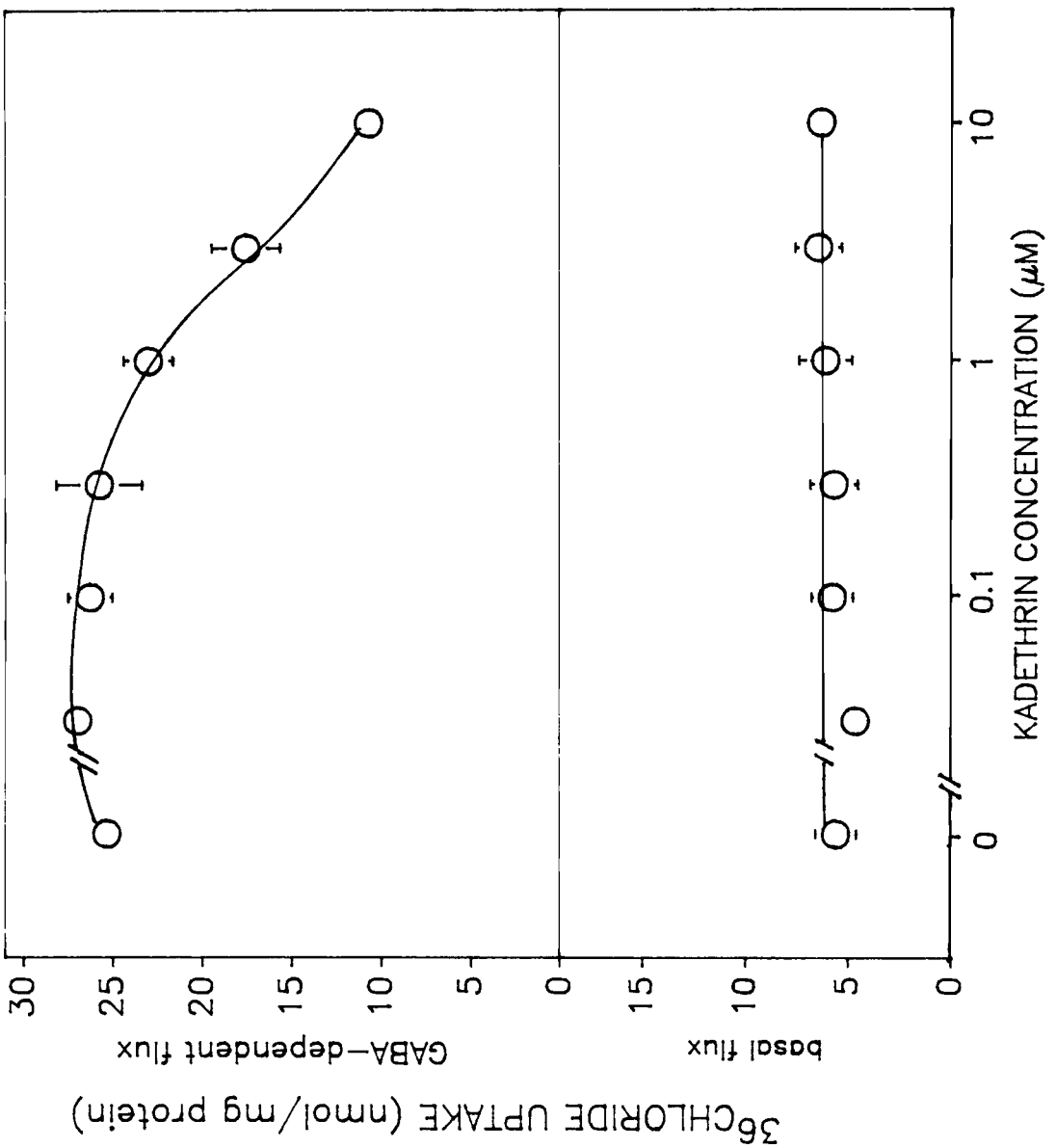

^{36}CHLORIDE UPTAKE (nmol/mg protein)
GABA–dependent flux
basal flux
30
25
20
15
10
5
0
15
10
5
0
0
0.1
1
10
KADETHRIN CONCENTRATION (μM)

Figure 23. Inhibition of 50 μM GABA-gated chloride influx by the Type I pyrethroid, permethrin. 1R,cis permethrin is the insecticidally active isomer with 1S,cis permethrin lacking inseticidal activity. N= 2 separate experiments of three runs each.

FIGURE 23

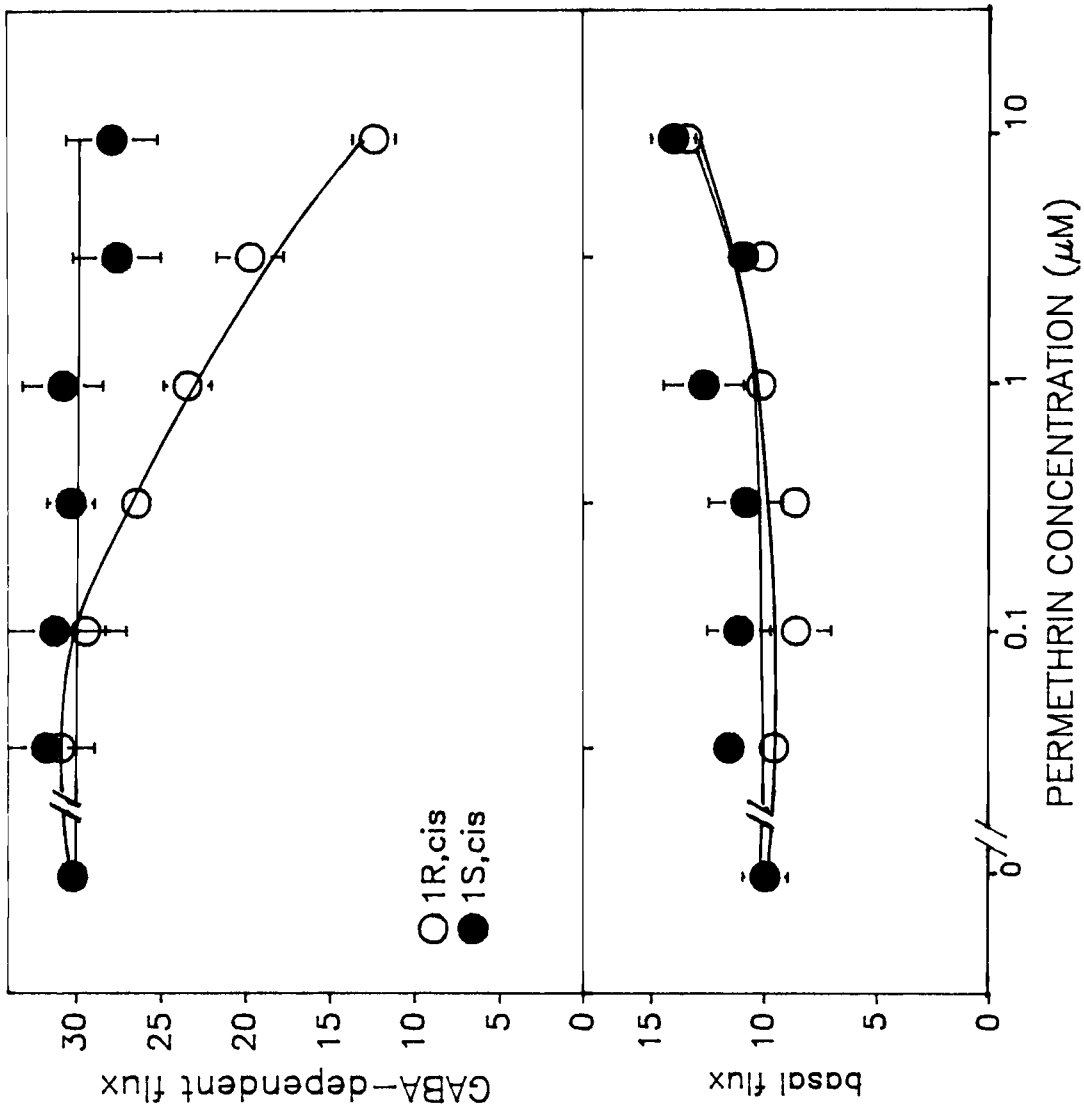

GABA–dependent flux
basal flux
^{36}CHLORIDE UPTAKE (nmol/ mg protein)
1R,cis
1S,cis
PERMETHRIN CONCENTRATION (μM)
0
5
10
15
20
25
30
0.1
1

Figure 24. Modulation of the deltamethrin-induced inhibition of 50 μM GABA-gated chloride influx by 300 nM PK 11195. N= 2 experiments of three runs each.

FIGURE 24

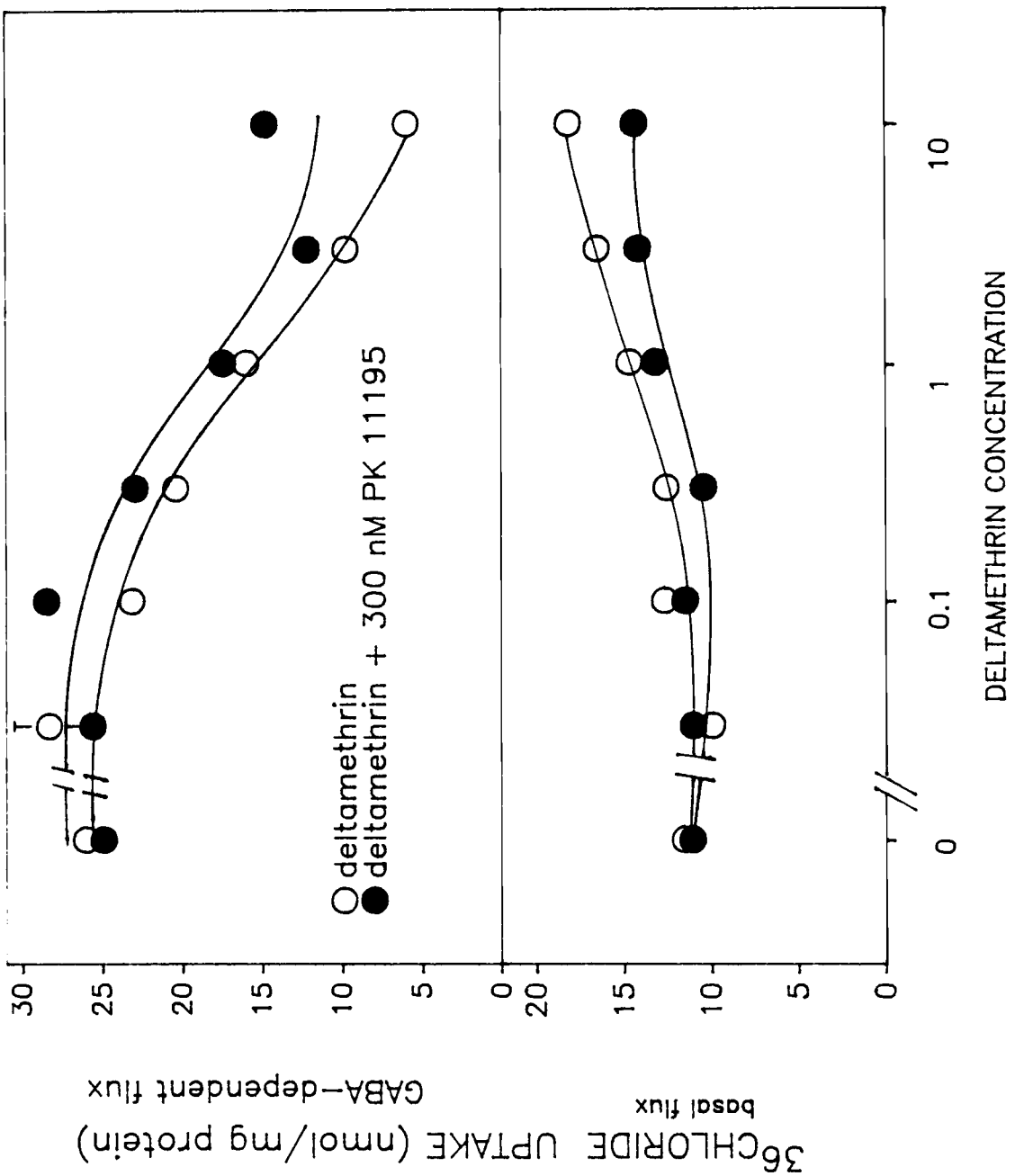

^{36}CHLORIDE UPTAKE (nmol/mg protein)
GABA-dependent flux
basal flux
deltamethrin
deltamethrin + 300 nM PK 11195
30
25
20
15
10
5
0
20
15
10
5
0
0
0.1
1
10
DELTAMETHRIN CONCENTRATION

Figure 25. Antagonism of the deltamethrin-induced inhibition of 50 μM GABA-gated chloride influx by 1 μM tetrodotoxin. N= 2 separate experiments of three runs each.

FIGURE 25

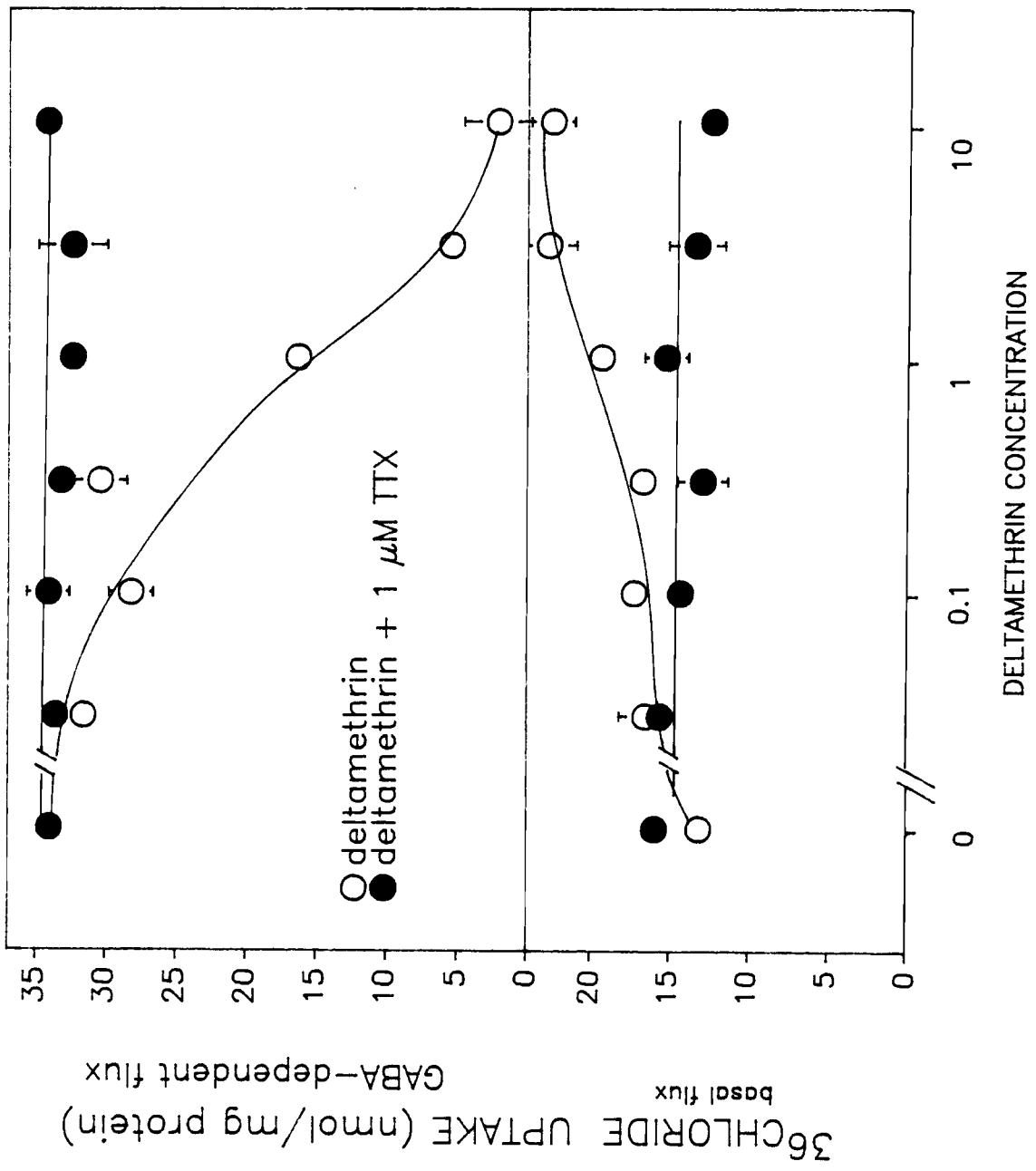

^{36}CHLORIDE UPTAKE (nmol/mg protein)
GABA-dependent flux
basal flux
deltamethrin
deltamethrin + 1 μM TTX
DELTAMETHRIN CONCENTRATION
0
0.1
1
10
0
5
10
15
20
25
30
35

Figure 26. Antagonism of the veratridine-induced inhibition of 50 μM GABA-gated chloride influx by 1 μM tetrodotoxin. N= 1 experiment of three runs.

FIGURE 26

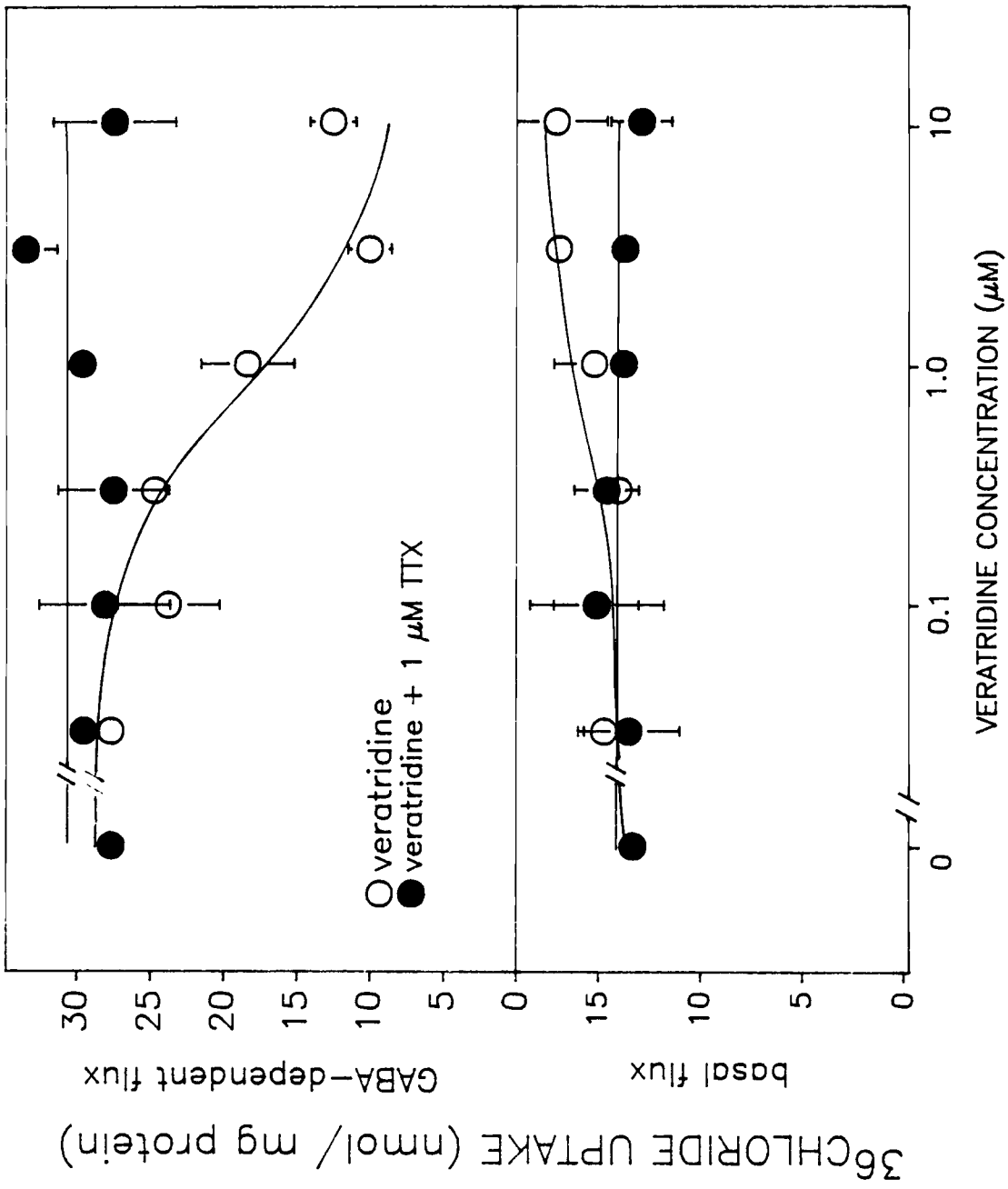
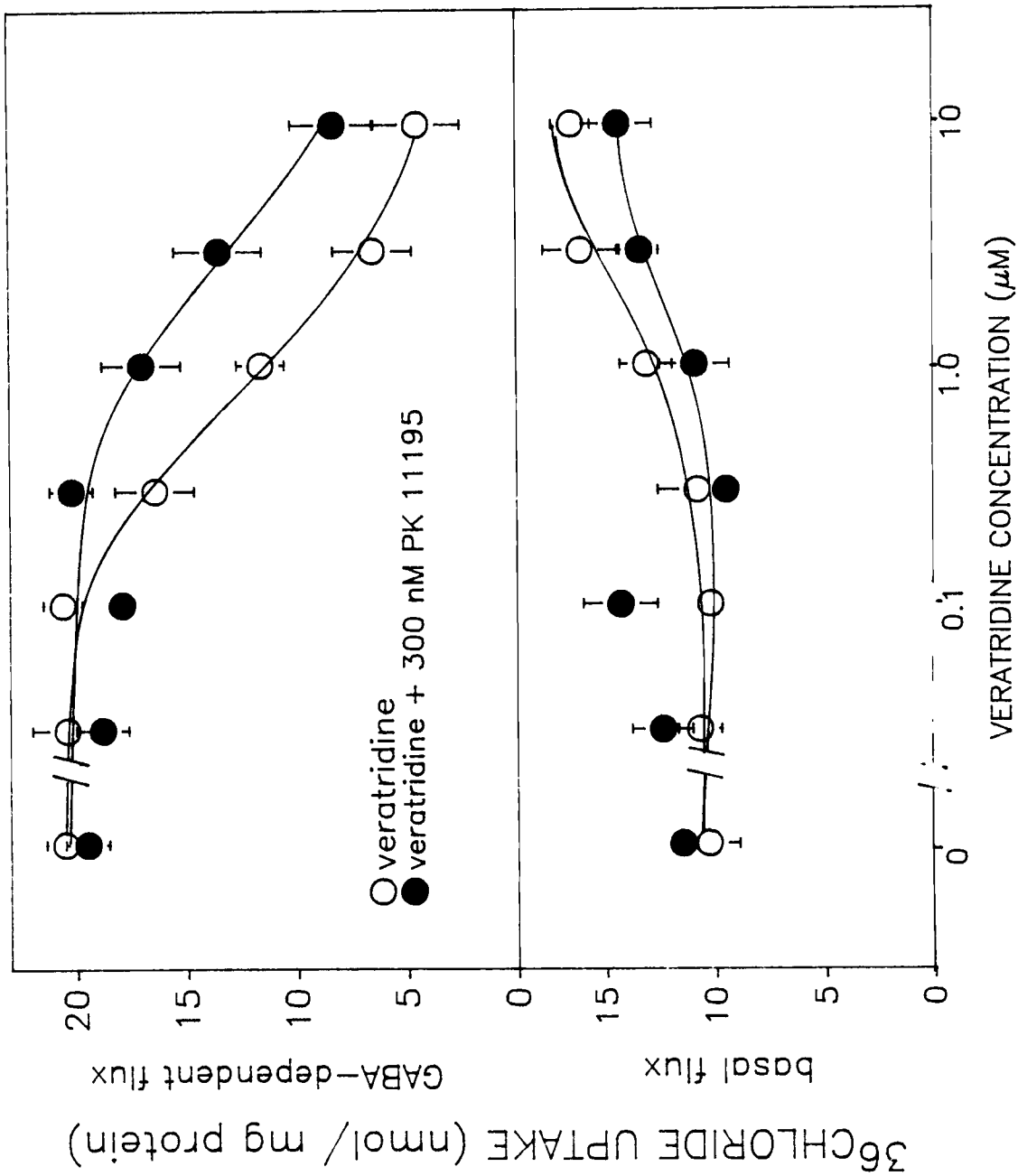

^{36}CHLORIDE UPTAKE (nmol/ mg protein)
GABA–dependent flux
basal flux
veratridine
veratridine + 1 μM TTX
VERATRIDINE CONCENTRATION (μM)
0
5
10
15
20
25
30
0.1
1.0
10

Figure 27. Modulation of veratridine-induced inhibition of 50 μM GABA-gated chloride influx by 1 μM PK 11195. N=2 separate experiments of three runs each.

FIGURE 27


^{36}CHLORIDE UPTAKE (nmol/ mg protein)
GABA–dependent flux
basal flux
20
15
10
5
0
veratridine
veratridine + 300 nM PK 11195
0
0.1
1.0
10
VERATRIDINE CONCENTRATION (μM)

CHAPTER 5 DIFFERENTIAL REGULATION OF SPECIFIC [^{35}S]TBPS BINDING TO RAT BRAIN MEMBRANES BY PYRETHROID INSECTICIDES AND RO5-4864

Leslie L. Devaud and Thomas F. Murray

ABSTRACT

Pyrethroid insecticides and Ro5-4864 were found to modulate specific [^{35}S]TBPS binding in a GABA-dependent manner. Ro5-4864 inhibitied binding in the absence of GABA but induced enhancement of [^{35}S]TBPS binding in the presence of either 5 or 10 μM GABA. Pyrethroid insecticides inhibited [^{35}S]TBPS binding, with increased potency when GABA levels were raised. GABA is a potent noncompetitive inhibitor of [^{35}S]TBPS binding and the present results indicate that pyrethroid insecticides and Ro5-4864 modulate the influence of GABA on [^{35}S]TBPS binding in an opposing manner. The effects of Ro5-4864 were not antagonized by Ro15-1788 but were sensitive to PK 11195, suggesting that Ro5-4864 interacts with an allosteric site on the $GABA_A$ receptor distinct from the classical benzodiazepine binding site. Pyrethroids inhibit [^{35}S]TBPS binding in a GABA-like fashion. In an interaction study, deltamethrin was shown to enhance the potency of GABA as an inhibitor of [^{35}S]TBPS binding. These findings imply that while both Ro5-4864 and

pyrethroids are potent proconvulsants, this action is not mediated by an interaction of these compounds with the [^{35}S]TBPS binding site.

INTRODUCTION

Picrotoxinin (PTX) is a convulsant and proconvulsant which acts noncompetitively to block GABAergic neurotransmission (Simmonds, 1980; Leeb-Lundberg et al, 1980). The PTX receptor, located in the chloride ionophore of the $GABA_A$ receptor, had been investigated with [^{3}H]dihydropicrotoxinin (DHP) (Ticku et al, 1978;Leeb-Lundberg and Olsen, 1980). However, it was difficult to obtain definitive results with this radioligand due to its low affinity for the PTX receptor with very high levels of non-specific binding (Leeb-Lundberg and Olsen, 1980).

Certain cage convulsants were found to inhibit the binding of [^{3}H]DHP to its site at the $GABA_A$ receptor (Ticku and Olsen, 1979; Olsen and Leeb-Lundberg, 1981). Synthesis of a radiolabelled cage convulsant, [^{35}S]t-butylbicyclophosphorothionate (TBPS) has provided a much better probe for investigation of the picrotoxinin receptor (Squires et al, 1983; Wong et al, 1984).

Since the development of this ligand, there has been intensive research into $GABA_A$ receptor function. Modulation of [^{35}S]TBPS binding has been employed as a means to discern the influences on $GABA_A$ receptor activity by benzodiazepine, barbiturates and many convulsant and proconvulsant compounds. However, characterization of [^{35}S]TBPS binding has given varied results (Wong et al, 1984; Squires

et al, 1983). As an example, Wood and coworkers reported the stimulation of [^{35}S]TBPS binding induced by classical benzodiazepine agonists in a well-washed membrane preparation (Wood et al, 1984). Others have reported the inhibition of [^{35}S]TBPS binding elicited by the benzodiazepines flunitrazepam and clonazepam (Gee et al, 1986). Gee also reported that generally GABA-positive ligands inhibit [^{35}S]TBPS binding while GABA-negative compounds act in an opposing manner (Gee et al, 1986; Gee, 1987).

These conflicting results can be reconciled with the recent evidence showing the predominant effects of GABA on the direction and magnitude of modulation exerted by ligands influencing [^{35}S]TBPS binding to its receptor. Not only does GABA itself noncompetitively inhibit the binding of [^{35}S]TBPS but GABA antagonists reverse its inhibitory activity with potencies dependent on the level of GABA present (Seifert and Casida, 1985; Squires and Saederup, 1987). In the presence of GABA, classical benzodiazepine agonists inhibit [^{35}S]TBPS binding while inverse agonists enhance [^{35}S]TBPS binding (Seifert and Casida, 1985; Gee et al, 1986; Gee, 1987).

Without addition of exogenous GABA, Ticku and coworkers reported that Ro5-4864 (4'chlor-diazepam) inhibited the binding of [^{35}S]TBPS with an IC_{50} of approximately 20 μM. Ro5-4864 is a ligand which has been used to characterize the peripheral-type benzodiazepine receptor (PTBR) as it has low affinity for the GABA-coupled benzodiazepine site and very high affinity for the PTBR (Schoemaker et al, 1983). However, these findings suggest that Ro5-4864 may interact with the $GABA_A$ receptor, possibly at the TBPS site. Ro5-4864 is a convulsant and proconvulsant and thus displays

physiological effects directly opposite to the classical benzodiazepines (Benavides et al, 1984; File et al, 1984; Devaud et al, 1986). In agreement with this, Gee (1987) has reported the ability of Ro5-4864 to enhance [^{35}S]TBPS binding in the presence of GABA.

Pyrethroid insecticides have also been shown to noncompetitvely displace [^{35}S]TBPS binding with a complete correlation between mouse icv toxicity and [^{35}S]TBPS binding inhibition (Lawrence and Casida, 1983). Furthermore, increasing the amount of GABA present in the assay enhances the potency of the Type II pyrethroid 1RαS,cis cypermethrin as an inhibitor of [^{35}S]TBPS binding (Seifert and Casida, 1985).

Both Ro5-4864 and pyrethroid insecticides are proconvulsants and previous investigations have suggested an involvement of pyrethroids with an Ro5-4864 binding site in that PK 11195, an antagonist of the PTBR blocks the proconvulsant activity of pyrethroids (Devaud et al, 1986; Devaud and Murray, 1988). Recent investigations have also demonstrated the ability of Ro5-4864 and pyrethroids to inhibit GABA-gated chloride influx (Eldefrawi and Eldefrawi, 1987; Bloomquist and Soderland, 1985; this volume, Chapter 4). Thus it was of interest to further assess the interaction of Ro5-4864 and pyrethroids as modulators of [^{35}S]TBPS binding to its site at the $GABA_A$ receptor.

In addition, while there are several reports on the inhibition of [^{35}]TBPS binding by pyrethroids, there has not been a thorough pharmacological characterization of their GABA-dependent inhibitory potency and efficacy. Therefore, the present investigation also

examined the influence of both Type I and Type II pyrethroids on [^{35}S]TBPS binding in the presence of GABA.

METHODS

Materials: [^{35}S]TBPS (specific activity 96.5 Ci/mmol) was purchased from DuPont-NEN, Boston, MA. Ro5-4864 was generously supplied by Dr. Peter Sorter, Hoffman-LaRoche, Inc., Nutley NJ. PK 11195 was a generous gift of Dr. G. LeFur, Pharmaku Laboratories, Genevilliers, France. Pyrethroids: 1RαS,cis cypermethrin, 1SαR,cis cypermethrin, 1R,cis permethrin, 1S,cis permethrin and allethrin were gifts from Dr. Arthur Ramsey, FMC Corporation, Princeton, NJ. Kadethrin, cismethrin and deltamethrin were gifts from Dr. P. Foulhoux, Roussel-Uclaf, Romainville, France. Tetramethrin and resmethrin were obtained from the EPA, Research Triangle Park, NC.

Membrane preparation: Two preparations were used during the present investigation. The P_2 pellet suspension was prepared according to the method of Gee (Gee et al, 1986). Briefly, cerebral cortices from male Sprague-Dawley rats, 160-180 g, (Simonson Labs, Gilroy, CA) were rapidly dissected over ice. The membranes were homogenized in 20 volumes of 0.32 M sucrose with 10 passes using a teflon-glass homogenizer. The homogenate was centrifuged at 1000 x g for 10 minutes and the resulting supernatant was centrifuged at 9000 x g for 20 minutes. The resulting pellet was resuspended in 20 volumes ice cold 50 mM Na-KPB buffer, pH 7.4, yielding a final protein concentration of 1.5 - 1.8 mg/ml (as determined by the method

of Lowry following solubilization with 0.5 N NaOH) (Lowry et al, 1951).

A second membrane suspension, a total particulate fraction, was prepared by the rapid dissection of Sprague-Dawley rat cerebral cortices over ice with homogenization in 80 volumes of 10 mM Tris-citrate buffer for 20 s at a Polytron setting of 7. The homogenate was centrifuged at 40,000 x g for 10 minutes. The resulting pellet was resuspended in 80 volumes of buffer and centrifuged again at 40,000 x g for 10 minutes. The resulting pellet was subjected to a snap freeze of 5 minutes, allowed to thaw at room temperature, diluted with 80 volumes of buffer and then washed twice more. The final pellet was resuspended in 80 volumes (original wet weight) of assay buffer (50 mM Na-KP), pH 7.4, for a final protein concentration of 0.5 - 0.6 mg/ml as determined by the method of Lowry, following solubilization with 0.5 N NaOH (Lowry et al, 1951). The two membrane preparations were used to compare the effects of endogenous GABA on the interactions of pyrethroids, Ro5-4864, and PK 11195 with GABA on [^{35}S]TBPS binding.

[^{35}S]TBPS binding assay: 240 μl ice cold 50 mM assay buffer, 10 μl buffer only or buffer with the appropriate concentration of GABA, 200 μl homogenate and 1 μl 100 % DMSO or DMSO with inhibitor were added in order. 50 μl of 1 nM [^{35}S]TBPS was added to start the incubation. Assay tubes were incubated at 25^{o}C for 90 minutes. Non-specific binding was determined in the presence of 100 μM picrotoxinin and was less than 20% in either membrane preparation. The incubation was terminated by rapid filtration through Schleicher & Schuell 32 glass fiber filters using a Brandel Cell Harvester

followed by 4 4-ml washes with ice cold assay buffer. After elution, radioactivity was measured by liquid scintillation spectroscopy.

Data analysis: Parameter estimates were obtained by using the non-linear curve fitting routine, LIGAND (Munson and Rodbard, 1980) for pyrethroid inhibition of specific [^{35}S]TBPS binding or MK MODEL Plus (Holford, 1983) for Ro5-4864 modulation of [^{35}S]TBPS binding.

RESULTS

The present investigation employed two different membrane preparations to better elucidate the activity of GABA both as an inhibitor of [^{35}S]TBPS binding and as it influences the interactions with other compounds on [^{35}S]TBPS binding. P_2 membrane preparations typically contain high levels of endogenous GABA, on the order of 200 μM (Gardner et al, 1981). A well-washed membrane preparation with a lower concentration of protein will have considerably less GABA, though detectable amounts will still be present. Use of these two preparations proved helpful in delineating the GABA-dependent activities of Ro5-4864 and pyrethroids as modulators of specific [^{35}S]TBPS binding.

In agreement with previous reports, Ro5-4864 was seen to modulate [^{35}S]TBPS binding in a GABA-dependent manner (Figure 28). As shown in Figure 28, Ro5-4864 produced a modest inhibition of [^{35}S]TBPS binding without addition of exogenous GABA; it did not effect [^{35}S]TBPS binding in the presence of 2.5 μM added GABA and enhanced [^{35}S]TBPS binding with the inclusion of 5 μM GABA. The

enhancement of [^{35}S]TBPS binding by Ro5-4864 was seen as a 50-75% increase over control levels of [^{35}S]TBPS bound. The enhancement of [^{35}S]TBPS is actually a reversal of the inhibitory effect of GABA not an absolute increase in [^{35}S]TBPS binding over that seen in the absence of either GABA or Ro5-4864. This becomes apparent when data are presented quantitatively as fmol [^{35}S]TBPS bound/ mg protein instead of as percent of control.

From these results it is impossible to distinguish whether the modulation of [^{35}S]TBPS binding elicited by Ro5-4864 was mediated via an interaction with the classical benzodiazepine receptor site or through an Ro5-4864 site purportedly allosterically-coupled with the $GABA^A$ receptor (Gee, 1987; Gee et al, 1988). The addition of 1 μM Ro15-1788, a classical benzodiazepine antagonist, had no effect on [^{35}S]TBPS binding by itself. It also did not influence the Ro5-4864 enhancement of [^{35}S]TBPS binding (Figure 29, Table 6). The lack of antagonism by Ro15-1788 of the Ro5-4864-induced enhancement of [^{35}S]TBPS binding supports the suggestion of an interaction of Ro5-4864 with a site distinct from the classical benzodiazepine binding site

PK 11195, an antagonist of the PTBR, inhibited greater than 95% of specific [^{35}S]TBPS binding with an IC_{50} value of 10.21 ± 0.99 μM in the presence of 5 μM GABA (Figure 30). A test of the PK 11195 sensitivity of the Ro5-4864 enhancement of [^{35}S]TBPS binding showed that 1 μM PK 11195 was able to antagonize the Ro5-4864-induced increase in [^{35}S]TBPS binding (Table 6, Figure 31). Addition of 300 nM PK 11195 increased the EC_{50} value for Ro5-4864 enhancement from 1.08 ± 0.09 μM to 2.23 ± 0.12 μM ($p < 0.05$) with no shift in the E_{max}

value (Table 6). The presence of 1 μM PK 11195 slightly reduced calculated maximal response induced by Ro5-4864 in the total particulate membrane preparation from 69.2 ± 2.3 % to 63.0 ± 2.5 % enhancement of [^{35}S]TBPS binding. These results suggest that PK 11195 attenuates the effects of Ro5-4864 and that at high doses of PK 11195 there may be additional, non-competitive influences on the Ro5-4864-induced modulation of [^{35}S]TBPS binding.

To further investigate the GABA-dependence of the Ro5-4864-induced modulation of [^{35}S]TBPS binding, the effect of 1 μM Ro5-4864 on the GABA dose-dependent inhibition of [^{35}S]TBPS binding was assessed. As shown in Figure 32, the biphasic nature of the effect of Ro5-4864 on [^{35}S]TBPS binding was quite evident. At low doses of GABA, Ro5-4864 elicited a modest enhancement of ^{35}S]TBPS binding. This effect peaked at 1 μM GABA and thereafter Ro5-4864 attenuated the inhibition of [^{35}S]TBPS binding induced by GABA, increasing the IC_{50} value from 3.95 ± 0.29 μM to 6.17 ± 1.11 μM. PK 11195 by itself did not influence the inhibition of [^{35}S]TBPS binding elicited by GABA (IC_{50} value of 3.86 ± 0.05). The addition of either 300 nM or 1 μM PK 11195 did not influence the Ro5-4864-induced antagonism of the inhibition of [^{35}S]TBPS binding elicited by GABA.

The Type II pyrethroid deltamethrin was also found to affect the Ro5-4864 modulation of [^{35}S]TBPS binding by increasing the IC_{50} value for RO5-4864 from 428 ± 43.5 nM to 576 ± 54.6 nM ($P < 0.05$) with the addition of 1 μM deltamethrin (Figure 33) with no effect on the E_{max} value for Ro5-4864. The presence of 100 nM deltamethrin had a more modest effect on the enhancement of [^{35}S]TBPS binding elicited by Ro5-4864, shifting the EC_{50} value from 394.4 ± 51.8 nM to 444.4 ±

69.1 nM, also with no effect on the maximal response. The activity of deltamethrin was most noticeable on basal levels of [^{35}S]TBPS binding, reducing the amount of [^{35}S]TBPS bound from 21.2 ± 1.5 fmol/ mg protein to 10.7 ± 1.7 fmol/mg protein (Figure 33).

Pyrethroid insecticides have also been shown to modulate [^{35}S]TBPS binding to its site in the chloride ionophore (Seifert and Casida, 1985; Lummis et al, 1987; Crofton et al, 1987). These compounds inhibit rather than enhance [^{35}S]TBPS binding, also in a GABA-dependent manner. As shown in Figure 34, deltamethrin was able to inhibit specific [^{35}S]TBPS binding only in the presence of exogenous GABA. It was most potent in this regard at 5 μM GABA, reducing the IC_{50} value for deltamethrin inhibition from 2.66 ± 0.45 μM in the presence of 2.5 μM GABA to 1.57 ± 0.34 μM at 5 μM GABA. In this measure, inclusion of 10 μM GABA did not further enhance the potency of deltamethrin as an inhibitor of [^{35}S]TBPS binding. As further evidence of the GABA-dependency of this effect, the addition of 1 μM bicuculline totally abolished the inhibitory activity of deltamethrin on [^{35}S]TBPS binding in the presence of 5 μM GABA (data not shown).

Thus it appears that while both Ro5-4864 and pyrethroids such as deltamethrin modulate [^{35}S]TBPS binding to rat brain membranes in a GABA-dependent manner, they do so in opposing directions. With these actions in mind, an investigation into the effect of several concentrations of deltamethrin on the GABA-induced inhibition of [^{35}S]TBPS binding was undertaken. Due to the prominent effects of deltamethrin as an inhibitor of basal [^{35}S]TBPS binding in the presence of endogenous GABA, the total particulate membrane

suspension was employed for this assay. As depicted in Figure 35, deltamethrin was able to increase the potency of GABA as an inhibitor of [^{35}S]TBPS binding in a dose-dependent manner: the addition of 300 nM deltamethrin induced a non-significant reduction in the IC_{50} value for GABA from 5.86 ± 0.62 μM to 4.90 ± 0.44 μM. At a concentration of 1 μM, deltamethrin further increased the affinity of GABA as an inhibitor of [^{35}S]TBPS binding, shifting the IC_{50} value from 5.38 ± 0.64 μM to 3.62 ± 0,52 μM ($p < 0.005$). The presence of 3 μM deltamethrin also significantly reduced the IC_{50} value from 4.11 ± 0.24 μM to 1.59± 0.11 μM ($p < 0.001$) for GABA (Table 7). These results further support the differing effects of Ro5-4864 and deltamethrin on GABA-modulated [^{35}S]TBPS binding.

As it has been previously demonstrated that PK 11195 antagonizes certain actions of deltamethrin, it was of interest to determine the effect of PK 11195 on the inhibition of [^{35}S]TBPS binding elicited by deltamethrin. A dose of 1 μM PK 11195, which did not inhibit binding by itself, appeared to enhance the deltamethrin-induced inhibition of [^{35}S]TBPS binding by reducing the deltamethrin IC_{50} from 2.59 ± 1.9 μM to 1.32 ± 1.1 μM without affecting the maximal inhibition (data not shown). Inclusion of 300 nM PK 11195 with deltamethrin also enhanced deltamethrin's potency as an inhibitor of [^{35}S]TBPS binding by shifting the IC_{50} from 1.97 ± 0.27 μM to 1.66 ± 0.14 μM. Thus, PK 11195 and deltamethrin appear to modulate [^{35}S]TBPS binding in a similar manner and in opposition to the influence exerted by Ro5-4864.

A broad range of pyrethroids have been shown to exert potent proconvulsant effects in the rat (Devaud et al, 1986; Devaud and

Murray, 1988) and to inhibit the functional coupling of GABA to its receptor (Chapter 4). Therefore, it was important to measure the pharmacological profile of pyrethroid inhibition of [^{35}S]TBPS binding. All pyrethroids tested were found to inhibit [^{35}S]TPBS binding with efficacies approaching 100% (Table 8). However, as evident in Figure 36, the range of affinities between the most potent and the least potent pyrethroid was small, from about 1 μM to slightly higher than 10 μM, whether measured in the P_2 pellet membrane preparation or the total particulate membrane preparation (Table 8). Furthermore, the stereospecificity of pyrethroid inhibition of [^{35}S]TBPS binding was not of the same order as had been noted in other measures of pyrethroid activity. While the insecticidally active isomer of cypermethrin, 1RαS,<u>cis</u>, did inhibit [^{35}S]TBPS binding with a greater potency than its insecticidally active isomer, the difference in potency between the two isomers was less than 10-fold regardless of the level of GABA in the assay. Similar results were observed for the two permethrin isomers. Therefore, while pyrethroid insecticides are able to inhibit the specific binding of [^{35}S]TBPS to rat brain membranes generally by greater than 90%, neither the potency range from Type II to Type I compounds nor the stereoisomeric preference seen in this measure are of the extent observed in their insecticidal or proconvulsant activity or as inhibitors of [^{3}H]Ro5-4864 binding.

DISCUSSION

The present investigation adds further support to the reports of the complex regulation of [^{35}S]t-butylbicyclophosphorothionate binding to the picrotoxinin site in the chloride channel of the $GABA_A$ receptor. Ro5-4864, a ligand reported to be selective for the peripheral-type benzodiazepine receptor, does exert significant effects at the $GABA_A$ receptor with the direction of Ro5-4864 modulation of [^{35}S]TBPS binding being absolutely dependent on the presence of GABA. As evident in Figure 27, with low or no GABA present, Ro5-4864 will inhibit the specific binding of [^{35}S]TBPS to rat brain membranes. Addition of 5 μM or greater GABA results in a 50-75% enhancement of [^{35}S]TBPS binding. What is also apparent in Figure 27 is that Ro5-4864, while acting to increase the level of [^{35}S]TBPS binding, is doing so by antagonizing the potent inhibition elicited in the presence of GABA.

Wood and coworkers have reported that in assay conditions with low GABA, classical benzodiazepine agonists enhance [^{35}S]TBPS binding while inverse agonists inhibit it (Wood et al, 1984). It has also been reported that in the presence of GABA contrasting results are obtained: inhibition of [^{35}S]TBPS binding by benzodiazepine agonists, such as flunitrazepam, with benzodiazepine inverse agonists enhancing [^{35}S]TBPS binding greater than two-fold (Gee et al, 1986).

Considered together with the results of the present investigation it appears that Ro5-4864 behaves similarly to benzodiazepine inverse agonists in the presence of GABA.

That this Ro5-4864 site is distinct from the benzodiazepine binding site recognized by classical benzodiazepine agonists is suggested by the findings of Gee and coworkers in a recent report. Their findings show that the inhibitory effects of clonazepam on [^{35}S]TBPS binding are abolished by the irreversible labelling of the classical benzodiazepine binding sites by Ro-15-4513 with no apparent effect on the enhancement of [^{35}S]TBPS binding elicited by Ro5-4864 (Gee et al, 1988). In support of these findings, the present investigation, has shown that the classical benzodiazepine antagonist, Ro15-1788, did not influence Ro5-4864 modulation of [^{35}S]TBPS binding. However, the enhancement of[^{35}]TBPS binding elicited by Ro5-4864 was antagonized by PK 11195, further supporting the presence of a $GABA_A$ receptor site recognized by both PTBR ligands.

Previous investigations have shown an interaction of pyrethroid insecticides with an Ro5-4864 binding site (Gammon and Sander, 1985; Lawrence et al, 1985; Devaud et al, 1986; Devaud and Murray, 1988). In support of these findings, the present study has shown that the Type II pyrethroid, deltamethrin, antagonized the Ro5-4864-induced increases in [^{35}S]TBPS binding (Figure 31) implicating an interaction of this compound with a $GABA_A$ receptor-coupled Ro5-4864 site as well as at the PTBR.

Even though both Ro5-4864 and pyrethroid insecticides are

proconvulsants, pyrethroids did not influence [^{35}S]TBPS binding in the same manner as did Ro5-4864. While requiring the presence of GABA, these compounds inhibited the specific binding of [^{35}S]TBPS to rat brain membranes with potencies in the low micromolar range in agreement with prior reports (Seifert and Casida, 1985a,b; Crofton et al, 1987).

The inhibition of [^{35}S]TBPS binding elicited by pyrethroid insecticides does not show the wide range in potencies typically observed between the Type II and Type I compounds nor do the insecticidally active and inactive isomers display the same magnitude of stereoisomeric preference typically seen in other physiological and biochemical assays (Lawrence and Casida, 1983; Devaud et al, 1986; Devaud and Murray, 1988; this volume, Chapter 4).

As further support of the opposing nature of interaction of Ro5-4864 and deltamethrin on [^{35}S]TBPS binding, while Ro5-4864 antagonized GABA's inhibition of [^{35}S]TBPS binding, deltamethrin, in a dose-dependent manner, enhanced the potency of GABA as an inhibitor of specific [^{35}S]TBPS binding. Thus, while pyrethroid insecticides and Ro5-4864 share similar physiological activities as proconvulsants, it is not likely that they exert their proconvulsant activities via an interaction with the [^{35}S]TBPS site on the $GABA_A$ receptor chloride ionophore.

In conclusion, this series of investigations has shown that pyrethroids and PTBR ligands appear to interact with sites on the $GABA_A$ receptor with the manner of their interactions being highly regulated by GABA.

Table 6 PK 11195- and Ro15-1788-sensitivity of the Ro5-4864 modulation of specific [^{35}S]TBPS binding

Treatment	E_{max}[a]	EC_{50}[b]	N
A. P_2 pellet preparation with 5 μM GABA			
control	54.6±0.7	506.3±61.2	1.14±0.12
+1μM PK 11195	49.8±0.3	607.5±36.3*	1.33±0.08
B. Total particulate preparation with 5 μM GABA			
1. control	70.0±2.1	1078.1±84.6	1.24±0.09
+300 nM PK 11195	69.5±14.8	2230.8±119.5*	1.08±0.34
2. control	69.2±2.3	969.5±86.7	1.34±0.12
+1 μM PK 11195	63.0±2.5	1162.9±117.0*	1.40±0.15
C. Total particulate preparation with 5 μM GABA			
control	70.3±1.9	950.1±67.0	1.34±0.09
+1 μM Ro15-1788	69.7±4.7	1072.6±188.6	1.51±0.29

[a] % enhancement of [^{35}S]TBPS binding above basal levels (in the absence of Ro5-4864); [b] nM; *significant difference in treatment versus control values, $p < 0.05$.

Table 7 Deltamethrin modulation of the GABA dose-dependent inhibition of specific [^{35}S] TBPS binding.

Treatment	IC_{50} (μM)	nH	% displacement
A. control	5.86±0.62	1.34±0.16	97.0±1.9
+ 300 nM dm	4.90±0.44	1.34±0.12	97.7±1.3
B. control	5.38±0.64	1.25±0.04	98.5±0.9
+ 1 μM dm	3.62±0.52*	1.32±0.12	98.2±1.1
C. control	4.11±0.24	1.33±0.10	99.6±0.4
+ 3 μM dm	1.59±0.11*	1.10±0.11	99.7±0.4

dm = deltamethrin. The total particulate membrane preparation with 5 μM GABA was employed in this series of experiments.
* $p < 0.05$.

Table 8: Pyrethroid inhibition of [^{35}S]TBPS binding to rat brain membrane.

	A: P_2 pellet			B: total particulate		
	IC_{50}	n_H	% displacement[a]	IC_{50}	n_H	% displacement
1RαS,<u>cis</u> cypermeth.	2.91 ± 0.7	1.17 ± 0.14	97.7 ± 2.0	1.31 ± 0.22	1.49 ± 0.25	98.2 ± 1.2
deltamethrin	2.48 ± 0.37	1.02 ± 0.04	89.2 ± 1.2	1.65 ± 0.41	1.11 ± 0.13	97.5 ± 0.9
kadethrin	3.26 ± 0.26	0.84 ± 0.08	82.7 ± 1.4	2.28 ± 0.76	1.54 ± 0.51	90.1 ± 3.4
1R,<u>cis</u> permethrin	8.27 ± 1.23	0.96 ± 0.09	78.5 ± 1.7	3.52 ± 1.23	1.21 ± 0.33	97.9 ± 1.0
cismethrin	3.89 ± 0.34	1.22 ± 0.05	94.3 ± 2.9	6.39 ± 1.14	1.04 ± 0.19	82.8 ± 4.0
allethrin	7.62 ± 1.23	2.18 ± 0.32	96.8 ± 1.2	5.20 ± 1.07	1.25 ± 0.38	85.2 ± 1.2
tetramethrin	3.66 ± 0.39	1.44 ± 0.04	97.8 ± 0.7	3.70 ± 1.25	1.72 ± 0.71	98.6 ± 0.8
resmethrin	8.10 ± 1.28	1.24 ± 0.17	83.9 ± 2.9	5.75 ± 2.07	0.93 ± 0.10	86.4 ± 1.0
1SαR,<u>cis</u> cypermeth.	10.00 ± 1.35	1.12 ± 0.16	86.3 ± 3.4	6.34 ± 1.75	1.69 ± 0.39	75.8 ± 2.0
1S,<u>cis</u> permethrin	inactive	---	---	11.64 ± 3.72	0.97 ± 0.22	74.6 ± 3.1

A: P_2 pellet membrane suspension with 5 μM GABA, n=3

B: Total particulate membrane suspension with 10 μM GABA, n=3

Pyrethroids were tested as inhibitors over a concentration range of 10 nM to 30 μM. Higher concentrations were not employed due to the limited aqueous solubility of these compounds

[a] maximal % of specific [^{35}S]TBPS binding inhibited

Figure 28. GABA-dependency of the Ro5-4864 modulation of specific [^{35}S]TBPS binding to rat brain membranes. GABA concentration: 0 (○), 2.5 μM (●), 5 μM (△). The P_2 pellet membrane preparation was used in this series of experiments.

FIGURE 28

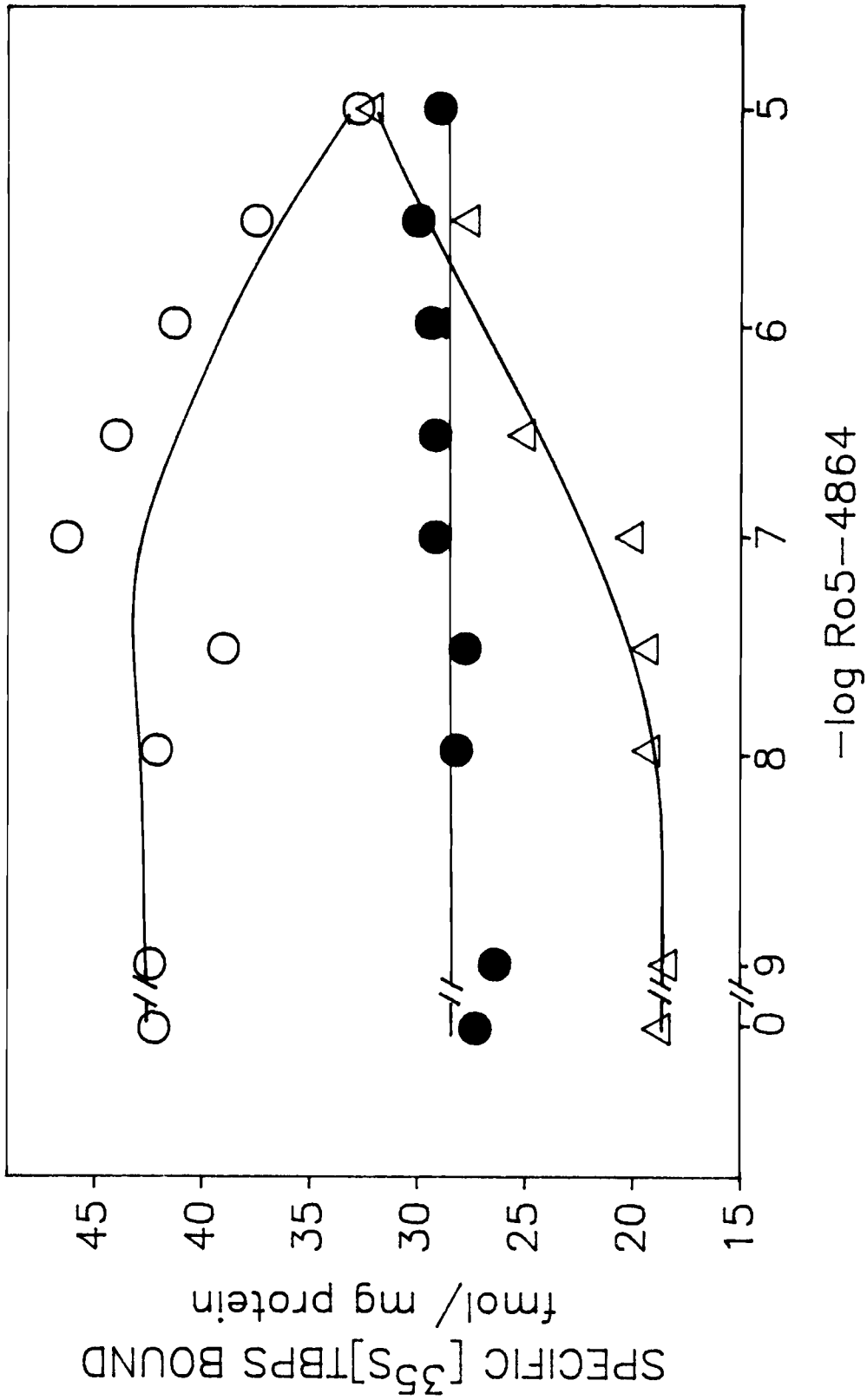

SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
45
40
35
30
25
20
15
5
6
7
8
9
0
-log Ro5-4864

Figure 29. Effect of the classical benzodiazepine antagonist, Ro15-1788, on the Ro5-4864-induced enhancement of specific [^{35}S]TBPS binding. With 5 μM GABA using the total particulate membrane preparation. Data presented is from a representative experiment. N=3.

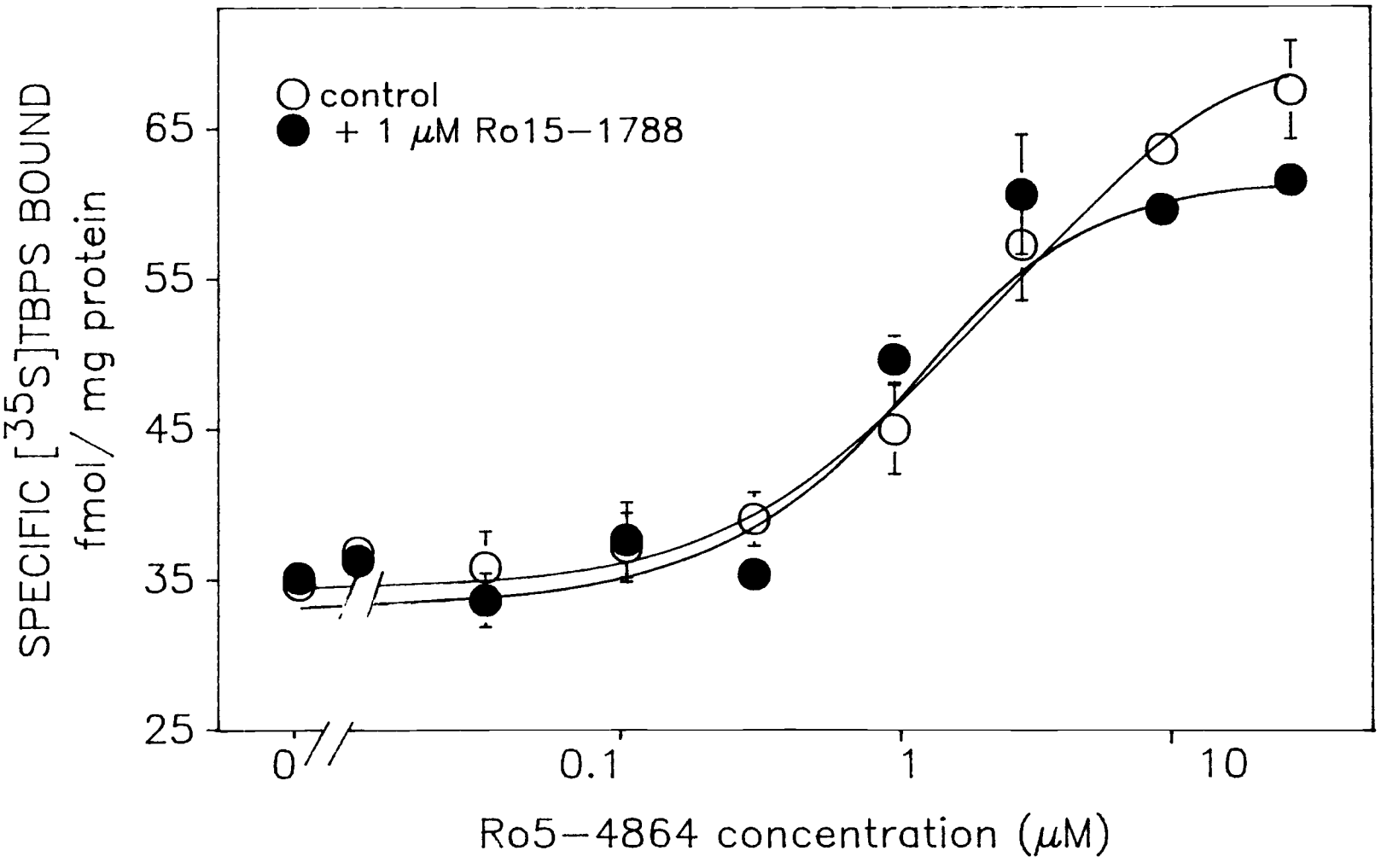

control
+ 1 μM Ro15–1788
SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
65
55
45
35
25
0
0.1
1
10
Ro5–4864 concentration (μM)

FIGURE 29

Figure 30. Dose-dependent inhibition of [^{35}S]TBPS binding by PK 11195. In the total particulate membrane preparation with 5 μM GABA. Data presented is from a representative experiment. N=3.

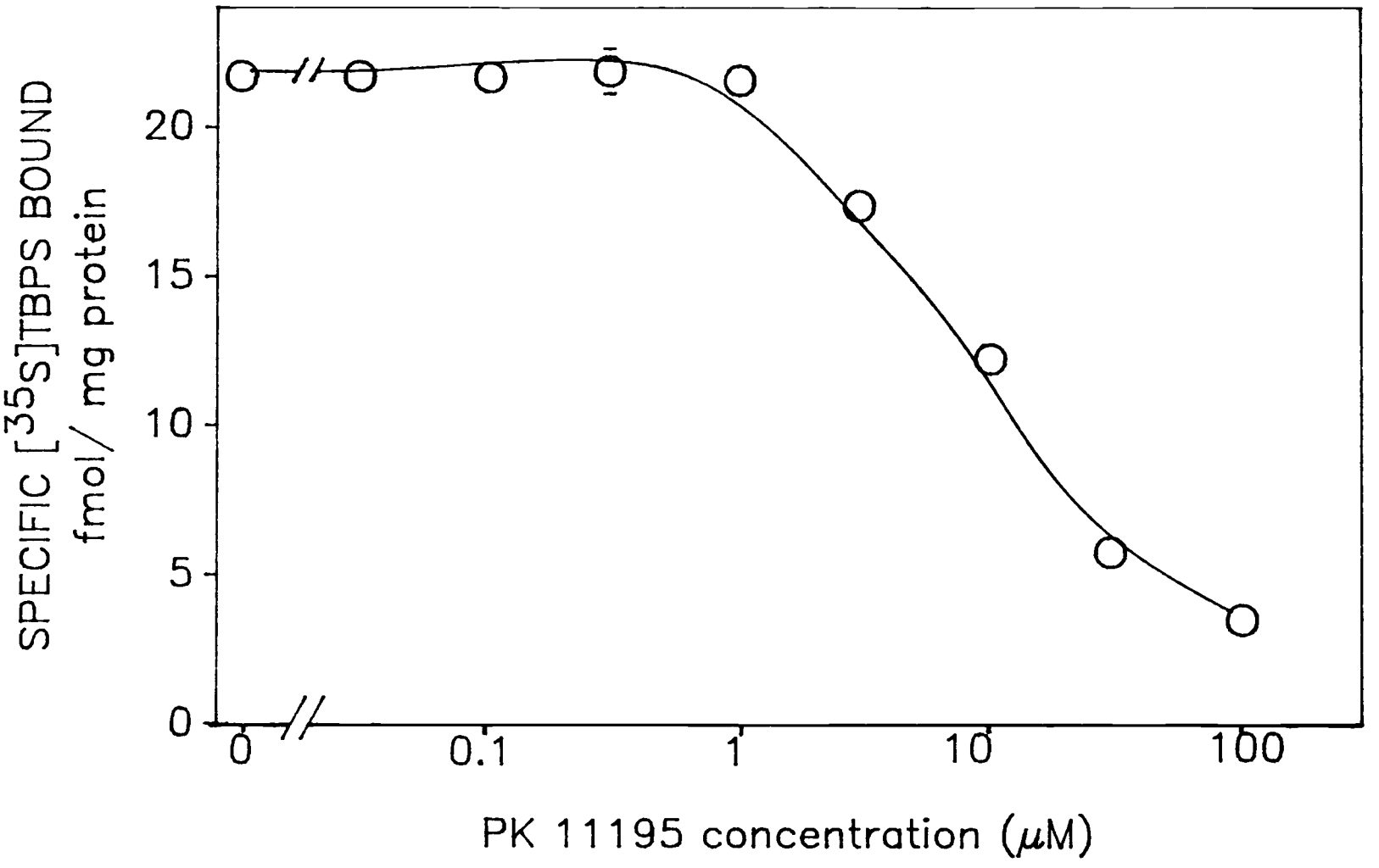

SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
20
15
10
5
0
0
0.1
1
10
100
PK 11195 concentration (μM)

FIGURE 30

Figure 31. Effects of PK 11195 on the Ro5-4864-induced enhancement of [^{35}S]TBPS binding. With 5 μM GABA using the total particulate membrane preparation. Data presented is from a representative experiment. N=3.

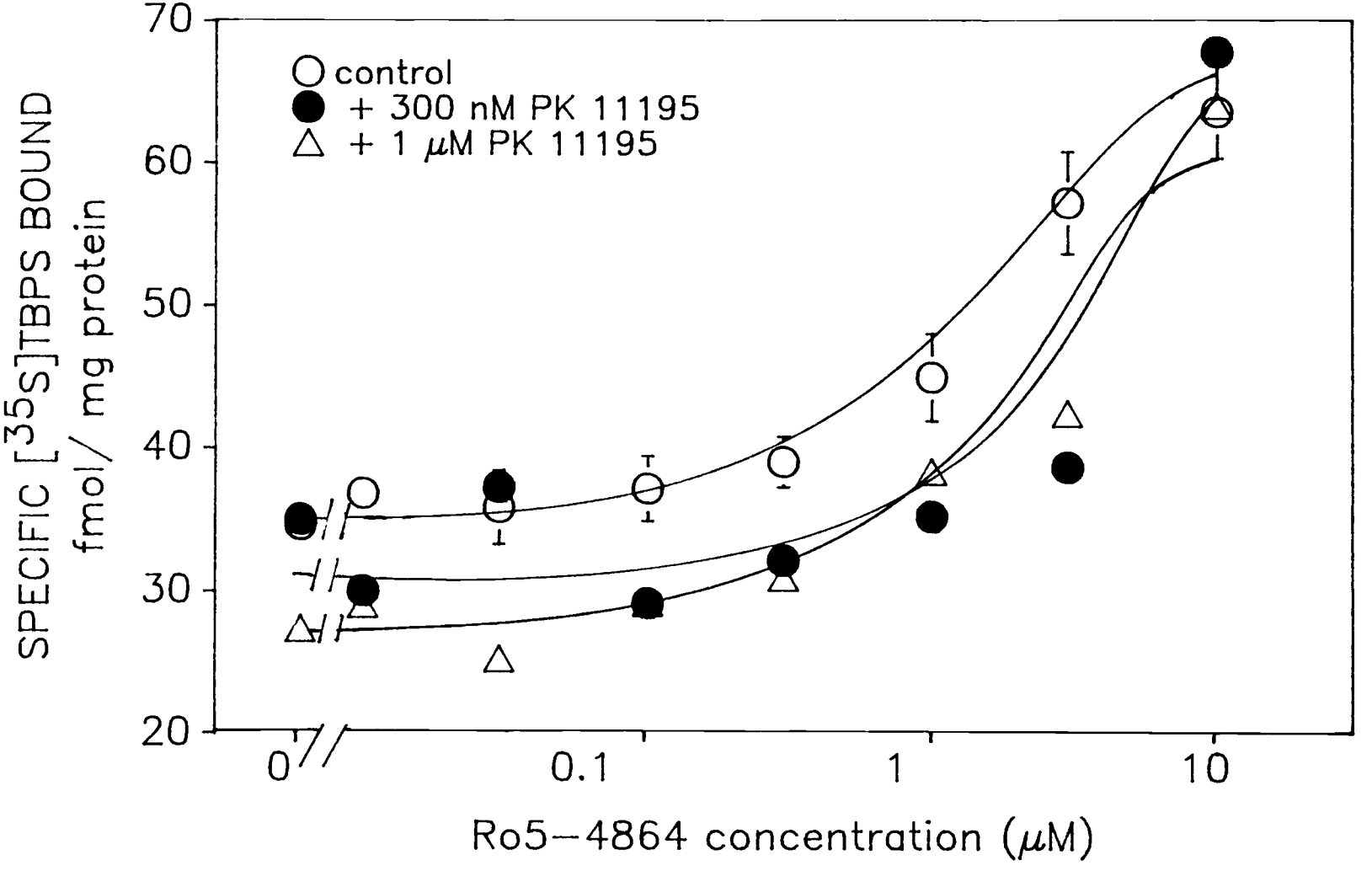

control
+ 300 nM PK 11195
+ 1 μM PK 11195
SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
70
60
50
40
30
20
0
0.1
1
10
Ro5-4864 concentration (μM)

FIGURE 31

Figure 32. Ro5-4864 modulation of the GABA dose-dependent inhibition of [^{35}S]TBPS binding. In the total particulate membrane preparation with 5 μM GABA. Data presented is from a representative experiment. N=3.

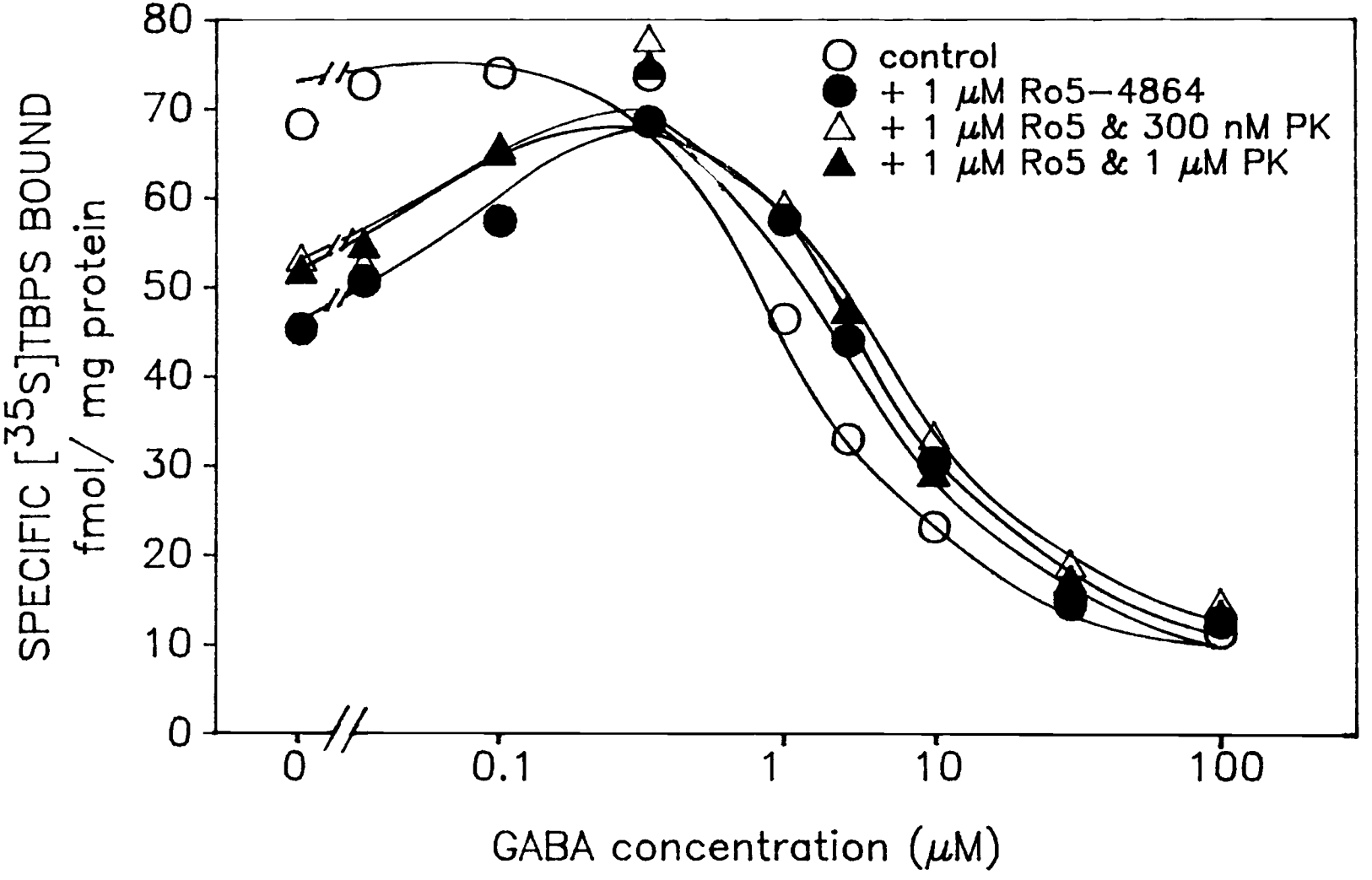

SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
GABA concentration (μM)
control
+ 1 μM Ro5–4864
+ 1 μM Ro5 & 300 nM PK
+ 1 μM Ro5 & 1 μM PK

FIGURE 32

Figure 33. Deltamethrin antagonism of Ro5-4864-induced enhancement of [^{35}S]TBPS binding. In the P_2 pellet membrane preparation. N=3.

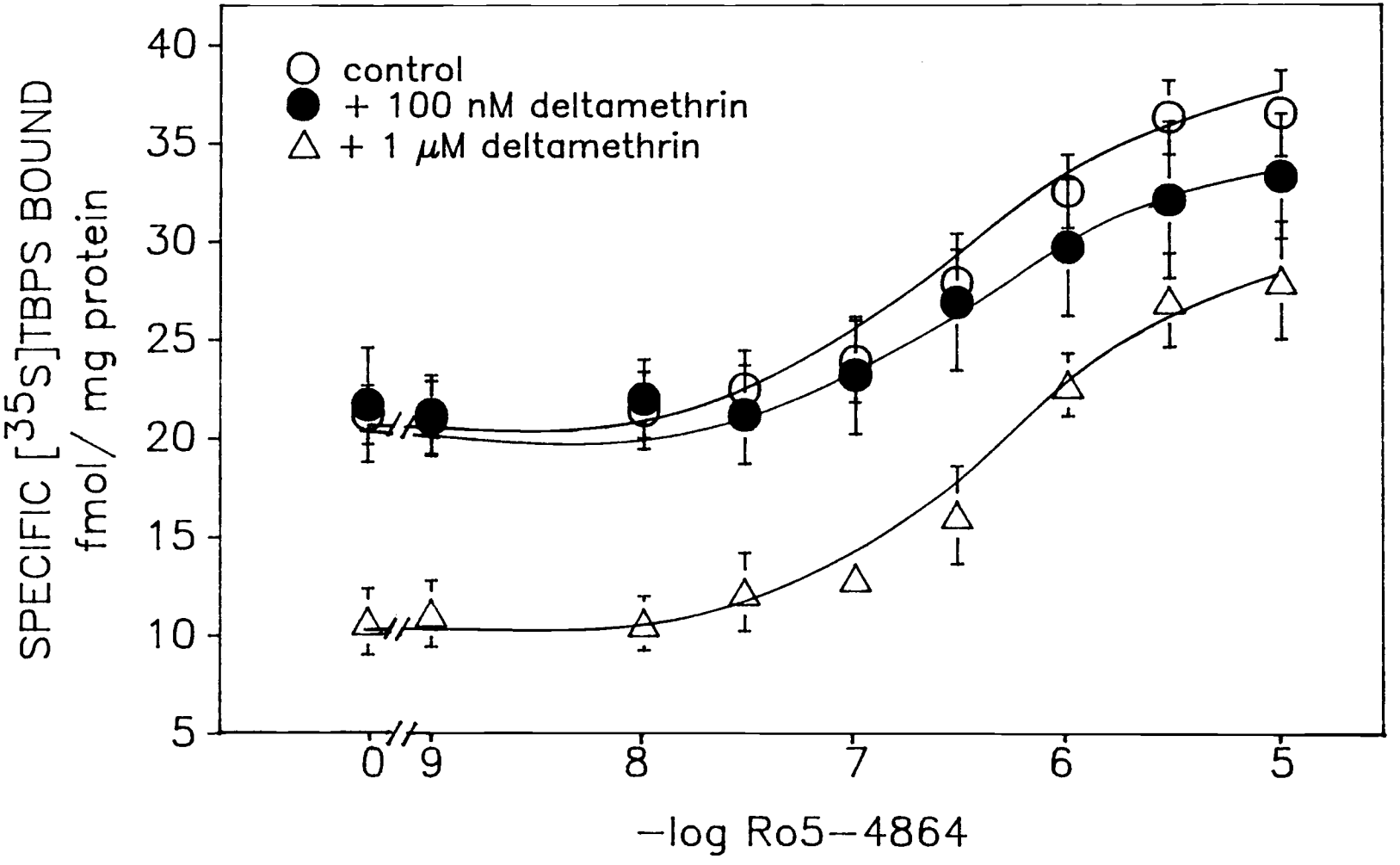

control
+ 100 nM deltamethrin
+ 1 μM deltamethrin
SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
40
35
30
25
20
15
10
5
0
9
8
7
6
5
−log Ro5−4864

FIGURE 33

Figure 34. GABA-dependency of the deltamethrin-induced inhibition of [^{35}S]TBPS binding. In the total particulate membrane preparation. 0 μM GABA (○), 2.5 μM GABA (●), 5 μM GABA (△), and 10 μM GABA(▲).

FIGURE 34

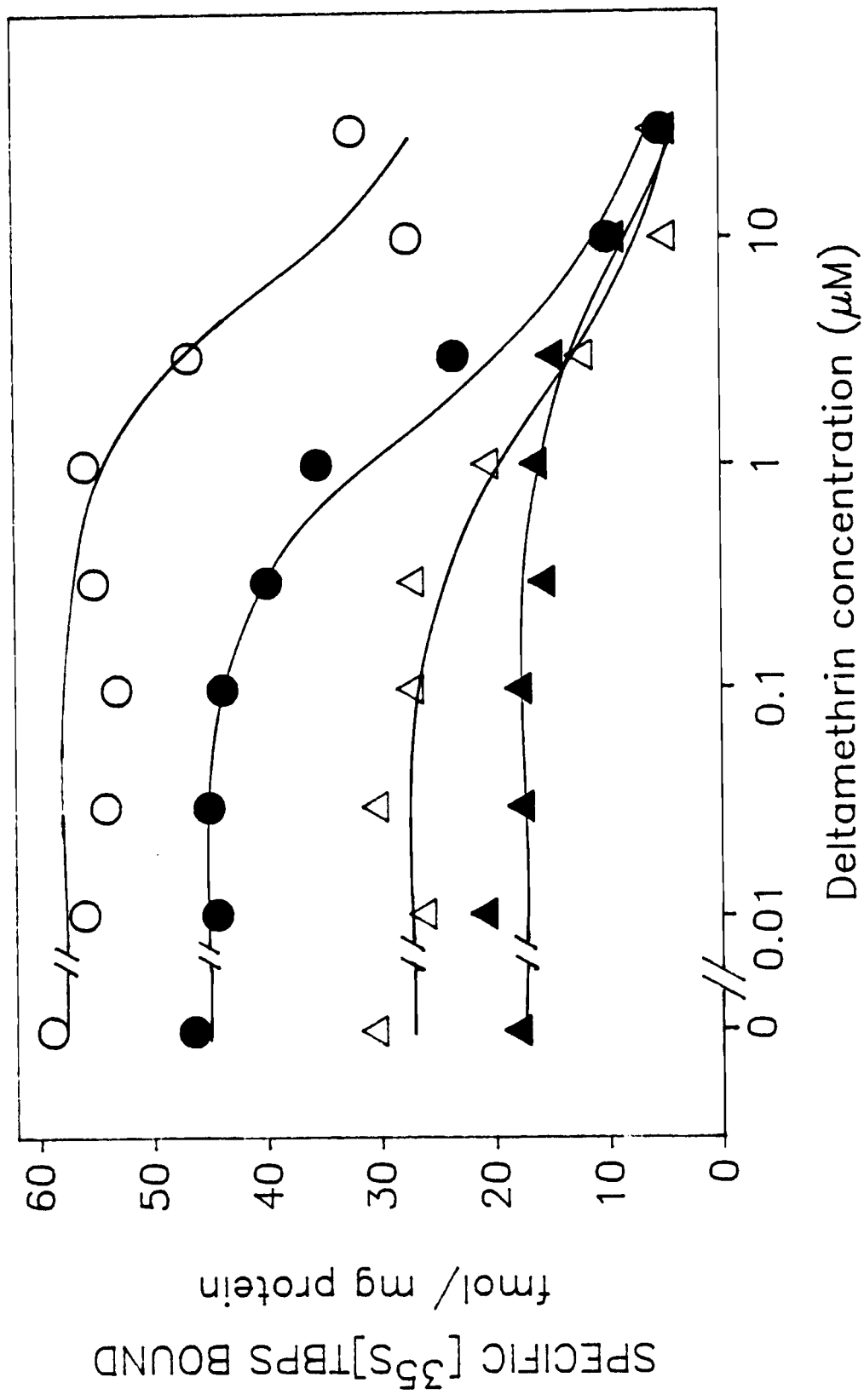

SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
0
10
20
30
40
50
60
0
0.01
0.1
1
10
Deltamethrin concentration (μM)

Figure 35. Deltamethrin-induced enhancement of the inhibition of [^{35}S]TBPS binding by GABA. Using the total particulate membrane preparation.

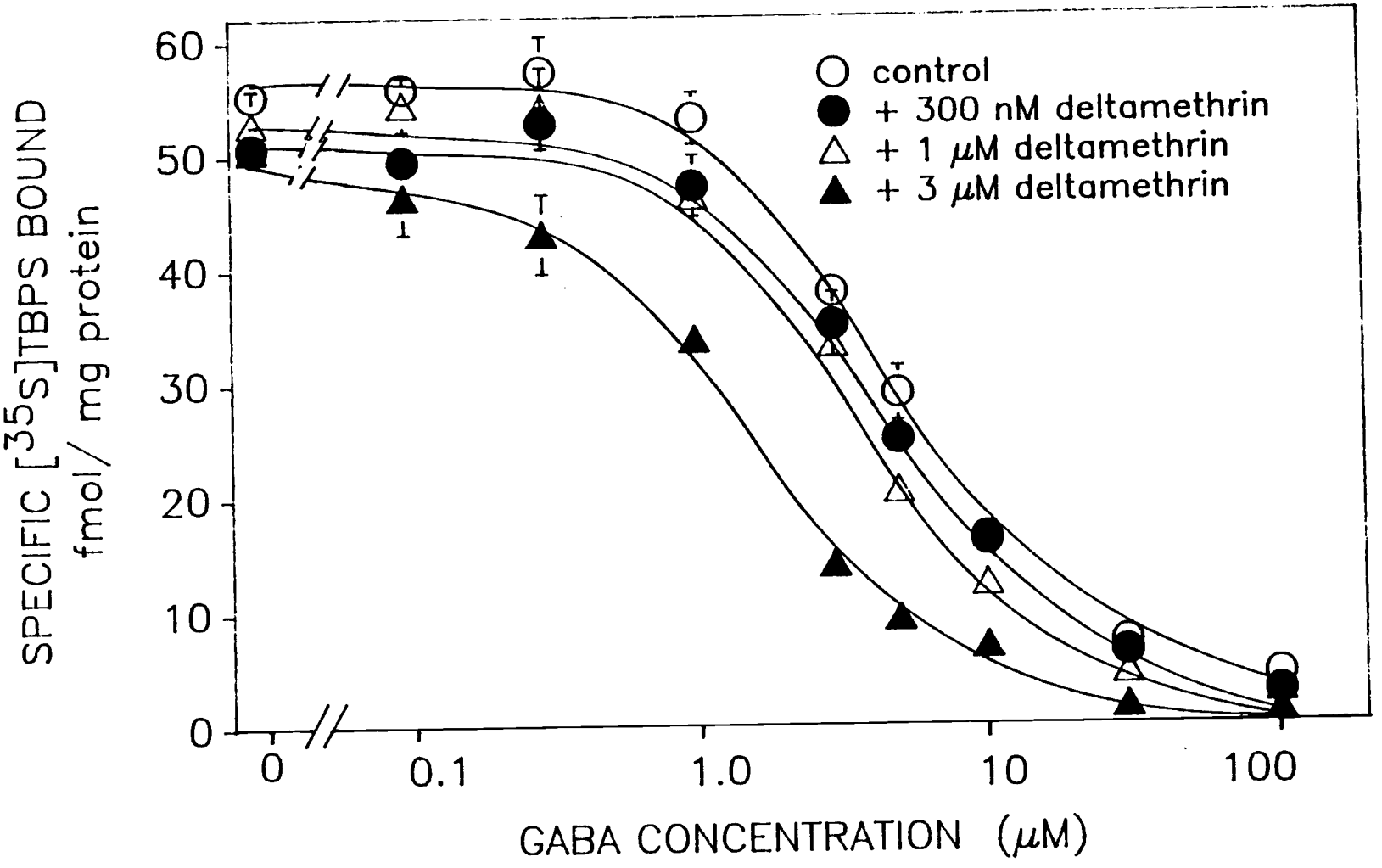
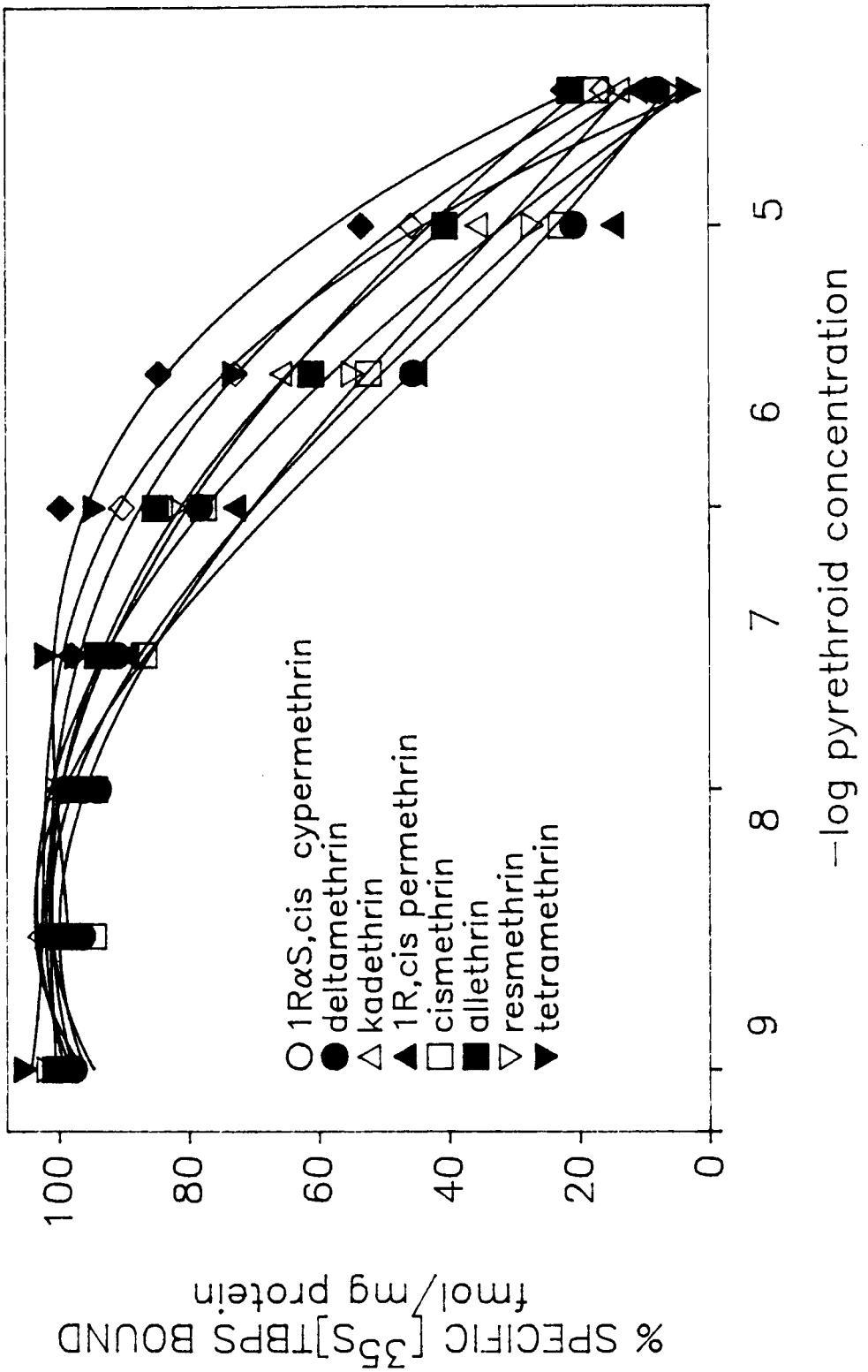

SPECIFIC [35S]TBPS BOUND
fmol/ mg protein
60
50
40
30
20
10
0
0
0.1
1.0
10
100
GABA CONCENTRATION (μM)
control
+ 300 nM deltamethrin
+ 1 μM deltamethrin
+ 3 μM deltamethrin

FIGURE 35

Figure 36. Pyrethroid inhibition of specific [^{35}S]TBPS binding to rat brain membranes. Data presented was obatined using the P_2 pellet membrane preparation with 5 μM GABA. N=3. A similar grouping of pyrethroids was also present when measure in the total particulate membrane preparation. Pyrethroids: 1RαS,<u>cis</u> cypermethrin (○), deltamethrin (●), kadethrin (△), 1R,<u>cis</u> permethrin (▲), cismethrin (◖), allethrin (◯), tetramethrin (▽), resmethrin (▼), and1SαR,<u>cis</u> cypermethrin(◆).

FIGURE 36


% SPECIFIC [35S]TBPS BOUND
fmol/mg protein
100
80
60
40
20
0
9
8
7
6
5
−log pyrethroid concentration
1RαS,cis cypermethrin
deltamethrin
kadethrin
1R,cis permethrin
cismethrin
allethrin
resmethrin
tetramethrin

Chapter 6 CONCLUSION

The results of these investigations have shown the importance of $GABA_A$ receptors and Ro5-4864 binding sites as loci of action for pyrethroid insecticides. With the increased use of pyrethroid insecticides and the development of newer, more potent and persistent pyrethroids, there has been a more immediate need to elucidate the precise mechanisms of action of pyrethroids and to discern any undesirable effects on non-target species.

While much research has focused on the neurotoxic effects of pyrethroids, there had been no investigations into the acute effects of acute, low doses of these compounds. Chapters 2 and 3 detail the potent proconvulsant actions of pyrethroid insecticides in rats using a sensitive measure of seizure susceptibility. It has also been shown that PK 11195, a ligand purported to be selective at the peripheral-type benzodiazepine receptor, blocked the proconvulsant activity of both Type I and Type II compounds. In addition, it was demonstrated (Figure 38) that PK 11195 antagonizes the neurotoxic symptomology elicited by a moderate dose of the Type II pyrethroid, deltamethrin.

[^{3}H]Ro5-4864 and [^{35}S]TBPS were utilized as probes in an attempt to discern the specific receptor interactions involved with the proconvulsant activity of pyrethroid insecticides. Pyrethroid

insecticides inhibited binding to both the peripheral-type benzodiazepine receptor (Chapter 3) and to the picrotoxinin site of the $GABA_A$ receptor (Chapter 5). However, there was a much better correlation between the observed proconvulsant activity of pyrethroids and their potencies as inhibitors of binding to the PTBR than to the [^{35}S]TBPS site.

At this time the question has not been answered as to how interactions with the PTBR are translated physiologically into the proconvulsant activity displayed by pyrethroid insecticides and Ro5-4864. Recent evidence implicates a localization of the PTBR to the outer mitochondrial membrane and a high affinity of protopohyrins for this site (Anholt et al, 1986; Verma et al, 1987). It is difficult to reconcile these findings with the physiological manifestation of proconvulsant activity displayed by pyrethroids and Ro5-4864.

Ro5-4864 has recently been suggested to interact with the $GABA_A$ at a site distinct from the classical benzodiazepine binding site. The present investigation measuring the interactions of pyrethroids and ligands for the PTBR with the [^{35}S]TBPS binding site (Chapter 5) supports these findings and also raises the possibility of relevant pyrethroid interactions with an allosteric site on the $GABA_A$ receptor.

Investigations into the functional significance of interactions of pyrethroids and Ro5-4864 with the $GABA_A$ receptor used the ^{36}chloride influx methodology (Chapter 4). The results from this study showed that pyrethroids do indeed exert potent effects at the $GABA_A$ receptor and that PK 11195 antagonizes this interaction. This

investigation has also raised questions about the significance of membrane potential on the modulation of the $GABA_A$ receptor as both Type II pyrethroids and veratridine, a potent sodium channel activator, increased chloride influx in the absence of added GABA. Moreover, modulation of both GABA-dependent and GABA-independent chloride influx were prevented by the presence of tetrodotoxin. Akaike and coworkers (1986) have reported that at low concentrations of GABA, the kinetics of activation show a voltage dependency. Additional investigations need to be undertaken to distinguish the nature of this effect and the specificity of the antagonism exerted by PK 11195.

The very important and complex nature of the modulation of the $GABA_A$ receptor complex was evident in the results determined in both the ^{36}chloride flux assay (Chapter 4) and the [^{35}S]TBPS binding study (Chapter 5). GABA exerts a very powerful and diverse control on ligand interactions with the $GABA_A$ receptor, even to influencing the direction of effect. The insights gained from this series of investigations are a step forward in understanding the complex regulation of the $GABA_A$ receptor and will be useful for designing future investigations into the interactions of compounds with the $GABA_A$ receptor.

CHAPTER 7 REFERENCES

Abalis, I.M., Eldefrawi, M.E. and Eldefrawi, A.T.: Effects of insecticides on GABA-induced chloride influx into rat brain microsacs, J. Toxicol. Environ. Hlth., 18: 13-23 (1986).

Abbasy, M.A., Eldefrawi, M.E. and Eldefrawi, A.T.: Allethrin interactions with the nicotinic acetylcholine receptor channel, Life Sci., 31: 1547-1552 (1982).

Akaike, N., Inoue, M. and Krishtal, O.A.: "Concentration-clamp" study of γ-aminobutyric acid-induced chloride current kinetics in frog sensory neurones, J. Physiol., 379: 171-185 (1986).

Allan, A.M. and Harris, R.A.: γ-aminobutyric acid agonists and antagonists alter chloride flux across brain membranes, Mol. Pharm., 29: 497-505 (1986).

Anholt, R., Pederson, P., DeSouza, E.B., and Snyder, S.H.: The peripheral-type benzodiazepine receptor localization to the mitochondiral outer membrane, J. Biol. Chem., 261: 576-583 (1986).

Arunlakshana, O. and Schild, H.O.: Some quantitative uses of drug antagonists, Br. J. Pharm., 14:48-58 (1959).

Barnard, E.A., Darlison, M.G. and Seeburg, P.: Molecular biology of the $GABA_A$ receptor: the receptor/channel superfamily, Trends Neuro., 10: 502-509 (1987).

Bloomquist, J.R., Adams, P.M. and Soderlund, D.M.: Inhibition of γ-aminobutryic acid-stimulated chloride flux in mouse brain vesicles by polychlorocycloalkane and pyrethroid insecticides, Neurotoxicol., 7: 11-20 (1985).

Benavides, J., Guilloux, F, Allam, D., Uzan, A., Mizoule, J., Renault, C., Dubroeucq, M., Gueremy, C., and LeFur, G: Opposite effects of an agonist, Ro5-4864, and an antagonist, PK 11195, of the peripheral type benzodiazepine binding sites on audiogenic seizures in DBA/2J mice, Life Sci., 34:2613-2620 (1984).

Bolger, G.T., Mezey, E., Cott, J., Weissman, B.A., Paul, S.M. and Skolnick, P: Differential regulation of 'central' and 'peripheral' benzodiazepine binding sites in the rat olfactory bulb, Eur. J. Pharmacol., 105:143-148 (1984).

Braestrup, C. and Squires, R.F.: Specific benzodiazepine receptors

in rat brain characterized by high affinity [^{3}H]diazepam binding, Proc. Nat'l. Acad. Sci., USA, 74:3805-3809 (1977).

Casida, J.E.: Pyrethrum flowers and pyrethroid insecticides, Environ. Hlth. Persp., 34: 189-202 (1980)

Casida, J.E., Gammon, D.W., Glickman, A.H. and Lawrence, L.J.: Mechanisms of selective actions of pyrethroid insecticides, Ann. Rev. Pharmacol. Toxicol., 23:413-438 (1983).

Casida, J.E. and Lawrence, L.J.: Structure-activity correlations for interactions of bicyclophosphorus esters and some polychlorocycloalkaane and pyrethroid insecticides with the brain specific t-butylbicyclophosphorothionate receptor, Environ. Hlth. Persp., 61: 123-132 (1985).

Cole, L.M. and Casida, J.E.: Pyrethroid toxicology in the frog, Pest. Biochem. Physiol., 20: 217-224 (1983).

Creveling, C.R., McNeal, E.T., McCulloh, D.H., and Daly, J.W.: Membrane potentials in cell-free preparations from guinea pig cerebral cortex: effect of depolarizing agents and cyclic nucleotides, J. Neurochem., 35: 922-932 (1980).

Crofton, K.M., Reiter, L.W., and Mailman, R.B.: Pyrethroid insecticides and radioligand displacement from the GABA receptor chloride ionophore complex, Toxicol. Lett., 35:183-90 (1987).

Davies, J.H.: The pyrethroids: an historical introduction, in The Pyrethroid Insecticides ed. by J.P. Leakey, Taylor & Francis, Ltd., pp 1-31 (1985).

Devaud, L.L. and Murray, T.F.: Involvement of peripheral-type benzodiazepine receptors in the proconvulsant actions of pyrethroid insecticides, J. Pharmacol. Exp. Ther., 247: in press (1988).

Devaud, L.L., Szot, P. and Murray, T.F.: Antagonism of pyrethroid-induced proconvulsant activity, Eur. J. Pharmacol., 120:269-273 (1986).

Eldefrawi, M.E., Sherby, S.M., Abalis, I.M. and Eldefrawi, A.T.: Interactions of pyrethroids and cyclodiene insecticides with nicotinic acetylcholine and GABA receptors, Neurotoxicol., 6:47-52 (1985).

Eldefrawi, A.T. and Eldefrawi, M.E.: Receptors for -aminobutyric acid and voltage-dependent chloride channels as targets for drugs and toxicants, FASEB J., 1: 262-271 (1987)

Elliot, M., Janes, N.F. and Potter, C.: The future of pyrethroids in insect control, Ann. Rev. Entomol., 23: 443-469 (1978)

Elliot, M.: Established pyrethroid insecticides, Pestic. Sci., 11: 119-128 (1980).

File, S., Green, A.R., Nutt, D.J. and Vincent, N.D.: On the convulsant action of Ro5-4864 and the existence of a micromolar benzodiazepine binding site in rat brain, Psychopharmacology, 82:199-202 (1984).

Gammon, D.W., Brown, M.A. and Casida, J.E.: Two classes of pyrethroid action in the cockroach, Pest. Biochem. Physiol., 15:181-191 (1981).

Gammon, D.W., Lawrence, L.J. and Casida, J.E.: Pyrethroid toxicology: protective effects of diazepam and phenobarbital in the mouse and the cockroach, Tox. Appl. Pharmacol., 66:290-296 (1982).

Gammon, D.W. and Sander, G.: Two mechanisms of pyrethroid action: electrophysiological and pharmacological evidence, Neurotoxicol., 6:63-86 (1985).

Gardner, C.R., Klein, J. and Grove, J.: Endogenous GABA determines the characteristics of [^{3}H]GABA-binding, Europ. J. Pharmacol., 75: 83-92 (1981).

Gee, K.W.: Phenylquinolines PK 8165 and PK 9084 allosterically modulate [^{35}]t-butylbicyclophosphorothionate binding to a chloride ionophore in rat brain via a novel Ro5-4864 binding site, J. Pharmacol. Exp. Ther., 240:747-753 (1987).

Gee, K.G., Lawrence, L.J. and Yamamura, H.I.: Modulation of the chloride ionophore by benzodiazepine receptor ligands: influence of γ-aminobutyric acid and ligand efficacy, Mol. Pharmacol., 30: 218-225 (1986).

Gee, K. W., Brinton, R.E. and McEwen, B.S.: Regional distribution of a Ro5-4864 binding site that is functionally coupled to the γ-aminobutyric acid/benzodiazepine receptor complex in rat brain, J. Pharmacol. Exp. Ther., 244: 379-383 (1988).

Ghiasuddin, S.M. and Soderlund, D.M.: Pyrethroid insecticides: potent, sterospecific enhancers of mouse brain sodium channel activation, Pest. Biochem. Physiol., 24: 200-206 (1985).

Hamill, O.P., Bormann, J. and Sakmann, B.: Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA, Nature, 305: 805-808 (1983).

Harris, R.A. and Allan, A.M.: Functional coupling of γ-aminobutyric acid receptors to chloride channels in brain membranes, Science, 228: 1108-1110 (1985).

Hayes, W.: Pesticides and human toxicity, Ann. N.Y. Acad. Sci., 160:

40-54 (1971).

Holford, N.: MK MODEL PLUS An extended least squares modelling program, Dep. Pharmacol. Clin. Pharmacol., University of Auckland, School of Medicine, Auckland, New Zealand (1983)

Hoyer, D., Reynolds, E.E., Molinoff, P.B.: Agonist-induced changes in the properties of beta-adrenergic receptors on intact S49 lymphoma cells. Time-dependent changes in the affinity of the receptor for agonists. Mol. Pharmacol. 25:209-218 (1984).

Johnson, M.K.: The target for initiation of delayed neurotxicity by organophosphorus esters: biochemical studies and toxicological applications, Rev. Biochem. Toxicol., 4: 141-212 (1982).

Lawrence, L.J. and Casida, J.E.: Pyrethroid toxicology: mouse intracerebral structure-toxicity relationships, Pest. Biochem. Physiol., 18:9-14 (1982).

Lawrence, L.J., and Casida, J.E.: Stereospecific action of pyrethroid insecticides on the γ-aminobutyric acid receptor-ionophore complex, Science, 221:1399-1401 (1983).

Lawrence, L.J., Gee, K.W. and Yamamura, H.I.: Interactions of pyrethroid insecticides with chloride-ionophore associated binding sites, Neurotoxicol, 6:87-94 (1985).

Leeb-Lundberg, F. and Olsen, R.W.: Picrotoxinin binding as a probe of the GABA postsynaptic membrane receptor-ionophore complex. In Psychopharmacology and Biochemistry of Neurotransmitter Receptors , Yamamura, H.I., Olsen, R.W. and Usdin, E., Ed.s, Elsevier, New York, pp 593-606 (1980).

LeFur, G., Perrier, M.L., Vaucher, N., Imbault, F., Flamier, A., Benavides, J., Uzan, A., Renault, C., Dubroeucq, M.C. and Gueremy, C.: Peripheral benzodiazepine binding sites: Effect of PK 11195, 1-(2-chlorphenyl)-N-methyl-N-1-methylpropyl)-3-isoquinolinecarboxamide, I. in vitro studies, Life Sci. 32:1839-1847 (1983).

LeFur, G.F., Guilloux, F., Rufat, P., Benavides, J., Uzan, A., Renault, C., Dubroeuq, M.C. and Gueremy, C: Peripheral benzodiazepine binding sites: Effect of PK 11195, 1-(2-chlorophenyl)-N-methyl-(1-methylpropyl)-3 isoquinolinecarboxamide. II In vivo studies, Life Sci. 32: 1849-1953 (1983)

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.A.: Protein measurement with the folin phenol reagent, J. Biol. Chem., 193:265-275 (1951).

Lummis, S., Chow, S. Holan, G. and Johnston, G.: γ-aminobutyric acid receptor ionophore complexes: differential effects of deltamethrin, dichlorophenyltrichloroethane and some novel insecticides in a rat

brain membrane preparation, J. Neurochem., 48:689-694 (1987).

Lund, A.E. and Narahashi, J.: Kinetics of sodium channel modification as the basis for the variation in the nerve membrane effects of pyrethroids and DDT analogs, Pest. Biochem. Physiol., 20:203-216 (1983).

Lundeen, J.E. and Gordon, J.H.: Computer analysis of binding data: In O'Brien B. (ed) Receptor binding in drug research. Marcel Dekker, New York, pp. 31-49 (1985).

Marangos, P.J., Patel, J., Boulenger, J.P. and Clark-Rosenburg, R.: Characterization of peripheral-type benzodiazepine binding sites in brain using [^{3}H]Ro5-4864, Mol. Pharmacol., 22:26-32 (1982).

Mohler, H. and Okada, T.: Benzodiazepine receptors: demonstration in the central nervous system, Science, 198:849-851 (1977).

Munson, P.J. and Robard, D.: LIGAND: a versatile computerized approach for characterization of ligand binding systems, Analyt. Biochem., 107:220-239 (1980).

Murray, T.F., Sylvester, D., Schultz, C.S. and Szot, P.: Purinergic modulation of the seizure threshold for pentylentetrazol in the rat, Neuropharmacol., 24:761-766 (1985).

Narahashi, T: Mode of action of pyrethroids, Bull Wld Hlth Org., 44:337-345 (1971).

Narahashi, T: Neurophysiological study of pyrethroids: molecular and membrane mechanism of action, Pesticide Chemistry: Human Welfare and the Environment, Vol. 2, Natural Products, Ed. by Miyamoto, J. and Kearney, P.C., Pergamon Press, Oxford, pp 109-114 (1983)

Nutt, D.J., Cowen, P.J., and Green, A.R.: On the measurement in rats of the convulsant effects of drugs and the changes which follow electroconvulsive shock, Neuropharmacol., 19:1017-1023 (1980).

Nutt, D.J., Cowen, P.J., and Green, A.R.: Studies on the post-ictal rise in seizure threshold, Eur. J. Pharmacol., 71:287-295 (1981).

O'Brien, R.D.: Insecticides, Action and Metabolism, Academic Press, Inc., N.Y. (1967).

Olsen, R.W. and Leeb-Lundberg, F.: Convulsant and anticonvulsant drug binding sites related to GABA-regulated chloride ion channels, Adv. Biochem. Psychopharmacol., 26: 93-102 (1981).

Richards, J.G. and Mohler, H.: Benzodiazepine receptors, Neuropharmacol., 23:233-242 (1984).

Schofield, P.R., Darlison, M.G., Fujita, N., Burt, D.R., Stephenson,

F.A., Rodrigues, H., Rhee, L.M., Ramachandran, J., Reale, V., Clencourse, T.A., Seeburg, P.H., and Barnard, E.A.: Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family, Nature, 328: 221-227 (1987).

Schwartz, R.D. and Mindlin, M.: Inhibition of the GABA receptor-gated chloride ion channel in brain by noncompetitive inhibitors of the nicotinic receptor-gated cation channel, J. of Pharmacol. Exp. Ther., 244: 963-970 (1988).

Schoemaker, H., Boles, R., Horst, W.D. and Yamamura, H.I.: Specific high-affinity binding sites for [^{3}H]Ro5-4864 in rat brain and kidney, J. Pharmacol. Exp. Ther., 225:61-69 (1983).

Seifert, J. and Casida, J.E.: Regulation of [^{35}S]t-butylbicyclo-phosphorothionate binding sties in rat brain by GABA, pyrethroid and barbiturate, Eur. J. Pharmacol., 115:191-198 (1985a).

Seifert, J. and Casida, J.E.: Solubilization and detergent effects on interactions of some drugs and insecticides with the t-butylbicyclophosphorothionate binding site with the γ-aminobutyric acid receptor-ionophore complex, J. Neurochem., 44: 110-116 (1985b).

Snyder, S.H., Verma, A., and Trifiletti, R.R.: The peripheral-type benzodiazepine receptor: a protein of mitochondrial outer membranes utilizing porphyrins as endogenous ligands, FASEB J., 1: 282-288 (1987).

Sokol, R.R. and Rohlf, F.J.: Kendall's coefficient of rank correlation, Biometry, pp. 533-535 (1969).

Squires, R.F., Casida, J.E., Richardson, M. and Saederup, E.: [^{35}S]t-butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ-aminobutyric acid-A and ion recognition sites, Mol. Pharmacol., 23: 326-336 (1983).

Squires, R.F. and Saederup, E.: $GABA_A$ receptor blockers reverse the inhibitory effects of GABA on brain specific [^{35}S]TBPS binding, Brain Res., 414: 357-364 (1987).

Staatz, C., Bloom, A.S. and Lech, J.L.: A pharmacological study of pyrethroid neurotoxicity in mice, Pest. Biochem. Physiol., 17:287-292 (1982).

Tamkun, M.M. and Catterall, W.A.: Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles, Mol. Pharmacol., 19: 78-86 (1981).

Tjcku, M.K., Ban, M. and Olsen, R.W.: Binding of [^{3}H]dihydropicrotoxinin, a γ-aminobutyric acid synaptic antagonist, to rat brain membranes, Mo. Pharmacol., 14: 391-402 (1978).

Ticku, M.K. and Olsen, R.W.: Cage convulsants inhibit picrotoxinin binding, Neuroppharmacol., 18: 315-318 (1979).

Ticku, M.K. and Ramanjaneyulu, R: Ro5-4864 inhibits the binding of [^{35}S]t-butylbicyclophosphorothionate to rat brain membranes, Life Sci. 34:631-638 (1984).

Verma, A., Nye, J.S., and Snyder, S.H.: Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor, Proc. Natl. Acad. Sci., 84: 2256-2260 (1987).

Verschoyle, R.D. and Aldridge, W.N.: Structure-activity relationships of some pyrethroids in rats, Arch. Toxicol., 45:325-329 (1980).

Vijverberg, H.P., van der Zalm, J.M. and van den Bercken, J.: Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves, Nature, 295: 601-603 (1982)

Vijverberg, H. P., and van den Bercken, J.: Action of pyrethroid insecticides on the vertebrate nervous system, Neuropath. Appl. Neurobiol., 8: 421-440 (1982).

Weissman, B.A., Cott, J., Hommer, D., Quirion, R., Paul, S. and Skolnick, P: Pharmacological, electrophysiological and neurochemical actions of the convulsant benzodiazepine Ro5-4864 (4'chlor diazepam), in: Benzodiazepine Recognition Site Ligands: Biochemistry and Pharmacology, ed. by Biggio and E. Costa, pp 139-150 (1983).

Weissman, B.A., Cott, J., Jackson, J.A., Bolger, G.T., Weber, K.H., Horst, W.D., Paul, S.M. and Skolnick, P.: "Peripheral-type" binding sites for benzodiazepines in brain: relationship to the convulsant actions of Ro5-4864, J. Neurochem., 44:1494-1499 (1985).

Wong, D.T., Threlkeld, P.G., Bymaster, F.P. and Squires, R.F.: Saturable binding of ^{35}S-t-butylbicyclophosphorothionate to the sites linked to the GABA receptor and the interaction with GABAergic agents, Life Sci., 34: 853-860 (1984).

Wood, P.L., Loo, P., Braunwalder, A., Yokoyama, N. and Cheney, D.L.: In vitro characterization of benzodiazepine receptor agonists, antagonists, inverse agonists and agonist/antagonists, J. Pharmacol. Exp. Ther., 231: 572-575 (1984).