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A COMPARATIVE STUDY OF AVERMECTIN B_{1a} AND OTHER MODULATORS OF THE γ-AMINOBUTYRIC ACID RECEPTOR·CHLORIDE ION CHANNEL COMPLEX

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Abstract

The interactions of the anthelmintic agent avermectin B_{1a}, the anticonvulsant pentobarbital, and the anxiolytic tracazolate with the γ-aminobutyric acid (GABA) receptor·chloride ion channel complex in rat brain membrane were studied. The results indicated that they all potentiated ligand binding to the GABA and benzodiazepine receptors. The stimulatory effects of avermectin B_{1a} and pentobarbital, but not tracazolate, on GABA receptor binding were inhibited by picrotoxin. The effect of avermectin B_{1a} was not additive with those of tracazolate and pentobarbital. On the other hand, the stimulatory effect of GABA on benzodiazepine binding was additive with those of avermectin B_{1a} and pentobarbital, but tracazolate and pentobarbital inhibited the effect of avermectin B_{1a}. In receptor heat inactivation experiments, avermectin B_{1a} and clonazepam protected GABA receptors, whereas avermectin B_{1a} and GABA protected benzodiazepine receptors. Tracazolate, pentobarbital, and picrotoxin did not protect either receptor. These findings suggest that the recognition sites for the benzodiazepines, avermectin B_{1a}, tracazolate, pentobarbital, and picrotoxin are coupled allosterically to the GABA receptor·chloride ion channel complex in different ways. The binding site for avermectin B_{1a} may be partially shared by picrotoxin, pentobarbital, and tracazolate.

γ-Aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the mammalian central nervous system and in the invertebrate peripheral nervous system (Krnjevic, 1974; Takeuchi, 1976). Evidence accumulated from studies of GABAergic function in the mammalian central nervous system indicates that compounds which activate the GABA receptor·chloride ion channel complex have the potential to be developed as useful drugs for treating anxiety, convulsion, epilepsy, and a variety of CNS disorders (Meldrum, 1978; Enna, 1980). This hypothesis has gained support recently since several classes of anxiolytic compounds have been shown to interact with the GABA receptor complex. This complex is believed to consist of multiple recognition sites, including the binding sites for GABA and the benzodiazepines (Enna and Snyder, 1975; Squires and Braestrup, 1977; Möhler and Okada, 1977; Tallman et al., 1979). Avermectin B_{1a}, an anthelmintic (Egerton et al., 1979) and insecticidal agent (Ostlind et al., 1979), has been shown to affect the GABAergic nervous system of invertebrates (Fritz et al., 1979; Kass et al., 1980) and to potentiate the muscle relaxant effect of diazepam in mice (Williams and Yarbrough, 1979). It enhances the receptor binding of the benzodiazepines (Williams and Yarbrough, 1979; Pong, 1980; Pong et al., 1981) and GABA (Pong and Wang, 1980, 1982). The anesthetic barbiturates have been reported recently to enhance GABA binding (Willow and Johnston, 1981; Olsen, 1981) and to potentiate GABA-enhanced benzodiazepine binding (Skolnick et al., 1980). Another class of anxiolytic compounds, the pyrazolopyridines such as tracazolate, also has been shown to potentiate GABA binding (Placheta and Karobath, 1980; Meiners and Salama, 1980) as well as benzodiazepine binding (Beer et al., 1978; Williams and Risley, 1979; Salama and Meiners, 1980; Supavilai and Karobath, 1981). Furthermore, the convulsant picrotoxin is known to interact with the GABA·benzodiazepine receptor complex (Ticku and Olsen, 1978; Olsen, 1981; Pong and Wang, 1980, 1982). It inhibits the effects of avermectin B_{1a} on lobster neuromuscular junction (Fritz et al., 1979), on *Ascaris* nervous system (Kass et al., 1980), and on GABA binding to rat brain membranes (Pong and Wang, 1982). In the present study, we examined the interactions of avermectin B_{1a}, tracazolate, pentobarbital, and picrotoxin

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with the GABA and benzodiazepine receptors in rat brain membranes to delineate further the mechanism of avermectin B_{1a} modulation of the GABA receptor·chloride ion channel complex.

Materials and Methods

Avermectin B_{1a} and sodium pentobarbital were obtained from the Merck Institute for Therapeutic Research, Rahway, NJ. Tracazolate was obtained from Dr. B. A. Meiners, ICI Americas, Inc., Wilmington, Delaware. [^{3}H]Flunitrazepam (79.3 Ci/mmol) and [^{3}H]GABA (30 Ci/mmol) were purchased from New England Nuclear. Clonazepam was a gift from Hoffmann-LaRoche, Inc. Picrotoxin was obtained from ICN Pharmaceuticals, Inc., Plainview, NY.

Preparation of rat brain synaptic membranes. The brains of the male adult Wistar rats were homogenized with a Teflon homogenizer at 0°C in 15 vol of 0.32 M sucrose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $NaHCO_3$ and centrifuged at 1,500 × *g* for 10 min, and the supernatant was recentrifuged at 17,000 × *g* for 10 min. The pellets were frozen for at least 3 hr, resuspended in 10 mM sodium phosphate buffer, pH 7.4, and centrifuged at 25,000 × *g* for 30 min. These steps were repeated five times and the final pellets were stored at −20°C.

Benzodiazepine receptor binding. The pellets were resuspended in 10 mM sodium phosphate buffer, pH 7.4, and 50-μl aliquots (25 μg of protein) were incubated in triplicate at 0°C with 0.5 nM [^{3}H]flunitrazepam with or without drug in 0.95 ml of 50 mM Tris-Cl, pH 7.4, for 90 min. At the end of the incubation, the membranes were filtered on Whatman GF/B filters and washed two times with 5.0 ml of the ice cold Tris-Cl buffer. The radioactivity retained on the filter was determined in Aquasol 2 (New England Nuclear). Nonspecific binding was estimated in the presence of 10 μM unlabeled clonazepam and was subtracted from total binding to obtain the value for specific binding.

TABLE I

Effects of avermectin B_{1a}, tracazolate, pentobarbital, and clonazepam on [^{3}H]GABA binding to rat brain synaptic membranes

Well washed rat brain membranes were prepared and assayed for [^{3}H]GABA binding as described under "Materials and Methods" using 10 nM [^{3}H]GABA. Data are expressed as the mean ± SD. The experiments were repeated three times in quadruplicate.

Compound	Concentration	Percent Stimulation over Control
	μM	
Avermectin B_{1a}	1	11.6 ± 1.7
	2	34.2 ± 2.7
	7	64.0 ± 3.6
Tracazolate	5	15.4 ± 2.8
	10	32.7 ± 4.3
	33	43.1 ± 6.0
Pentobarbital	200	9.2 ± 2.1
	500	32.7 ± 4.8
Clonazepam	1	1.4 ± 2.1
	10	3.2 ± 2.9

TABLE II

Effects of picrotoxin on the enhancement of [^{3}H]GABA binding by avermectin B_{1a}, tracazolate, and pentobarbital

Well washed rat brain membranes were prepared and assayed for [^{3}H]GABA binding as described under "Materials and Methods." Data are expressed as the mean ± SD. The experiments were repeated three times in quadruplicate in two separate preparations of synaptic membranes.

Treatment	Percent Stimulation over Control Binding
Picrotoxin (100 μM)	1.4 ± 4.1
Avermectin B_{1a} (7 μM)	88.2 ± 5.6
Avermectin B_{1a} + picrotoxin (100 μM)	17.6 ± 6.4
Tracazolate (33 μM)	86.7 ± 8.5
Tracazolate + picrotoxin (100 μM)	76.4 ± 7.3
Pentobarbital (500 μM)	45.6 ± 4.2
Pentobarbital + picrotoxin (100 μM)	4.8 ± 2.7

GABA binding. The pellets were resuspended in 10 mM sodium phosphate buffer, pH 7.4, and 50-μl aliquots (50 μg of protein) were incubated in quadruplicate at 0°C with 10 nM [^{3}H]GABA with or without drug in 0.95 ml of 50 mM Tris-Cl, pH 7.4, for 15 min. The incubation mixture then was filtered rapidly under vacuum through a Whatman GF/B filter and immediately washed with 7 ml of the ice cold Tris buffer. The radioactivity retained on the filter was determined in Aquasol 2. Nonspecific GABA binding was determined in the presence of 10^{-3} M unlabeled GABA and normally contributed about 15 to 25% of the total binding.

Results

Interaction with GABA receptor binding. Avermectin B_{1a} and tracazolate markedly enhanced specific [^{3}H]GABA binding to rat brain synaptic membranes in a dose-dependent manner (Table I). At 7 μM, avermectin B_{1a} increased the binding of [^{3}H]GABA up to 80% over control (average, 55 ± 5%; n = 15). Tracazolate had a somewhat smaller effect than avermectin B_{1a} at a higher concentration (33 μM). Pentobarbital had an even smaller effect at very high concentrations (200 to 500 μM) and it was variable in different membrane preparations (10 to 45%). Clonazepam had no apparent effect on [^{3}H]GABA binding. Picrotoxin did not have any effect on control binding, but it blocked 80% and 91% of the stimulatory effects of avermectin B_{1a} and pentobarbital, respectively (Table II). Under similar conditions, picotoxin inhibited the tracazolate stimulation of [^{3}H]GABA binding by only 9%. This suggests that avermectin B_{1a} and tracazolate may not act via a common site. The combination of avermectin B_{1a} and either tracazolate or pentobarbital, each at the concentrations resulting in maximal stimulation, did not increase GABA binding further, suggesting that avermectin B_{1a} may share, at least partially, the binding sites of pentobarbital and tracazolate (Table III).

Protection of GABA receptor from heat inactivation. To explore the specificity of the interactions of these compounds with GABA receptors, the synaptic mem-

TABLE III

Combination effects of avermectin B_{1a} with pentobarbital and tracazolate on [^{3}H]GABA binding

Well washed rat brain membranes were prepared and assayed for [^{3}H]GABA binding as described under "Materials and Methods" using 10 nM [^{3}H]GABA. Data are expressed as the mean ± SD. The experiment was repeated three times in quadruplicate in two separate preparations of synaptic membranes.

Treatment	Percent Stimulation over Control
Avermectin B_{1a} (7 μM)	53.5 ± 7.5
Pentobarbital (500 μM)	26.4 ± 5.2
Avermectin B_{1a} (7 μM) + pentobarbital (500 μM)	45.3 ± 6.9
Tracazolate (33 μM)	42.8 ± 3.6
Avermectin B_{1a} (7 μM) + tracazolate (33 μM)	54.1 ± 4.6

branes were resuspended and incubated at 60°C in 50 mM Tris-Cl, pH 7.4, for various times in the presence of avermectin B_{1a}, clonazepam, tracazolate, pentobarbital, or picrotoxin, and the specific binding of GABA then was determined at 0°C. In the absence of any drug, GABA binding was decreased to only 15% of the original level after 5 min of incubation at 60°C. Avermectin B_{1a} protected GABA receptors from denaturation (Fig. 1). At the end of 5 min at 60°C, 60 to 70% of the receptors were still capable of binding [^{3}H]GABA. Although clonazepam did not stimulate GABA binding, it nevertheless effectively protected GABA receptors from heat inactivation. In contrast, tracazolate and pentobarbital were not able to protect GABA receptors from heat inactivation despite their stimulatory effects on GABA receptor binding. Picrotoxin was also incapable of protecting GABA receptors.

Interactions with benzodiazepine receptor binding. Avermectin B_{1a} was the most potent compound tested in enhancing [^{3}H]flunitrazepam binding to synaptic membranes. Its EC_{50} value was approximately 50-fold lower than that of GABA and 100- and 1000-fold lower than tracazolate and pentobarbital, respectively. Avermectin B_{1a} also gave the greatest magnitude of enhancement of binding. Its enhancement was 100% more than that by GABA and 500% more than that by tracazolate or pentobarbital (Fig. 2). Picrotoxin did not have any effect on [^{3}H]flunitrazepam binding.

To delineate further the mechanisms of potentiation by these compounds, combination experiments were performed (Table IV). At concentrations which potentiate [^{3}H]flunitrazepam binding maximally, the combination of GABA (100 μM) with avermectin B_{1a} (7 μM) or with pentobarbital (500 μM) were nearly additive but the effect of tracazolate (33 μM) was not consistently additive with that of GABA. However, the effects of pentobarbital and tracazolate were not additive with that of avermectin B_{1a}. In fact, tracazolate inhibited the effect of avermectin B_{1a}. The effects of pentobarbital and tracazolate were not additive.

Protection of benzodiazepine receptor binding from heat inactivation. To study further the interactions of these compounds with benzodiazepine receptors, the synaptic membranes were heated to 60°C for various periods of time in 50 mM Tris-Cl, pH 7.4. The survival of [^{3}H] flunitrazepam binding then was determined at 0°C. In

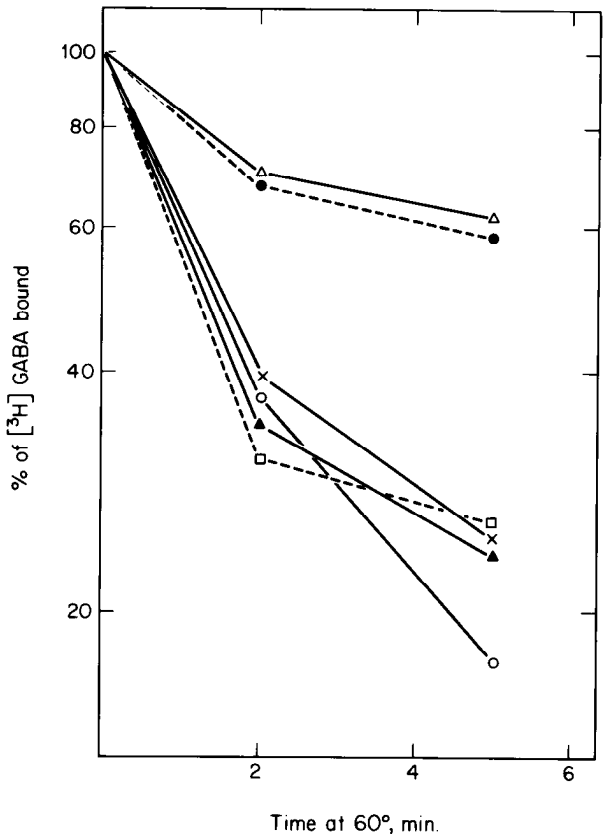

Figure 1. Protection of GABA receptors from heat inactivation by avermectin B_{1a}, clonazepam, tracazolate, picrotoxin, and pentobarbital. Synaptic membranes were incubated at 60°C in the presence or absence of drugs for various periods of time as indicated, and the binding of [^{3}H]GABA to membranes then was determined at 0°C. Control, ○——○; 7 μM avermectin B_{1a}, △——△; 10 μM clonazepam ●- - -●; 500 μM pentobarbital, ×——×; 100 μM picrotoxin, ▲——▲; 33 μM tracazolate, □- - -□.

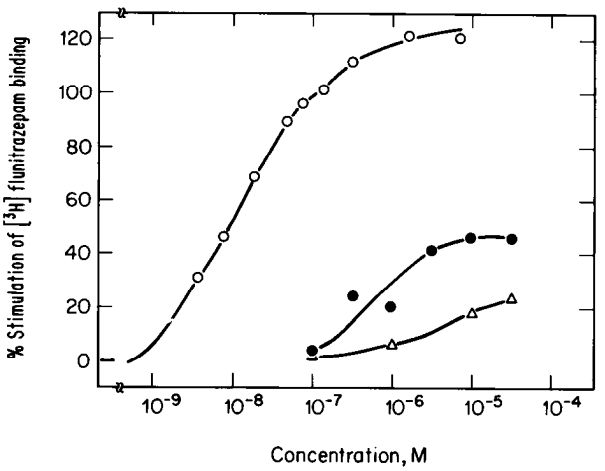

Figure 2. The effect of various concentrations of avermectin B_{1a}, GABA, and tracazolate on specific [^{3}H]flunitrazepam binding. Avermectin B_{1a}, ○——○; GABA, ●——●; tracazolate, △——△.

control samples, benzodiazepine binding was inactivated with a $t_{1/2}$ of 1.9 ± 0.2 min (Fig. 3). The presence of avermectin B_{1a} and GABA protected benzodiazepine receptors from heat inactivation. The $t_{1/2}$ in the presence

TABLE IV

Combination effects of avermectin B_{1a}, GABA, tracazolate, and pentobarbital on specific binding of [^{3}H]flunitrazepam

Well washed rat brain membranes were prepared and assayed for [^{3}H]flunitrazepam binding as described under "Materials and Methods" using 0.5 nM [^{3}H]flunitrazepam. The mean ± SEM for four separate experiments is given. Stimulations were standardized relative to stimulation by avermectin B_{1a} plus GABA which varied from 134.2 to 160.8%.

Compound	Percent Stimulation
GABA (100 μM)	38.8 ± 5.7
Pentobarbital (500 μM)	19.4 ± 3.0
GABA (100 μM) + pentobarbital (500 μM)	62.4 ± 8.0
Avermectin B_{1a} (7 μM)	80.7 ± 2.3
Avermectin B_{1a} (7 μM) + pentobarbital (500 μM)	71.0 ± 11.3
Avermectin B_{1a} (7 μM) + GABA (100 μM)	100
Tracazolate (33 μM)	11.0 ± 5.0
Tracazolate (33 μM) + GABA (100 μM)	42.5 ± 8.3
Tracazolate (33 μM) + avermectin B_{1a} (7 μM)	33.6 ± 11.7
Tracazolate (33 μM) + pentobarbital (500 μM)	20.1 ± 7.5

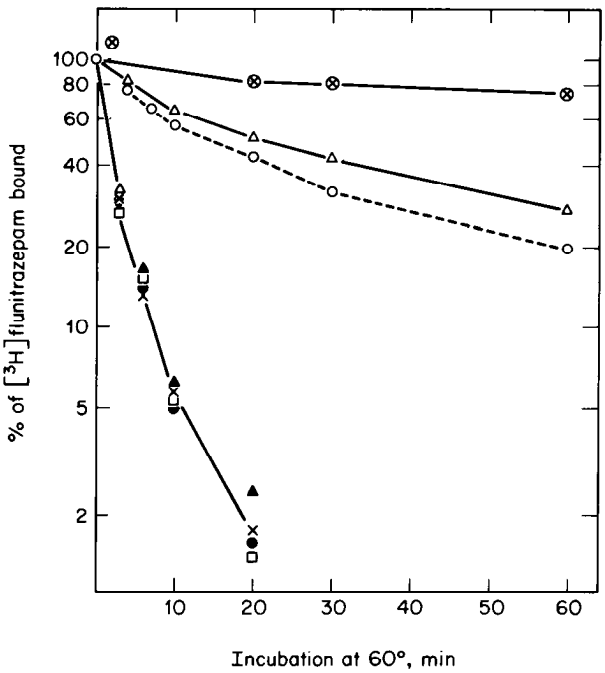

Figure 3. Protection of benzodiazepine receptors from heat inactivation by avermectin B_{1a}, GABA, tracazolate, picrotoxin, and pentobarbital. Synaptic membranes were incubated at 60°C in the presence or absence of drugs for various periods of time as indicated, and [^{3}H]flunitrazepam binding then was determined at 0°C. Control, ●——●; avermectin B_{1a}, △——△; 100 μM GABA, ○- - -○; 500 μM pentobarbital, ▲——▲; 33 μM tracazolate, □——□; 100 μM picrotoxin, ×——×; 7 μM avermectin B_{1a} + 100 μM GABA, ⊗——⊗.

of avermectin B_{1a} was 28.4 ± 6.9 min. The $t_{1/2}$ in the presence of GABA was 16.0 ± 3.3 min. The receptor was remarkably more stable in the presence of the combination of avermectin B_{1a} and GABA with a $t_{1/2}$ more than 6 hr. In contrast, tracazolate, picrotoxin, and pentobarbital did not protect benzodiazepine receptors from inactivation.

Discussion

The present study indicates that the recognition sites for GABA, the benzodiazepines, avermectin B_{1a}, tracazolate, pentobarbital, and picrotoxin are all associated with the GABA receptor·chloride ion channel complex (also see Olsen, 1981, for review). The findings are in agreement with the observation that the benzodiazepines, avermectin B_{1a}, pentobarbital, and tracazolate all possess anticonvulsant and antianxiety activities *in vivo*. The potentiating effects of these compounds on GABA and benzodiazepine receptor binding ultimately could lead to a prolonged opening of the chloride ion channel, thus allowing an increase of chloride ion influx to postsynaptic nerve terminals and inhibition of neurotransmission.

Avermectin B_{1a}, tracazolate, and pentobarbital all stimulated GABA binding. The combination of avermectin B_{1a} with tracazolate or pentobarbital did not increase GABA binding further, suggesting that the binding site for avermectin B_{1a} may be partially shared by pentobarbital and tracazolate. The sites of interaction of tracazolate and avermectin B_{1a} are apparently different since, at concentrations which enhance binding to the same extent, avermectin B_{1a} protected the GABA receptor from heat inactivation, while tracazolate did not. Their stimulatory effects on GABA binding also were affected differentially by picrotoxin which blocked the effect of avermectin B_{1a} by 80 to 85% and that of tracazolate by only 9 to 12%. The site of action of avermectin B_{1a} thus may interact with part of the site of action of picrotoxin. It is unclear whether the small inhibitory effect of picrotoxin on tracazolate stimulation of GABA binding may be due to a partial sharing of binding sites by these two drugs. Etazolate, which has a pharmacological profile similar to tracazolate, competes with the binding of [^{3}H] dihydropicrotoxin (Olsen, 1981). Conformational changes of the GABA receptor complex may occur when the receptor binds different ligands under various experimental conditions and this could affect the interaction of picrotoxin with the GABA receptor complex. The occupancy of GABA or benzodiazepine receptors may affect the mode of binding for avermectin B_{1a} or picrotoxin. Picrotoxin blocks the stimulatory effect of avermectin B_{1a} on GABA binding but not that on benzodiazepine binding (Pong et al., 1981). Conceivably, the occupation of benzodiazepine receptors by the ligand may lead to a conformation change such that the binding of picrotoxin may no longer hinder the effect of avermectin B_{1a}. A temperature dependence of the actions of picrotoxin on the GABA·benzodiazepine receptor complex has been reported (Karobath et al., 1981).

It has been shown that picrotoxin abolishes the enhancement of GABA binding by pentobarbital (Table II; Willow and Johnston, 1981; Olsen, 1981). In the present study, the potentiating effect of avermectin B_{1a} on GABA binding also was inhibited by picrotoxin. However, the binding sites of avermectin B_{1a} and pentobarbital are apparently different since, in heat inactivation experiments, GABA receptors are protected by avermectin B_{1a} but not by pentobarbital. Furthermore, picrotoxin did not protect GABA receptors from heat inactivation. Unlike avermectin B_{1a}, clonazepam was effective in the

protection of GABA receptors from heat inactivation without potentiating GABA receptor binding. This result suggests that the GABA receptor detected in the present study is coupled to a benzodiazepine receptor. Protection of GABA receptors by clonazepam and GABA has been reported (Gavish and Snyder, 1980).

Avermectin B_{1a} has potent stimulatory effects on benzodiazepine receptor binding by reducing the rate constant of dissociation between benzodiazepine and the receptor (Pong et al., 1981), an action which might be related to its potentiation of the muscle relaxant effect of diazepam in mice (Williams and Yarbrough, 1979). In a digitonin-solubilized fraction, avermectin B_{1a} stimulation of benzodiazepine binding was blocked by the GABA antagonist bicuculline, suggesting that the same macromolecule or complex possesses the recognition sites for avermectin B_{1a}, GABA, and the benzodiazepines (Pong et al., 1981). This notion is supported by the evidence that avermectin B_{1a} stimulates GABA binding and the stimulatory effect is inhibited by picrotoxin (Pong and Wang, 1982). GABA and avermectin B_{1a} appear to have a very similar mechanism of action in the potentiation of benzodiazepine binding (Pong et al., 1981), but GABA could elevate the avermectin B_{1a} enhancement of benzodiazepine binding further, suggesting that the two compounds do not share the same site of action.

It appears that avermectin B_{1a} may interact with the benzodiazepine receptor through sites independently recognized by tracazolate and pentobarbital. The effect of pentobarbital is additive with that of GABA but not with that of avermectin B_{1a}. Tracazolate decreased the potentiation effect of avermectin B_{1a} on benzodiazepine binding. The additive effect of etazolate, a pyrazolopyridine with a structure similar to that of tracazolate, with that of GABA was reported by others (Olsen and Leeb-Lundberg, 1981). In the present study, no reproducible additive effect between GABA and tracazolate was observed. It is unclear whether this can be attributed to instability of part of the receptor complex in different membrane preparations (Willow and Johnston, 1981; Olsen, 1981) or to the basic chemical difference between these two pyrazolopyridines. Nevertheless, the interactions of avermectin B_{1a} and GABA with the benzodiazepine receptor are quite different. This difference can be distinguished in combination experiments with tracazolate and pentobarbital. Furthermore, the enhancement of benzodiazepine binding by GABA is not chloride ion dependent (Pong et al., 1981), whereas the enhancements of avermectin B_{1a} (Pong et al., 1981), etazolate (Supavilai and Karobath, 1981), and pentobarbital (Leeb-Lundberg et al., 1980) are dependent upon chloride ion. This observation indicates that the binding sites of avermectin B_{1a}, pentobarbital, and tracazolate may be functionally closer to a chloride ion channel than GABA binding sites.

The protection of benzodiazepine receptors from heat inactivation by avermectin B_{1a} and GABA and the protection of GABA receptors by avermectin B_{1a} and benzodiazepine indicate that the recognition sites for GABA, avermectin B_{1a} and the benzodiazepines may be distinct but closely linked. Neither pentobarbital nor tracazolate protected the benzodiazepine receptor from heat inactivation even though both potentiated benzodiazepine binding. Picrotoxin did not potentiate benzodiazepine binding and did not exert any protection of benzodiazepine receptors from heat inactivation. It is possible that avermectin B_{1a}, GABA, and benzodiazepine produce more stable conformations of the GABA receptor complex following allosteric interaction. Alternatively, the recognition sites of avermectin B_{1a}, GABA, and benzodiazepine may be located on the same peptide or in a more tightly coupled complex than the others; therefore, only these three ligands are able to produce the protective effects in the heat inactivation. However, it remains controversial whether the GABA and benzodiazepine recognition sites reside in two separate protein molecules (Massotti et al., 1981; Gavish and Snyder, 1981). In a previous paper, we reported bicuculline-sensitive avermectin B_{1a} stimulation of benzodiazepine binding in a digitonin-solubilized fraction (Pong et al., 1981). It thus appears that, even if the receptors of GABA and benzodiazepine reside in separate peptides, these two peptides may be coupled tightly in membranes.

In conclusion, our study supports the hypothesis that GABA receptors and benzodiazepine receptors are part of the GABA receptor·chloride ion channel complex. This complex appears to contain separate sites for GABA, the benzodiazepines, avermectin B_{1a}, pentobarbital, tracazolate, and picrotoxin. However, some overlapping may occur among the recognition sites of avermectin B_{1a}, pentobarbital, tracazolate, and picrotoxin.

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