AVERMECTIN MODULATION OF GABA BINDING TO MEMBRANES OF RAT BRAIN, BRINE SHRIMP AND A FUNGUS, *MUCOR MIEHEI*

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High affinity [^{3}H]GABA (γ-aminobutyric acid) binding sensitive to muscimol and bicuculline was detected in membranes derived from rat brain and brine shrimp. Avermectin stimulated this GABA binding with maximum stimulation seen in these membranes at 400 and 40~80 ng/ml, respectively. This avermectin stimulation of GABA binding was Cl^--dependent, bicuculline and picrotoxin-sensitive and was associated with an increase in B_m but not K_d of the systems. The membranes from *Mucor miehei* also exhibited high affinity [^{3}H]GABA binding that was insensitive to classical neuronal GABA receptor agonists/antagonists and other agents. This novel GABA receptor was sensitive to Na^+ and extremely sensitive to low levels of avermectin (apparent K_i 20~40 ng/ml). This inhibition of GABA binding by avermectin was associated with a decrease in affinity (increase in K_d) and an increase in concentration of receptors (B_m). It is possible that these GABA receptors might play crucial roles in control of cell metabolism and that avermectin can prevent growth of this organism *via* interference in the receptor activity.

Avermectin is a macrocyclic lactone antibiotic produced during the fermentation of *Streptomyces avermitilis*[1]. This compound is a potent insecticide, acaricide, anthelmintic agent[2] that is proposed to act by interfering with the γ-aminobutyric acid (GABA) receptor in neuronal tissue[3]. This stimulation of GABA binding to its receptor is proposed to open up Cl^- ionophores that prevent passage of the nervous impulse in motor neurons resulting in paralysis[4,5]. While key experiments linking avermectin to chloride ion permeability and paralysis have been performed using avermectin sensitive organisms[4,5], all studies of the effect of avermectin on GABA receptors have been performed using an avermectin-resistant organism, the rat[3,6].

In this paper, we present a formal repetition of previous work on the effects of avermectin on rat brain membranes and a comparison with the effects seen on GABA binding to membranes derived from avermectin-sensitive organisms, the brine shrimp *Artemia salina*, and the fungus, *Mucor miehei*. The latter organism, a fungus, which is sensitive to avermectin at 2 μg/ml, contains no nervous system and consequently, should contain no classical neuronal type GABA receptors.

Materials and Methods

Whole brains (cerebellum, cerebrum and brain stem) from 250~325 g Sprague-Dawley male rats were homogenized with a Brinkman tissue homogenizer (Switzerland) at 0°C in 15 volumes of 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ and centrifuged at 1,500$\times g$ for 10 minutes. The supernatants were centrifuged again at 60,000$\times g$ for 2 hours at 4°C after which the pellets were resuspended in 10 volumes of 10 mM sodium phosphate (pH 7.4) and incubated at 4°C for 30 minutes before freezing to −20°C for 1~2 hours and thawing. The ultracentrifugation, resuspension, incuba-

tion and freeze-thaw steps were repeated two more times. The resultant membrane preparation was resuspended in the phosphate buffer at 10~20 mg protein/ml. The procedure is similar to that described by PONG and WANG[3].

Membrane preparations were also obtained from whole *Artemia salina* (brine shrimp) and the fungus, *Mucor miehei* ATCC 16457 by the identical procedures described above except that the organisms were disrupted by passage through a French pressure cell (Aminco, Silver Springs, MD, USA) at 20,000 psi at 4°C.

GABA binding to well washed membranes was measured in a filtration assay similar to that described previously[3]. Routinely, 50~100 μl of membranes were added to 900~950 μl 50 mM Tris-HCl buffer (pH 7.4) at 4°C to which had been added an appropriate amount of drug or test compound dissolved in water, methanol or dimethyl sulfoxide (final concentration of solvent never attained more than 1% v/v) and 10 nM [^{3}H]GABA (31 Ci/mmol); although solvents never interferred with GABA binding at this concentration, control tubes were always treated with appropriate solvent. Reaction tubes were incubated at 4°C for 20~90 minutes after which the contents were filtered (Whatman GF/B filter) and the filter washed with 2×3.5 ml of the Tris-HCl buffer. The washed filters were added to 10 ml Biofluor (New England Nuclear, Boston, MA) and the ^{3}H levels assayed by a scintillation spectrophotometer. Non-specific GABA binding was determined in the presence of 10^{-3} M GABA (unlabelled) and never represented more than 20% of the total binding. Specific binding was determined by subtracting non-specific from total binding.

Protein was determined by the LOWRY phenol method[7] with bovine serum albumin, fraction V as standard. All biochemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Results

Specific GABA Binding

All three membrane preparations were capable of binding [^{3}H]GABA effectively, with 80% of this binding being displaceable by cold GABA. In order to characterize the GABA binding activity, the effects of a number of known GABA receptor agonists and antagonists as well as other potential inhibitors were examined. As shown in Table 1, the effects of agents on membranes derived from rat brain and brine shrimp were similar. At the concentrations tested, bicuculline and muscimol effectively inhibited GABA binding while picrotoxin was only weakly active; epinephrine, taurine and acetylcholine were essentially inactive. While benzodiazepine was ineffective at modulating GABA binding to rat brain membranes, it stimulated the binding to brine shrimp membranes. GABA binding to *Mucor* membranes was relatively unaffected by muscimol, picrotoxin, bicuculline, epinephrine, taurine, acetyl-

Table 1. Effect of various agents on GABA binding to membranes.

Agent	Concentration (M)	[^{3}H]GABA binding (% control)		
		Rat brain	Brine shrimp	*M. miehei*
Bicuculline	10^{-4}	23.0	2.1	96.9
Muscimol	10^{-4}	15.3	6.3	87.6
Picrotoxin	10^{-4}	44.7	66.2	84.3
Epinephrine	10^{-3}	106.4	100.6	91.4
Taurine	10^{-3}	100.2	123	110.3
Acetylcholine	10^{-3}	115.3	125	88.6
Benzodiazepine	3×10^{-4}	112.5	288	67.4
Nipecotic acid	10^{-3}	96.3	89.3	76.0
NaCl	50×10^{-3}	81.3	79.6	7.3

choline and benzodiazepine. Nipecotic acid (the GABA transport inhibitor)[5] did not significantly alter GABA binding to any of the membranes. At 50 mM, Na^+ (as NaCl) was inhibitory against GABA binding to *Mucor* membranes but not against GABA binding to the other membranes. This would indicate that in all three systems, GABA transport proteins were not being detected and that true GABA receptors were being assayed.

Effect of Avermectin on GABA Binding

Avermectin stimulated GABA binding to both rat brain and brine shrimp membranes with optima of 400 and 40~80 ng/ml, respectively (Fig. 1). At higher concentrations, the stimulatory effect was not seen. With *Mucor* membranes, avermectin decreased GABA binding with an apparent K_i of 20~40 ng/ml.

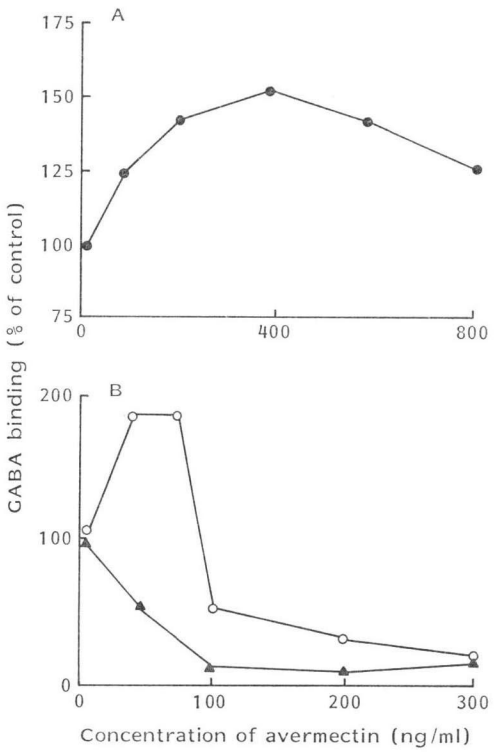
The avermectin stimulation of GABA binding to rat brain and brine shrimp membranes was dependent on Cl^- ions in the reaction mixture. The inhibitory effect of avermectin on GABA binding to *Mucor* membranes was partially repressed in the absence of Cl^- (Table 2). Picrotoxin and bicuculline inhibited the avermectin stimulation of GABA binding in the rat brain and brine shrimp membranes.

Further analysis of the binding of GABA to membranes and the effects of avermectin on this binding was performed using Scatchard analysis (Table 3). All three membrane systems exhibited high affinity GABA binding in the low nM range. Avermectin did not significantly alter the affinity of the rat brain or brine shrimp GABA receptors but did increase the apparant number of receptors present. However, avermectin decreased the affinity (increased the K_d) of *Mucor* receptors and increased the total number of receptors.

Fig. 1. Effect of avermectin on GABA binding to membranes derived from rat brain, brine shrimp and *M. miehei*.

Membranes were incubated at 4°C with various levels of avermectin and [^{3}H]GABA as described in the methods section. The membranes and bound GABA were recovered by filtration and then the total ^{3}H assayed by scintillation spectroscopy. Specific GABA binding was determined by subtracting non-specific (in the presence of 1 mM cold GABA) from total GABA binding.

Binding to rat brain membranes (●) in A and brine shrimp (○) and *M. miehei* membranes (▲) in B.



Discussion

In this study, we have attempted a formal repetition of the study by the Merck group[3,6] using membranes from the same organism and tissue, rat brain. In addition, since the rat is immune to the effects of avermectin, we wanted to extend the study to include the susceptible organisms, *A. salina*, the brine shrimp and *M. miehei*, a fungus. It is known that the latter contains no classical nervous system and thus should contain no neuronal-linked GABA receptors; yet it is avermectin sensitive[9]. However, it is conceivable that the fungus might possess unusual GABA receptors that could be associated with

Table 2. Effect of agents on avermectin-stimulated [^{3}H]GABA binding to membranes.

Agent	Avermectin*	[^{3}H]GABA binding to membranes (% control)		
		Rat brain	Brine shrimp	*M. miehei*
$+Cl^-$	−	100	100	100
	+	162	345	8.1
$-Cl^-$	−	106.6		84.2
	+	107.5	126	34.7
+Muscimol	−	100	100	nt
	+	148.3	227	nt
+Bicuculline	−	7.6	10.3	nt
	+	12.6	19.6	nt
+Picrotoxin	−	45.7	72.3	nt
	+	49.8	85.7	nt

* The concentrations of avermectin used in this study was 200 ng/ml for rat brain, 60 ng/ml for brine shrimp and 80 ng/ml for *Mucor* membranes.
nt, not tested.

Table 3. Effect of avermectin on the [^{3}H]GABA association constant (K_d) and the inoxim binding (B_m) of various membranes.

Membrane source	Presence of avermectin*	K_d (nM)	B_m (pmol/mg protein)	r^{2**}
Rat brain	−	14.6	1.28	0.992
	+	13.3	1.76	0.945
Brine shrimp	−	2.68	0.014	0.998
	+	2.81	0.024	0.986
M. miehei	−	27.0	0.0478	0.859
	+	85.1	0.0648	0.841

* Avermectin was added at 800 ng/ml for rat brain, 60 ng/ml for brine shrimp and 30 ng/ml for *M. miehei* membranes.

** Scatchard analysis of specific [^{3}H]GABA binding (2~100 nM) to various membranes in the absence or presence of avermectin. Linear regression analysis of the data, plotted as bound GABA/free GABA vs. specific binding, yielded K_d, B_m and linear regression coefficients (r^2).

cell growth and division.

Using membranes from adult rat brains that were prepared in a similar manner to those prepared by PONG and WANG[3], we were able to confirm a muscimol and bicuculline-sensitive, high affinity GABA binding activity that was stimulated by low levels of avermectin. This avermectin-stimulated GABA binding was Cl^--dependent and associated with an increase in the B_m, but not K_d, of the system. As predicted in the literature[10,11], GABA binding was not appreciably affected by other hormones or neurotransmitters. The GABA binding was neither sensitive to nipecotic acid nor stimulated by Na^+ ions, indicating that it was a classic receptor and not a transport protein. Essentially, we confirmed the data of PONG and WANG[3] with one exception. In our system, GABA binding was partially inhibited by picrotoxin while in theirs it was resistant.

The data obtained using brine shrimp membranes was essentially the same as that described for rat brain membranes indicating that the rat system was a valid model for an invertebrate GABA receptor system. The differences noted in the systems were a) the higher concentration of GABA receptors in the rat brain membranes compared with the brine shrimp system which is predictable since the brine shrimp preparations were obtained from whole organisms and not purified nervous tissue; b) benzodiazepine stimulated GABA binding with brine shrimp membranes. This stimulation has been observed in other systems and is dependent on the presence of a low molecular weight modulator protein that can be washed off membranes[10]. It seems likely that in the preparation of these membranes from

brine shrimp, the protein was more strongly adsorbed and was not removed; and c) the maximum stimulation of GABA binding to brine shrimp membranes was observed at 40~80 ng/ml avermectin *versus* 400 ng/ml for rat brain membranes. This higher concentration needed for rat brain membranes would indicate that the latter membrane receptor probably exhibited less affinity for the drug than the comparable brine shrimp preparation.

A high affinity (low K_d), nipecotic acid-resistant GABA binding was detected in *Mucor*, indicating that it was not a classical GABA transport protein. This GABA binding was resistant to muscimol, bicuculline, picrotoxin, and the other hormones and neurotransmitters indicating that the receptor studied was not a typical GABA receptor. The binding was extremely sensitive to Na^+ and avermectin (apparent K_i 20~40 ng/ml). This avermectin inhibition of GABA binding was essentially independent of Cl^-, indicating that it was not a classical Cl^- ionophore associated GABA receptor. The avermectin inhibition of binding was associated with an increase in K_d (decrease in affinity) and increase in B_m for the accessible sites. Again, the concentration of receptors was low and comparable to that seen for brine shrimp.

From this study it is evident that the rat brain membrane GABA binding system is a very good model for an analogous membrane system from a susceptible organism, in this case the brine shrimp. However, it points out that the GABA receptors observed in *Mucor* although affected by avermectin are very different from these seen in more complex organisms such as the rat or brine shrimp. The functions of these receptors in *Mucor* are clearly not associated with a classic nervous system but may serve other functions in controlling cell growth and division and might very well be the target in the organism for avermectin. Other studies are needed to characterize these receptors in more detail and to elucidate their role in cell metabolism.

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