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AVERMECTIN B_{1a}: AN IRREVERSIBLE ACTIVATOR OF THE γ-AMINOBUTYRIC ACID-BENZODIAZEPINE-CHLORIDE-IONOPHORE RECEPTOR COMPLEX

Steven M. Paul[1], Phil Skolnick[2], and Martin Zatz[3]

[1]Clinical Psychobiology Branch, [2]Laboratory of Bioorganic Chemistry, NIAMDD; Laboratory of Clinical Science, NIMH; National Institutes of Health, Bethesda, Maryland 20205

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SUMMARY

Avermectin B_{1a}, an antihelminthic macrocyclic lactone, has been previously shown to reduce muscle membrane resistance by stimulating γ-aminobutyric acid-mediated chloride conductance. Since the benzodiazepine receptor is coupled to a receptor for γ-aminobutyric acid and related chloride ionophore, the effects of Avermectin B_{1a} on [^{3}H]diazepam binding to the benzodiazepine receptor were studied. In well-washed membrane fragments from rat cerebral cortex, Avermectin B_{1a} markedly increased the binding of [^{3}H]diazepam to benzodiazepine receptors. This effect was qualitatively similar to that observed with either γ-aminobutyric acid or chloride ion and was partially reversed by the γ-aminobutyric acid receptor antagonist, bicuculline. In contrast to the effects of γ-aminobutyric acid and chloride, the enhanced binding of [^{3}H]benzodiazepine elicited by Avermectin B_{1a} was *not* reversed by extensive washing of the membrane preparation. Avermectin B_{1a} appears to irreversibly modify benzodiazepine receptors at a γ-aminobutyric acid-chloride recognition site and may be valuable in biochemical studies of the regulation of benzodiazepine receptor function.

Avermectin B_{1a} is an antihelminthic macrocyclic lactone derived from *Streptomyces avermectilis* (1). At low concentrations AVM rapidly and irreversibly paralyzes nematodes, presumably by an action on the central nervous system (2). Neurophysiological studies of the lobster neuromuscular junction have shown that AVM eliminates inhibitory postsynaptic potentials by reducing membrane resistance of the muscle fibers; the latter also results in reduction of excitatory post-synaptic potentials (3). The AVM-induced alteration in muscle membrane resistance is closely related to the opening of chloride channels that are normally regulated by γ-aminobutyric acid (3). Furthermore, extensive washing of such AVM-incubated muscle preparations failed to reverse the drugs' effect on membrane resistance (3). These results suggest that at the lobster neuromuscular junction, AVM selectively and irreversibly activates GABA-mediated chloride conductance,

Abbreviations used: AVM = Avermectin B_{1a}; GABA = γ-aminobutyric acid; CNS = central nervous system.

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and may therefore be a useful biochemical probe for studying such events in the mammalian central nervous system.

Since there is good evidence to suggest that some, if not all, benzodiazepine receptors in the mammalian CNS are functionally coupled to a GABA receptor and a corresponding chloride ionophore (4), we examined the effects of AVM on the binding of [^{3}H]diazepam to benzodiazepine receptors both *in vitro* and *in vivo*. We now report that, like GABA and various anions, AVM selectively increases the affinity of the benzodiazepine receptor for benzodiazepines. However, in contrast to both GABA and anions, the activating effects of AVM appear to be irreversible.

MATERIALS AND METHODS

Membrane preparation: Membrane fragments were prepared from the brains of adult male Sprague-Dawley rats (100-150 g). Freshly dissected tissue was homogenized in 50 vol (w/v) of either ice-cold Tris-maleate or Tris-HCl buffer (50 mM, pH 7.4) using a Brinkmann Polytron (setting 5, 30 sec) (Brinkman Inst., New York). Membranes were centrifuged at 20,000 x G for 10 min (4°). In order to remove endogenous GABA, the membranes were washed three times in 100 volumes of buffer followed by recentrifugation. Following the final wash, membranes were resuspended in buffer (100 vol) and frozen (-20°) for at least 12 hours. An aliquot of the membrane preparation was thawed prior to each experiment, centrifuged (20,000 x G, 20 min) and resuspended in Tris-HCl or Tris-maleate buffer. In some experiments AVM (1 mg/kg) was administered i.p. to male mice (NIH general purpose, 20-25 g). At 1 and 6 hours after injection, both AVM-treated and vehicle-treated mice were sacrificed and washed membrane fragments from whole brain were prepared as described above.

Binding assays: The binding of [^{3}H]diazepam (Sp. Act. 83.5 Ci/mmole, NEN, Boston, Mass.) to benzodiazepine receptors was carried out with minor modifications of previously described methods (5). Briefly, 250 µl of extensively washed membranes were incubated with 0.1-10 µM AVM (Merck, Sharp and Dohme, Rahway, NJ) in a total incubation volume of 0.5 ml. AVM was dissolved in a vehicle of propylene glycol/ethanol (95:5), the latter being incorporated into the assay at a final concentration of less than 1%. Control incubations contained an equal volume of vehicle. All other drugs were added to the assay after dilution in Tris buffer. GABA and strychnine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Picrotoxin and (+)bicuculline methiodide were purchased from K & K/ICN and Pierce Chemical Co., respectively.

Incubations were carried out for 60 min at 0-4°C and terminated by rapid filtration through Whatman GF/B glass fiber filters. Filters were washed 4 times with 5 ml ice-cold buffer, dried, placed in scintillation vials containing 10 ml Aquasol (NEN, Boston, Mass.) and counted in a liquid scintillation counter. Specific binding was defined as the total binding less the binding obtained in the presence of 3 µM diazepam (nonspecific). Nonspecific binding was routinely less than 10% of the total binding. In a typical experiment, specific binding ranged from 5,000 to 40,000 DPM, depending on the free concentration of [^{3}H]diazepam.

RESULTS

AVM at concentrations as low as 0.1 μM significantly increased the binding of [^{3}H]diazepam to benzodiazepine receptors. The AVM-induced enhancement of [^{3}H]diazepam binding was more pronounced in extensively washed membranes devoid of endogenous GABA (Fig. 1 and unpublished observations). In washed membrane preparations AVM produced a highly significant ($p < .001$) increase in both the apparent affinity of benzodiazepine receptors for [^{3}H]diazepam and in the density of binding sites (Fig. 1). However, the effect of AVM on receptor affinity was quantitatively more significant since the change in receptor density never exceeded 10% (Fig. 1 and unpublished observations).

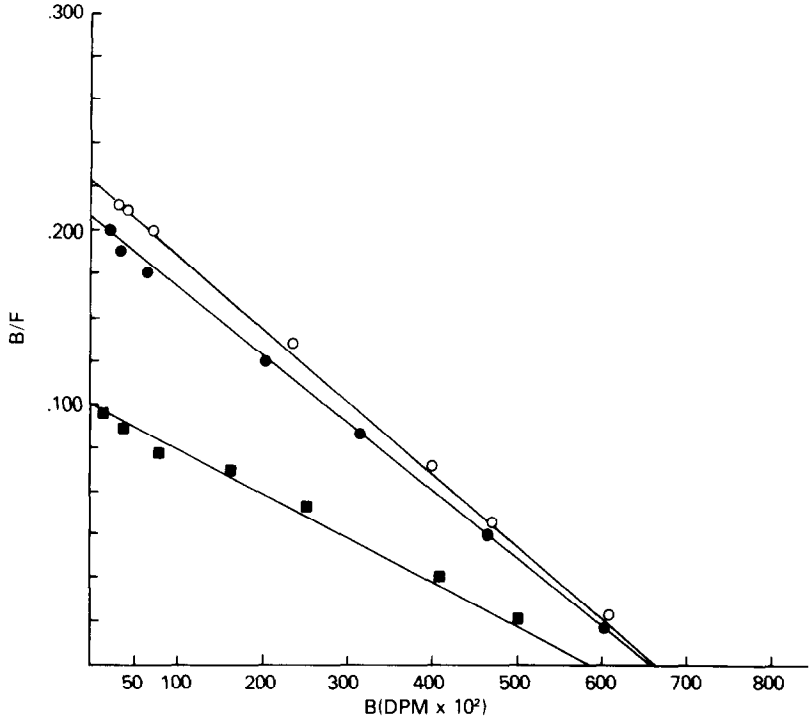

Figure 1. Scatchard analysis of [^{3}H]diazepam binding in well washed membranes with (O;●) and without (■) 1 μM AVM. One set of membranes was preincubated with 1 μM AVM (60 min, 0-4°C), washed 3 times in 100 vol buffer, and assayed as above (O). A significant increase in both the apparent affinity and B_{max} was observed in the AVM-incubated membranes. The AVM-stimulated enhancement of [^{3}H]diazepam binding was not reversed by washing (O). Concentrations of [^{3}H] diazepam between 0.5 and 16 nM were used. Each point represents the $\bar{X}$ of triplicate determinations. The experiment was repeated 4 times and a statistically significant increase in B_{max} and K_d ($p < .001$) was apparent when compared to incubations without AVM. B, [^{3}H]diazepam specifically bound (DPM/assay); F, free [^{3}H]diazepam (DPM/assay).

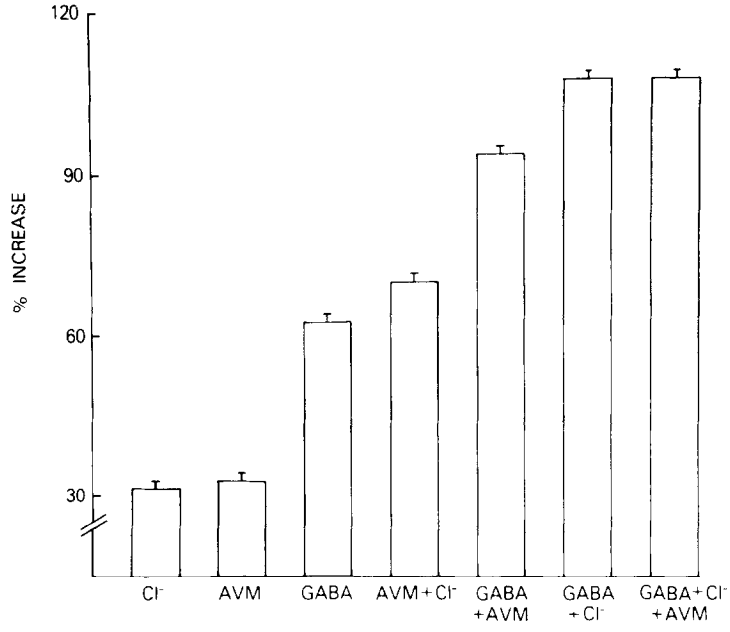

Figure 2. The effects of AVM (1 μM) with and without chloride, GABA, or chloride + GABA on [^{3}H]diazepam binding. Extensively washed membranes prepared in Tris-maleate buffer (50 mM, pH 7.4) were used. The AVM-induced stimulation of [^{3}H] diazepam binding (0.5 nM [^{3}H]diazepam) was additive with submaximally effective concentrations of either chloride (50 mM NaCl) or GABA (5 μM). However, AVM produced no further enhancement of maximally effective concentrations of both chloride (200 mM NaCl) and GABA (30 μM). Each bar represents the $\bar{X}$+S.E.M. of the % increase in [^{3}H]diazepam binding when compared to incubations containing Tris-maleate buffer alone (n = 3 experiments).

The stimulation of [^{3}H]diazepam binding by AVM was additive with submaximal concentrations of both GABA and chloride (Fig. 2). However, when maximally effective concentrations of GABA (30 μM) and chloride (200 mM) were used, AVM resulted in no further enhancement of benzodiazepine binding (Fig. 2).

Parenteral administration of AVM (1 mg/kg) to mice resulted in seizure activity characterized by repeated tonic-clonic movements lasting for at least 6 hrs and terminating in death. Animals sacrificed at 1 hr and 6 hrs following administration of AVM displayed a highly significant enhancement of [^{3}H]diazepam binding when compared to vehicle-treated controls (Fig. 3). The percent enhancement of [^{3}H]diazepam binding in brain membranes from AVM-treated mice was greater at low concentrations of ligand (Fig. 3) suggesting an increase in receptor affinity similar to that observed in the *in vitro* experiments.

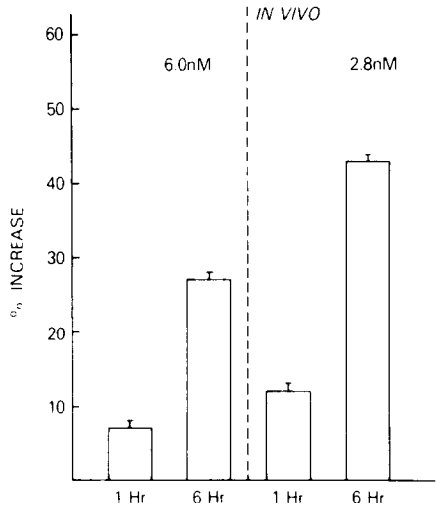

Figure 3. The effect of AVM (1 mg/kg) on [^{3}H]diazepam binding to membranes prepared 1 hr and 6 hr after i.p. administration. Membranes were assayed using two concentrations of [^{3}H]diazepam (2.8 and 6.0 nM). The % enhancement of binding when compared to vehicle-treated controls was greater at the low concentration of ligand (2.8 nM) suggesting an increase in receptor affinity. Each bar represents the $\bar{X}$+S.E.M. of the % enhancement observed in brain membranes from 6 AVM-treated mice and compared to the mean value for vehicle-treated controls (n=6). The increase in [^{3}H]diazepam binding in AVM-treated mice was statistically significant ($p < 0.01$) at both concentraions of ligand and at each time point.

To determine whether the AVM-induced stimulation of benzodiazepine receptors was irreversible, AVM- and GABA-incubated membranes were subjected to repeated washing (Fig. 4). Washing of GABA-incubated membranes completely reversed the enhancement observed with this compound (Fig. 4). In contrast, repeated washing

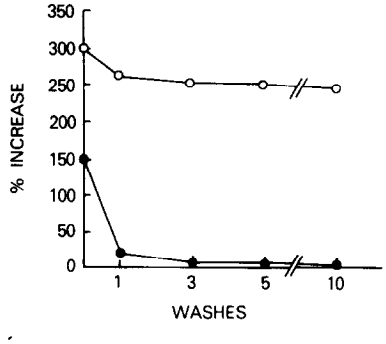

Figure 4. Effect of repeated washing on AVM- and GABA-stimulated [^{3}H]diazepam binding. Extensively washed membranes were incubated with AVM (10 μM) or GABA (10 μM) for 60 min at 0-4°C. An aliquot of the membrane preparation was removed and the membranes subjected to repeated washing in 100 vol of 50 mM Tris-HCl (pH 7.4) buffer. Membranes were assayed for [^{3}H]diazepam binding (0.5 nM) as described in the text. The GABA-stimulated enhancement of [^{3}H]diazepam binding (but not the AVM-stimulated) was reversed following repeated washing. Each point represents the $\bar{X}$ of triplicate determinations.

TABLE I

Effects of Bicuculline, Picrotoxin and Strychnine on AVM-Stimulated [^{3}H]Diazepam Binding

Compound	% Inhibition
Bicuculline	
50 μM	28
100 μM	60
Picrotoxin	
100 μM	0
Strychnine	
100 μM	0

Well washed membranes were preincubated for 30 min with the inhibitor. AVM (1 μM) or vehicle (propylene glycol) were then added. The specific binding of [^{3}H]diazepam (0.5 nM) was determined as described in the text. Results are from a typical experiment repeated three times with similar results.

of the AVM-incubated membranes (as many as 10 washings) resulted in only a slight decrease in the AVM-induced stimulation of [^{3}H]diazepam binding (Fig. 4).

Preincubation of membranes with the GABA antagonist bicuculline resulted in a significant inhibition of the AVM-mediated activation of benzodiazepine binding (Table 1). However, picrotoxin, a blocker of the chloride channel, or strychnine, a specific glycine antagonist, had no effect on AVM-stimulated binding (Table 1).

DISCUSSION

Our results suggest that AVM irreversibly activates the binding of [^{3}H] diazepam to benzodiazepine receptors by increasing receptor affinity in a manner similar to that of GABA and various anions. A previous report by Williams and Yarbrough (6) emphasized the effect of AVM in increasing receptor number (B_{max}). However, in this study it is likely that the modest effects of AVM on receptor affinity were due to the high concentrations of endogenous GABA present in their membrane preparations.

The AVM-induced activation of benzodiazepine receptors appears to involve a GABAergic mechanism, since bicuculline antagonized the effects of this compound. Furthermore, AVM failed to stimulate other classical neurotransmitter receptors such as the α- and β-adrenergic receptors (unpublished observations and ref. 6). The failure of AVM to further enhance the maximal effects of a combination of

GABA and chloride concentrations suggests that AVM activates benzodiazepine receptors through a site or mechanism common to both GABA and chloride ions.

Most significantly, extensive washing of AVM-incubated membranes failed to reverse the enhancement of [^{3}H]diazepam binding. The potent and irreversible activation of benzodiazepine receptor binding by AVM via a GABA-chloride linked mechanism should make it a valuable tool for studying GABAergic modulation of benzodiazepine receptors. Purification of the components of the GABA-benzodiazepine-chloride-ionophore receptor complex responsible may also be facilitated by use of AVM.

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BIBLIOGRAPHY

1. Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, S.A., Hartman, R., Kong, Y.L., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.B., Wallick, H., Stapley, E.O., Oiwa, R., and Omura, S. (1979) Antimicrobial Agents and Chemotherapy, 15, 361-367.
2. Egerton, J.R., Ostlind, D.A., Blair, L.S., Eary, C.H., Suhayda, D., Cifelli, S., Riek, R.F., and Campbell, W.C. (1979) Antimicrobial Agents and Chemotherapy, 15, 372-378.
3. Fritz, L.C., Wang, C.C., and Gorio, A. (1979) Proc. Natl. Acad. Sci. USA, 76, 2062-2066.
4. Tallman, J.F., Paul, S.M., Skolnick, P., and Gallager, D.W. (1980) Science, 207, 274-281.
5. Paul, S.M., and Skolnick, P. (1978) Science, 202, 892-894.
6. Williams, M., and Yarbrough, G. (1979) Eur. J. Pharmacol., 56, 273-276.