Pharmacology Biochemistry & Behavior, Vol. 14, pp. 839–844, 1981. Printed in the U.S.A.

An Endogenous Ligand to the Benzodiazepine Receptor: Preliminary Evaluation of Its Bioactivity

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Received 14 November 1980

DAVIS, L. G., H. McINTOSH AND D. REKER. *An endogenous ligand to the benzodiazepine receptor: Preliminary evaluation of its bioactivity*. PHARMAC. BIOCHEM. BEHAV. 14(6) 839–844, 1981.—Chromatographic separation of aqueous brain extracts yields a peptide containing fraction which competitively inhibits ^{3}H-diazepam binding to its receptor. An intracerebral-ventricular injection of this isolated fraction results in altered responses in pharmacological and behavioral tests which are similar to those observed when diazepam is administered in the same fashion. The most pronounced effect was obtained in the conflict test. Changes observed in other tests, such as blocking pentylenetetrazole convulsions, altering motility or reducing hyperthermia, were also consistent with the actions of diazepam. At the dose used, neither diazepam nor the brain extract altered muscular co-ordination in two ataxia evaluations. Thus, the animals' performance in the other paradigms would not be adversely influenced by immobilization side-effects. The results reported here support the notion that an endogenous factor does exist in brain which can act like the benzodiazepine drugs when tested for bioactivity in animal studies.

Anxiety Behavior Benzodiazepines Convulsions Diazepam Endogenous ligand Peptide

ALTHOUGH benzodiazepines are the most widely prescribed drugs in the United States [19,25], the mechanism of action for these anxiolytic minor tranquilizers is unknown. An accepted mode of action is an interaction with the gamma-aminobutyric-acid (GABA) system [2, 11, 12, 13, 18]. However, an extensive number of investigators have described benzodiazepine affects on other neurotransmitter systems [4, 10, 21, 22, 30, 44, 47]. Thus, the determination of their biochemical mechanisms of action, specifically as antianxiety agents, has been confounded with the mechanisms underlying other actions such as the anticonvulsant or ataxia effects. In 1977, binding sites for the benzodiazepine drugs were identified in brain tissue [6, 7, 34, 35]. The brain binding site has been shown to bind ^{3}H-diazepam in a high affinity, stereospecific manner [5, 6, 7, 32, 34, 35, 36]. Furthermore, many studies have evaluated alterations in these receptors after physiological or behavioral manipulations [27, 29, 39, 40, 42, 45]. These receptor studies have facilitated the understanding of how such antianxiety drugs might exert their therapeutic actions (for reviews, see [7, 11, 12]) and suggest that this drug binding site is physiological.

It was immediately suggested, similar to the proposal that followed the identification of an opiate binding site, that the brain might possess an endogenous ligand for these benzodiazepine binding sites [6, 7, 34, 35]. This reasoning has been supported, in part, by the specificity of the binding site and by the selective behavioral actions of the benzodiazepines [5, 7, 27, 29, 35, 36, 42]. The reports that a large number of known natural products were unable to block ^{3}H-diazepam binding [32,37] was not supportive for identifying an endogenous ligand to this receptor. However, none of these compounds were especially active in physiological tests for active benzodiazepine compounds. New candidate endogenous ligands typically are extracted from brain and tested for their ability to inhibit ^{3}H-diazepam binding to the brain receptor. The purines, inosine and hypoxanthine, were the first compounds to be identified as potentially active by such a benzodiazepine binding assay screen [1, 41, 42]. They were subsequently found to have activity, as would be expected of a putative endogenous ligand, in physiological tests [31, 42, 43] but at seemingly high doses. GABA modulin [46], which affects both GABA and benzodiazepine binding to their respective receptors [20], has aroused much interest in the interactions between these two neuronal receptors. A detailed hypothesis for the mechanism of action for ben-

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zodiazepines and for the interactions between these two receptors has been proposed [11,12]. Other reports of potential endogenous ligands have appeared [9, 17, 23] including the extensive purification from human urine of a β-carboline ligand for these receptors [8,38]. In our earlier studies [14,15] we have identified an endogenous ligand for the benzodiazepine receptor in aqueous brain extracts and human serum [16]. This fraction contained a putative natural ligand which was found to be a thermostable peptide (ca. 3000 daltons) that competed with ^{3}H-diazepam for its binding sites. Another endogenous factor from brain with characteristics similar to the above compound has been independently reported [33]. Although such pharmacological screening procedures, as the binding assay, can assist in the identification of a potential endogenous ligand for a specific receptor, it is also necessary to demonstrate that such compounds possess bioactive properties. The present report evaluates the activity of this brain peptide extract [14,15] as a putative natural ligand for the benzodiazepine receptors by comparing its activity to that of diazepam treatment and placebo conditions in typical behavioral and physiological tests for benzodiazepine compounds.

METHOD AND RESULTS

Endogenous Ligand Isolation

We have previously reported the isolation procedure of the endogenous factor that inhibits ^{3}H-diazepam binding in a radioreceptor assay [14,15]. Briefly, rat or bovine crude synaptosomal preparations (P_2) are osmotically shocked and freeze-fractured. The resulting supernatant is fractionated by gel filtration chromatography (Biogel P_{10}, 2.5×90 cm columns eluted with 0.01 M acetic acid). The active fraction (ca. 3000 daltons) is identified by its ability to inhibit ^{3}H-diazepam binding to rat brain receptors in a standard binding assay [6, 32, 34]. The identified fraction was purified further by elution from ion exchange columns. The resultant fraction that did not bind to either ion exchange column has been shown to be an active, competitive inhibitor of ^{3}H-diazepam binding to the benzodiazepine receptor [15]. This extracted material, which was shown to be a single fluorescamine staining spot in TLC (recent analytical HPLC studies indicate more than one component is present), was used in the present study. Prior to their intraventricular injection and testing in the behavioral and physiological explorations, the concentrations of this isolated brain extract and diazepam were adjusted by dilution with isotonic buffer to be approximately equipotent inhibitors, as determined in the benzodiazepine binding assay.

Cannula Implants and Intraventricular Injections

Male, 130–180 gram, albino rats were anesthetized with ketaset-plus (10 mg/kg) and prepared for surgery. Stainless steel cannula were chronically implanted following standard stereotaxic procedures with the cannula tip aimed at the right lateral ventricle according to skull markers and coordinates determined from a rat brain atlas [24]. All animals were allowed to recover with food and water ad lib in individual cages for at least 7 days. Their successful recovery was monitored by trained, licensed animal technicians.

Prior to the experimental protocol, animals were food and water deprived (48 hrs). Intracerebral-ventricular injections were through an inner cannula pre-matched for each implant and consisted of 5 μl (1 μl/minute) of a benzodiazepine solution (15 pmole/μl-diazepam) or the isolated endogenous factor or vehicle only. During this injection, the animals were awake but restrained. Each animal was returned to its own cage for 45 minutes (except where noted) prior to testing. Implant locations of the cannula were examined by routine visualization of sectioned paraffin blocks of Formalin fixed brain tissue.

TABLE 1
ATAXIA MEASUREMENTS AFTER INTRAVENTRICULAR INJECTION

	Placebo	Diazepam	Endogenous Ligand
Time rod held (mean sec±sem)	8.7±1.6	8.2±1.5	9.5±1.9
Number clinging to screen			
Full 30 sec	6	8	8
Climb up	1	2	2
Climb down	10	8	6
Fall off	0	0	0

Ataxia Studies

Two tests were used to measure for possible alterations in the muscular co-ordination of the animals receiving injections in this study. The first test measured the time an animal could cling to a 1 cm diameter bar while the second test determined the ability to hold onto or climb a 300 cm vertical screen (1 cm×1 cm grid). In neither test were any differences in the muscular abilities or co-ordination detected between animals receiving injections of placebo or diazepam or endogenous factor. Table 1 summarizes the data. Although diazepam when given orally is known to produce muscular relaxation, the doses we injected intraventricularly were ineffective in causing this effect. Whether this is due to the concentration of drug or the mode of administration is not known. The most important result obtained here is that little or no losses in muscular co-ordination occurred from injecting either of these compounds: It is important that this be determined before performing further test procedures. Thus, ataxia would not appear to be a factor that might interfere with the interpretation and performance in other specific tests.

Motility Studies

The animals were rated for their motility in Stoelting motility cages (Chicago, IL). Movement is monitored by detecting alterations in the established electromagnetic field and, not necessarily locomotion. Each cage recorder was standardized with a constant rotating pestle prior to the study. Animals were randomly selected and following a timed sequence of intraventricular injection of either diazepam, placebo or the endogenous factor, each was placed into one of the cages located in a secluded room with dimmed lighting according to the timed sequence. Following a 10 minute equilibration period from the handling, recordings were initiated and continued for 60 minutes with the automatic recording of movement units.

Two separate studies were performed with 5 animals in each group (Fig. 1.). In one study, the motility test was conducted immediately after the injection and in the second

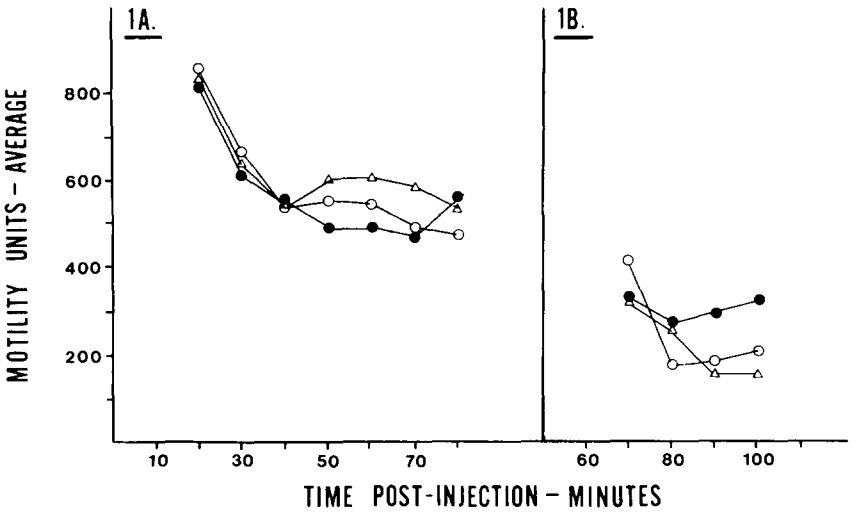

FIG. 1. Two separate experiments were conducted as described in the text: In the first group (n=5) animals were placed into the motility cages immediately after the intraventricular injection (1A). The second set were tested 60 minutes after the injection (1B). Movement units were automatically recorded continuously and tabulations were made every 5 minutes. Data are average of two successive 5 minute periods following the acclimation period (10 min). Placebo ●; Diazepam △; Endogenous Ligand ○.

study the test started 60 minutes after the injection. The results of these two experiments are presented in Fig. 1A and 1B. The dose used for the test compounds in this study did not markedly alter the motility of the animals when compared to the controls. However, 50–60 minutes after the injection, the animals receiving either diazepam or the endogenous factor did display a small but consistent increase in activity. Furthermore, at 90 minutes after injection a decrease in movement was observed for these two groups when compared to controls. Although these differences in activity between control and the experiment groups (diazepam and endogenous ligand) are not large, the magnitude and direction of the changes in the pattern suggests that the ligand is having an action similar to that of diazepam.

Hyperthermia Test

Effective benzodiazepine compounds have been shown to protect against L-tryptophan induced hyperthermia [26]. Animals received the MAO inhibitor tranylcypromine (5 mg/kg, intraperitoneally—IP), and 30 minutes later received L-tryptophan (100 mg/kg, IP) to cause an elevation of serotonin concentrations that induce hyperthermia [26]. The intraventricular injection of diazepam, endogenous factor or placebo occurred 60 minutes after the tryptophan injection. Rectal temperatures were measured and recorded every 30 minutes for two hours. Diazepam and the endogenous factor were only able to reduce slightly the temperature elevation (Fig. 2) and neither compound was able to completely block the increase in temperature as we had observed when IP injections of diazepam were evaluated (data not shown). The doses we injected intraventricularly might have been insufficient to reverse completely this whole body hyperthermia.

Protection Against Pentylenetetrazole (PTZ) Induced Convulsions

The anticonvulsant activity of benzodiazepines has been

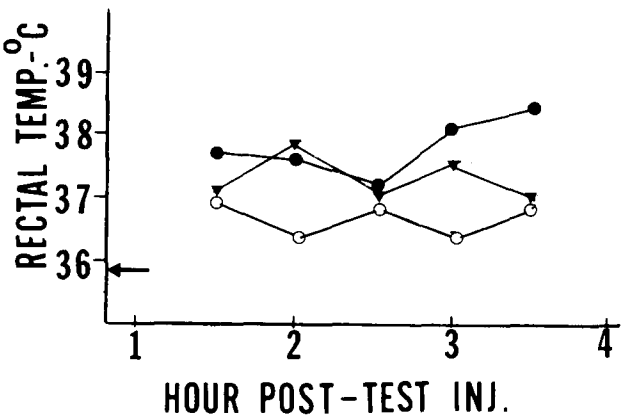

FIG. 2. Hyperthermia was induced by L-tryptophan after monoamine oxidase inhibition as detailed in text. Rectal temperatures were recorded every 30 minutes until animals became immobilized (n=3 each group). Placebo ●; Endogenous Ligand ▼; Diazepam ○. Arrow indicates average pre-experiment rectal temperature.

evaluated by their ability to retard the onset of PTZ induced convulsions [26, 28, 43]. In preliminary experiments, convulsions were consistently observed within two minutes of an IP injection of PTZ. Intraventricular injection (5 μl) of diazepam one hour before an IP administration of PTZ (69 mg/kg) was able to retard the onset of convulsions for up to 10 minutes. The injection of the endogenous factor retarded the convulsions up to 20 minutes and in one case only mild tremors were observed during a 90 minute observation period. Injection of placebo had no ability to retard or effect the convulsions in comparison to uninjected animals. The endogenous factor was apparently as potent as diazepam in its ability to retard PTZ induced convulsions (Table 2).

TABLE 2

PROTECTION AGAINST PTZ-INDUCED CONVULSIONS

	Placebo	Diazepam	Endogenous Ligand
Time to onset	1.3 min	5.5 min	15.3 min*
Time to death	15.8 min	46. min	44. min*

*One animal survived and is not included; n=3 each condition.

TABLE 3

CONFLICT TEST: SHOCK INDUCED AVERSION TO DRINKING

	No. licks pre-shock	No. shocks received	No. responses in shock period
Placebo	60.5±10.7	8.4± 1.3	32.4±11.5
Diazepam	67.0± 9.0	23.0± 4.5	109.1±31.6*
Endogenous Ligand	60.0± 9.2	25.1±11.8	87.1±36.1*

*Significant at $p<0.05$; n=8 each group; mean±sem.

Conflict Test

The modified Geller-conflict procedure, as described by Lippa, *et al.* [26,28], was used to test the ability of compounds to disinhibit the shock paired with drinking by thirsty animals. In this procedure, 48 hour water and food deprived animals were injected intraventricularly with either diazepam, buffer or endogenous ligand and tested in Skinner boxes 45 minutes later. These boxes were located in a separate room with white noise and the animals were observed on a closed circuit television. The animals, after locating the water spout (between 5–10 minutes with no group differences), were given 15 sec free access (without shock) to drink the water after which time the shock was manually turned on to an automatic 5 sec on/off fixed cycle program. The boxes were adjusted such that, a shock was automatically administered for each lick of the spout that an animal made. The shock (200 μA) was scrambled between the grids on the floor but activated only by licking the metal spout. Data were recorded on counters as number of licks during the free period, number of licks with the shock 'on' and total number of licks in test period (15 min).

Total number of responses in the shock period by the diazepam and the endogenous factor recipients was found to be statistically significant (Table 3) using the Mann-Whitney U-test when compared to the placebo controls. When animals were tested with lower doses of the endogenous fraction (1.0 μl injection) or at a shorter time (5 min after a 5 μl injection) the experimental and control animals showed no differences in the number of shocks they would accept simultaneously for a drink. Further dose response and time course experiments certainly need to be performed when the factor is eventually purified.

Each of the animals used in the above conflict-test were naive to the experimental procedures. In separate experiments 7–10 days later, the aversion to shock by the animals used in the original conflict test was extinguished by allowing three periods (one hour each) of free access to water from the drinking spout without shocks. These animals were then randomized and retested as above. The diazepam and endogenous ligand recipients again accepted more shocks than the controls (data not shown).

DISCUSSION

The demonstration in this report of bioactivity for an isolated brain extract supports the suggestion [6, 7, 34, 35] that an endogenous compound exists in brain for the benzodiazepine receptors. The results of this preliminary study also indicate that a fraction isolated from brain and reinjected into the cerebral ventricles of naive animals can alter their behaviors in certain tests which are similar to those changes caused by diazepam administration. This observation was especially true for the disinhibition (conflict) test and in the retardation of PTZ induced convulsions experiment. The endogenous fraction also was comparable in activity to diazepam in the other tests described. The equivalent potencies and actions of the isolated fraction and diazepam in all experiments would suggest that each of their actions were receptor mediated, especially since the administered doses had been pre-adjusted to be effectively equal in a *in vitro* benzodiazepine binding assay. The similar delay (40–60 minutes) in maximal activity for both of these active compounds is consistent with the invoking of receptor mediated events and strongly suggests that the site of action is distant from the lateral ventricle. This, of course, assumes that the compounds avoid degradation and implies that they may be neurohormonal *in vivo*. The alternative hypothesis is that diazepam and the endogenous factor are short-lived but induce a series of long-term adaptations that culminate in altered animal responses in the above physiological tests. These possibilities need to be investigated with future experiments.

The limited number of animals and conditions evaluated in each experiment and the lack of complete purity for the injected endogenous material cause some serious reservations about any conclusions that might be drawn concerning the existence of an endogenous ligand for the benzodiazepine receptor. The present results best serve as an encouragement for a continual searching and the purification of such an endogenous ligand. Subsequent studies are planned for the final purification of the presently identified factor and for its structural determination. The retesting of the purified factor and a synthetically identical compound in the physiological tests as described herein is necessary to answer the question of whether a "natural anti-anxiety" ligand does indeed exist.

ACKNOWLEDGEMENTS

The authors would like to especially thank Ms. Debbie Wilcox for her conscientious assistance during all phases of the testing, surgery and animal care and to thank Ms. Vicki Eichhorn for typing the manuscript. This research was supported by State of Missouri Intramural funds to the MO Institute of Psychiatry in St. Louis and performed in the Neuropharmacology Unit through the courtesy of Dr. W. Thompson, Director. The diazepam was a generous gift from Hoffman-La Roche (Nutley, NJ).

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