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IDENTIFICATION OF AN ENDOGENOUS PEPTIDE-LIGAND FOR THE BENZODIAZEPINE RECEPTOR

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SUMMARY: An inhibitor of ^{3}H-diazepam binding with characteristics that distinguish it from the other endogenous ligands reported for the benzodiazepine receptor was obtained from bovine brain. After isolation by gel filtration and ion exchange chromatography, the inhibitory factor was found to be a weakly charged molecule of approximately 3000 daltons. Although heat stable, the activity of the factor can be destroyed by treatment with papain. This factor, presumably peptide in nature, inhibited ^{3}H-diazepam binding competitively and in a concentration dependent fashion.

INTRODUCTION

Benzodiazepine binding sites, which are saturable, stereospecific, and possess a high affinity for a series of benzodiazepine derivatives, have been demonstrated to occur in neural tissue (1-6). The identification of benzodiazepine receptors led Squires and Braestrup to suggest the existence of an endogenous transmitter capable of interacting with the benzodiazepine receptors (1). In the ensuing search for such diazepam-like factors, many different endogenous compounds and psychotropic agents have been tested. Each failed to inhibit ^{3}H-diazepam binding by 50% when added at a concentration of 100 μM (6). Inosine and hypoxanthine, however, were described as competitive ligands for the benzodiazepine receptor (7-9). In behavioral tests, such as delaying the time of onset of pentylenetetrazole induced convulsions, inosine did retard the onset of convulsions (10) as would be expected for a diazepam-like ligand. However, the affinity of these purines for the receptor is low (7-9,11) and the dose

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required to mimic diazepam in behavioral tests is high (10). Other factors, isolated from brain, have also been reported to inhibit specific diazepam binding to varying degrees (12-16). Recently, investigations have focused on 'GABA modulin' (17). This 15000 dalton peptide modulates the binding of GABA to its receptor; after a pre-incubation period, 'GABA modulin' competitively inhibits diazepam binding as well (12, 17, 18). Conclusions drawn from these reports have prompted us to further examine brain tissue for a compound that can serve as the natural ligand of the benzodiazepine receptor. We now report the isolation from bovine cortical synaptosomes of a peptide-containing fraction which competitively inhibits specific ^{3}H-diazepam binding to its receptors.

MATERIALS AND METHODS

Diazepam and the ^{3}H-diazepam were generous gifts of Hoffman-LaRoche (Nutley, NJ) and New England Nuclear (Boston, MA), respectively. All other chemicals and materials were purchased from commercial sources.

Bovine brains were obtained from a local slaughter house and immediately placed on ice for transportation to the laboratory (20 minutes) during which time the dissection was accomplished. The preparation of the P_2 fraction from 48g brain cerebral gray by standard differential centrifugation procedures (19) was performed, as the initial step. This synaptosomal fraction was then lysed with 5 volumes of 50mM Tris-Cl buffer (pH 7.5), shaken on ice for thirty minutes, frozen, thawed, and again shaken before centrifugation at 10^{6} g-min. The supernatant obtained from the osmotically shocked synaptosomal fraction was freeze-dried, dissolved in 0.1 M acetic acid (4 ml) and applied to a Biogel P-10 gel filtration column (2.5x90cm). Sixty-five tubes (9.8 ml each) were collected by volume and their absorbance at 230 nm was determined. Seven peaks were observed and the tubes comprising each of them were combined. Following lyophilization and reconstitution in water, each of these fractions was tested for its ability to inhibit specific ^{3}H-diazepam binding.

Fraction #5 was further purified by applying it to ion exchange chromatography columns. The fraction, titrated to pH 3.5, was initially applied to a Dowex 50 (H^{+}) column and fractions were eluted with a linear gradient of increasing pH. Since the maximal activity was not retained by the column, the purification of the inhibitory factor was modified to include coupled columns of anionic (Dowex 1, Cl^{-}) and cationic (Dowex 50, H^{+}) exchange resins. Each column (1x20 cm) bound a portion of original material and these could be eluted from the resins with 0.2M NH_4OH and 0.25M HCl, respectively. One fraction was not bound to either resin. Thus,

three fractions were obtained. Each of these fractions was dried and tested in the binding assay for its ability to inhibit specific ^{3}H-diazepam binding.

Other extraction procedures (20) using acetone, acetic acid or ethanol as the solvent were also attempted.

The ^{3}H-diazepam binding assay was performed according to the procedures described by Möhler and Okada (4). Briefly, a crude synaptosomal membrane fraction was prepared as described above. This fraction was rehomogenized in 15 volumes of hypotonic 5mM Tris-Cl buffer (pH 7.5). Aliquots (300µl) of the membrane homogenate were added to plastic tubes containing 100µl of either buffer, unlabeled diazepam, or the fractions obtained from bovine brain. Addition of 100µl of ^{3}H-diazepam (1.6nM) initiated the binding assay. After 15 minutes of incubation, the mixture was centrifuged and the resultant pellets were solubilized and counted by scintillation spectroscopy. Binding was shown to be saturable, time dependent, reproducible, and specific, in agreement with the published reports (1-6).

The partially purified fraction containing the diazepam binding inhibitor was evaluated for homogeneity on Silica Gel G plates using two solvent systems. After allowing 5 hrs. for migration, the TLC plates were sprayed with fluorescamine (21) in order to visualize the spots under ultraviolet light.

Temperature stability was evaluated by incubating the fractions for 15 minutes at 100°C. The boiled fraction was tested in the binding assay either directly or after centrifugation to remove insoluble material.

The susceptibility of the factor to proteolytic destruction was determined by incubating it with 0.01 unit of papain for 15 minutes or with 0.1 unit for 16 hours under conditions previously described (22).

A determination of the amino acid composition was performed for the active fraction by gas-liquid chromatography (23) of TMS amino acid derivatives. Each identification is based upon the retention times when compared to known standards. Derivatization was carried out in closed tubes with bis(trimethylsilyl)trifluoro-acetamide-acetonitrile (1:1) at 150°C for 2.5 hours after hydrolysis in 6 N HCl for 24 hours. Column packing was 10 w/w% OV-11 on 100/120 mesh Supelcoport; 3 m x 2 mm I.D. glass. Initial temperature was 70°C, which was held for 5 min and then programmed at 4°C/min to 250°C.

Protein concentrations were estimated from the modified Folin procedure (24).

RESULTS AND DISCUSSION

Fractions that were obtained by the organic extraction procedures (20) failed to inhibit ^{3}H-diazepam binding. However, the concentrated aqueous supernatant obtained by osmotic lysis of a synaptosomal fraction inhibited specific ^{3}H-diazepam binding by 56.3% when tested in a benzodiazepine binding assay. Upon fractionation of this material by gel filtration and testing each of the fractions in the binding assay, it was determined that

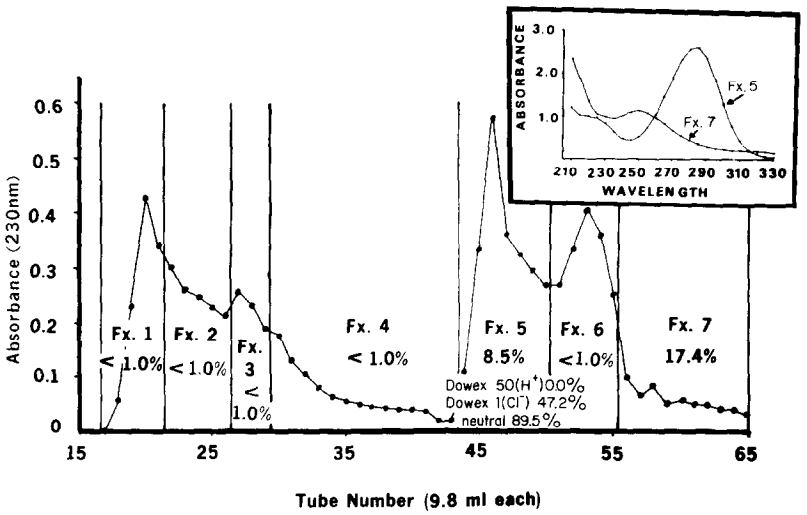

Figure 1. Chromatographic separation on a Biogel P-10 (2.5 x 90 cm) column of the soluble material derived from the synaptosomal (P_2) fraction. Blue dextran eluted in fractions equivalent to Fx 1 while chromate ions eluted after Fx 6. Sample fractions were eluted with 0.1M acetic acid and 9.8 ml fractions were collected. Each tube was monitored for absorbance at 230nM. Peaks were pooled as shown and each was tested in a benzodiazepine binding assay (4). The percent inhibition of ^{3}H-diazepam binding is indicated for each (Fx 1 through 7). The two peaks which inhibited the binding (Fx 5 and Fx 7) were scanned for their absorbancy at various UV wavelengths between 210 and 330nm (see insert).

Fx 5 was further purified by coupled anionic and cationic exchange chromatography (see methods section). The fraction, unbound to either column (designated neutral), was collected as were the fractions bound to each of the resins. After freeze-drying, all samples (Dowex 50 bound, Dowex 1 bound, and neutral) were reconstituted in water, pH checked 6.5 to 7.5, and tested in a benzodiazepine binding assay (4). Their percent inhibition of ^{3}H-diazepam binding is presented under Fx 5.

Each assay was performed in duplicate at least three times on separate preparations.

fractions #5 and #7, contained inhibitors of diazepam binding (Figure 1). Fraction #7, consisting of molecules less than 1000 daltons in size, presumably contained the purines (see insert in Figure 1) which have been previously reported to inhibit diazepam binding (7-9). The UV spectra of the inhibitory factor(s) present in fraction #5 (MW estimated 3000) differed from that of material in fraction #7 (see insert Figure 1). Further fractionation of fraction #5 by ion exchange chromatographies yielded three addition al fractions; the fraction which failed to bind to either column

contained a potent inhibitor (89.5%) of specific ^{3}H-diazepam binding (Figure 1).

Evaluation by thin layer chromatography of the composition of each of the fractions obtained indicated that the parent fractions (S_2P and Fx 5) and the inactive materials obtained after the ion exchange procedures each contained multiple areas of visible fluorescence. The active inhibitory fraction, however, provided only one resolvable spot (Figure 2). The factor was considered to be heat stable since 99% of the inhibitory activity remained when the boiled fraction was retested in the binding assay. Incubation with papain under mild conditions (15 minutes, 0.01 units) reduced the activity by 11%. Incubation for 16 hours (0.1 unit) reduced the factors ability to block ^{3}H-diazepam binding by >98%. Analysis of

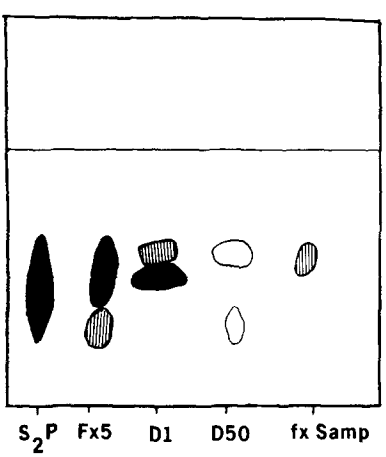

Figure 2. Schematic presentation of identifiable material after thin layer chromatography (TLC) migration according to standard procedures. Ten microliter sample aliquots were spotted on a 20 x 20 cm Silica Gel G TLC plate. The plate was placed into a chromatography tank containing n-butanol-acetic acid-ethyl acetate-water (1:1:1:1) as the eluting solvent for ascending chromatography. The plates were removed when the solvent front reached 5 cm from the top (about 5 hours) and dried at 110°C for 10 minutes. Upon cooling, the plates were stained with fluorescamine (21). After drying, the plates were examined under UV light. Spot densities are represented by relative darkened areas. Qualitatively similar results were also found when 1-butanol-pyridine-acetic acid-water (74:50:16:60) was used as the eluting solvent. Samples are from the left: synaptosomal supernatant (S_2P), Biogel column fraction 5 (Fx 5), fraction eluted from Dowex 1 (D1), elution from Dowex 50 (D50), and fraction unbound to either exchange column (fx Samp).

the amino acid composition of the partially purified peptide was performed following the procedures of Gehrke and Leimer (23). The presence of eight different amino acids (ALA, HIS, ISL, LEU, LYS, MET, TYR, VAL and 4 other minor peaks) was demonstrated. As the purines and other compounds with a similar ring structure (e.g. cyclic AMP, caffeine) have been shown to weakly inhibit diazepam binding (10,11), the noted presence of histidine, an amino acid with an imadozole ring, may explain the inhibitory effects (7-9) displayed by the purine ring structure.

Binding properties of the endogenous diazepam-inhibiting peptide indicate that the inhibition is concentration dependent (10 to 100% inhibition) between 2 and 60 μg of Lowry positive material (24). Moreover, double reciprocal plots demonstrate that it is a competitive inhibitor (Figure 3). Also, when unlabeled diazepam and the active peptide were added together, the inhibition of ^{3}H-diazepam binding by this mixture was both additive and competitive. The above data indicated that the isolated peptide reported here may well be an endogenous ligand for the benzodiazepine receptor.

The present identification of an apparent peptide ligand that differs in its size and properties from other reported ligands (6-10,12-18) and competes for the benzodiazepine receptors provides further support for the hypothesis (1) that such compounds exist. Nonetheless, the demonstration of pharmacological binding properties is not sufficient to claim that the endogenous ligand for benzodiazepine receptors has been found. For a definitive statement it needs to be demonstrated that the pharmacological, behavioral and biochemical effects are identical to those of diazepam itself. Preliminary investigations (Davis, Reker, McIntosh, Fikes, and Cohen, unpublished observations) have indicated that the purified diazepam-like peptide alters EEG activity in a manner

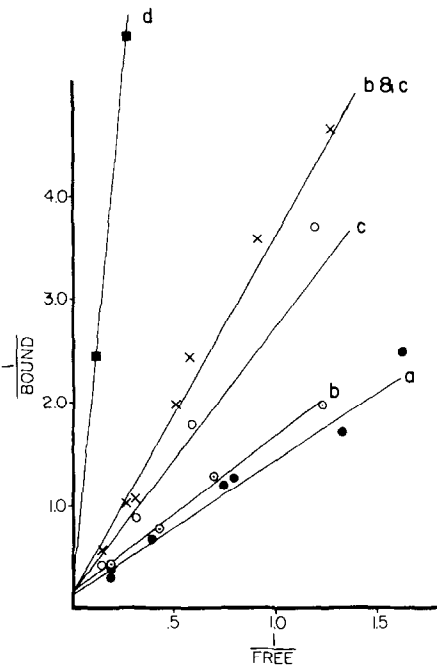

Figure 3. Analysis of competitive binding assays is depicted. These assays were performed with increasing amounts of ^{3}H-diazepam according to the assay procedures described by Möhler and Okada(4) in the absence and presence of the isolated active inhibitory fraction (Fx 5-neutral). After completion of the binding, samples were centrifuged in a Beckman Microfuge to separate the membrane pellets (bound) and the supernatants (free). Each individual sample was counted by liquid scintillation spectroscopy. The tested compounds include: buffer alone (a), 7.0 µg of Lowry positive (24) isolated inhibitory factor (b), 0.8 nM unlabeled diazepam (c), combination (b+c), and 100 µg of Lowry positive (24) isolated inhibitory factor (d). Each point represents an average of duplicate determinations, with the competitive binding assay having been performed on four different preparations. Each of these others when plotted gave similar presentations upon analysis. Units are pmole bound and nM free ^{3}H-diazepam.

that resembles that produced by diazepam. Both compounds induce slow rhythmic waves. Although, these electrophysiological results are only preliminary, they lend support to the notion that the brain contains a substance which mimics diazepam in binding and behavioral studies.

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