DIAZEPAM RECEPTOR CHARACTERIZATION: SPECIFIC BINDING OF A BENZODIAZEPINE TO MACROMOLECULES IN VARIOUS AREAS OF RAT BRAIN

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Received 31 August 1977

1. Introduction

Diazepam is an effective anti-anxiety drug or mild tranquilizer [1] which is also used as a muscle relaxant [2], a presurgery medication [3], an anti-epileptic agent and in alcohol withdrawal. It is a widely prescribed drug [4]. The toxicity of diazepam is remarkably low, as is its physical dependence [5]. Despite its widespread use, very little is known about the molecular basis of its action.

Recent investigations, concerning macromolecules that bind hormones or drugs, have been carried out in an attempt to correlate such binding with pharmacological actions. If a binding macromolecule is found for a drug, it is usually designated as a receptor, and it is assumed that an endogenous or naturally occurring ligand exists for the receptor. In the present study we found evidence for a highly specific, saturable binding of [^{3}H]diazepam to a purified synaptosomal fraction from rat forebrain and present some of the characteristics of this binding. (A note of caution should be interjected since there is a great deal of difference between a binding entity, a receptor and a receptor site. In the past, glass, plastics, and other inert materials have been shown to be effective 'binders' of ligands.) Such data should lead to a better understanding of the molecular action of diazepam. While this work was nearing completion, Squires and Braestrup [6] reported on benzodiazepine receptors in rat brain. In our report, using a higher specific activity of [^{3}H]diazepam and a more purified binding fraction, we give amplified details of the diazepam-binding site.

2. Materials and methods

[*methyl*-^{3}H]Diazepam was obtained from New England Nuclear Co., Boston, MA, and had a specific activity of 40 Ci/mmol. Pure unlabeled diazepam was a gift of Roche Laboratories, Nutley, NJ. The [^{3}H]-diazepam was supplied in ethanol; subsequent dilutions were made in this solvent. In all experiments 1 fmol [*methyl*-^{3}H]diazepam was equal to 19 cpm, using standard liquid scintillation counting procedures. Neuraminidase (EC 3.2.1.18), α-chymotrypsin (EC 3.4.4.5) and phospholipase A (EC 3.1.1.4) were purchased from Sigma Chemical Co., St Louis, MO. Papain (EC 3.4.4.10) was obtained from National Biochemicals Corp., Cleveland, OH and required 0.01 M EDTA for activation. Trypsin (EC 3.4.4.4) was obtained from Worthington Biochemical Corp., Freehold, NJ. All enzymes were crystalline and were used as weighed amounts in the assay.

Routinely, brain sections of 150–200 g Sprague-Dawley or Holtzman rats were homogenized in 10 vol. 0.32 M sucrose and the homogenate was centrifuged at 1000 × *g* for 10 min. Fractionation of the brain sections was as previously described [7–9]. The supernatant fluid was centrifuged at 10 000 × *g* for 20 min to yield a 'crude' P_2 synaptosomal pellet. To obtain a purer synaptosomal preparation, the combined P_2 pellets from 6 rat brains were resuspended in 60 ml 0.32 M sucrose and layered on a 1.20 M/0.80 M/0.32 M discontinuous sucrose gradient and centrifuged at 53 500 × *g* for 2 h [7–9]. This gradient system yields three fractions; A, B, and C. Fraction A consists primarily of microsomes, fraction B contained

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predominantly synaptosomes with some microsomal contamination, and fraction C contains mainly mitochondria [7–9]. The fractions were removed and washed in 4 vol. 50 mM Tris buffer solution, pH 7.5, before use.

To determine the optimal binding preparation, 500 μl samples of the P_2, A, B, and C fractions (resuspended in 50 mM Tris buffer) along with crude homogenates of the cerebellum, caudate nucleus, cerebral cortex, and whole forebrain (suspended in 0.32 M sucrose) were exposed to 5 nM [^{3}H]diazepam or [^{3}H]diazepam plus 3 μM unlabeled diazepam. The assay tubes were then incubated at 37°C for 15 min with shaking, followed by equilibration for 30 min in an ice bath. To collect the fraction binding the [^{3}H]-diazepam, 10 ml of ice-cold 50 mM Tris buffer, pH 7.5, was added to each incubation tube and the contents poured over Whatman GF/A glass fiber filters with suction. The filters were washed free of unbound diazepam with an additional 10 ml Tris buffer and placed in 5 ml Bray's solution. [^{3}H]Diazepam was measured in a Nuclear Chicago scintillation counter. Specifically-bound [^{3}H]diazepam, as given herein, was the activity in the assay tube incubated with only the [^{3}H]diazepam minus the activity in the appropriate assay tube with the [^{3}H]diazepam and the large excess of unlabeled diazepam, as is the convention in the determination of specific binding [10,11]. In most instances (except kinetic analyses) assays contained 5 nM [^{3}H]diazepam, since 16% of the available diazepam was bound at this concentration (fig.1); this allowed for greater or less binding to be measured in the various experiments, while still maintaining a 600 molar excess in the nonspecific assay tubes.

Because of its high binding activity (table 1) the B fraction was used for the kinetic studies. Aliquots, 500 μl, of the B fraction (4 mg protein/assay) were incubated with the [^{3}H]diazepam (or [^{3}H]diazepam plus unlabeled diazepam) at concentrations ranging from 0.01–500 nM.

Enzyme studies involved a preincubation of the purified synaptosomal preparation with the enzymes. Various concentrations of trypsin, papain, α-chymotrypsin, phospholipase A, and neuraminidase were incubated with the purified B fraction (4 mg protein/assay) for 30 min at 37°C. After the preincubation, specifically-bound [^{3}H]diazepam was determined as given above.

Protein was determined by the method of Lowry et al. [12].

3. Results and discussion

3.1. *Specific [^{3}H]diazepam binding in various regions of the brain*

The data in table 1 show that homogenates of several areas of the rat brain specifically bind [^{3}H]-diazepam; liver homogenates do not. Furthermore, boiling of the fractions abolished specific binding. Of the areas studied, the caudate nucleus and the cerebellum bound the least [^{3}H]diazepam, whereas the cerebral cortex and a P_2 homogenate of the forebrain bound greater amounts of the ligand. When the forebrain P_2 fraction was subfractionated, a 4.5-fold increase (to 90.0 fmol [^{3}H]diazepam bound/mg protein) in specific binding in the B (synaptosomal) fraction over the P_2 fraction was found. This strongly suggests a forebrain nerve-ending localization for the [^{3}H]diazepam. This purified synaptosomal fraction was used for all further studies.

3.2. *Kinetic studies*

Specific binding of [^{3}H]diazepam was concentration dependent and was saturable above 10^2 nM [^{3}H]diazepam (fig.1). The larger variation in the

Table 1
Specific binding of [^{3}H]diazepam in various rat brain sections and rat liver

Section	fmol/mg protein
Cerebellum	10.0
Caudate nucleus	8.5
Cerebral cortex	20.7
Total forebrain	
Crude homogenate	22.0
Fraction P_2	21.8
Fraction A	4.7
Fraction B	89.0
Fraction C	9.0
Liver	0

Homogenates of the various sections were incubated with 5 nM [^{3}H]diazepam as given in Materials and methods. Fractions A, B, C, and P_2 were isolated and incubated as described

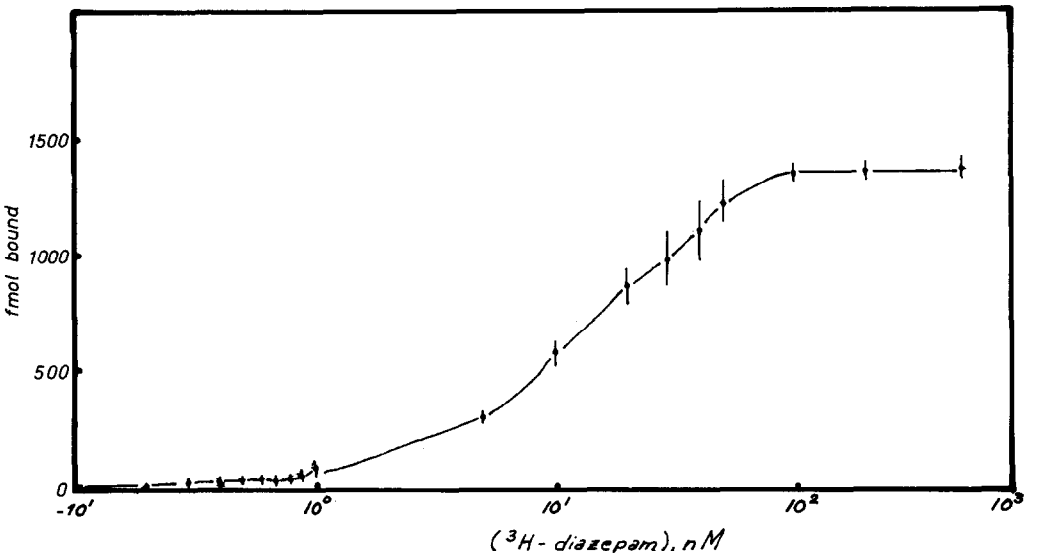

Fig.1. Binding of [^{3}H]diazepam to purified forebrain synaptosomal (nerve ending) macromolecules as a function of concentration of [^{3}H]diazepam. Each assay tube contained 4 mg purified synaptosomal protein and specific binding was determined as given in the text. Each point is the mean ± 1 SE.

middle portion of the log-linear curve may indicate that certain of the purified B fraction binding macromolecules have bound lesser or greater amounts of 'endogenous ligand'; at high [^{3}H]diazepam concentrations this variation would not be as evident, since the equilibrium would favor the [^{3}H]diazepam concentrations. A Scatchard analysis (fig.2) demonstrated that each of the specific binding sites had the same intrinsic apparent binding constant of $K_{d(app)}$ 14.8 nM. (This is usually interpreted to mean one binding site per 'macromolecule'.) Furthermore, it was found (figs.1 and 2) that maximal binding/4 mg synpatosomal protein was 1.45 pmol (or 363 fmol [^{3}H]diazepam/mg purified rat forebrain synaptosomal protein). By comparison, Squires and Braestrup [6] reported binding to membranes from 25 mg (wet wt) rat forebrain as one binding site with affinity constant K_d 2.6 nM.

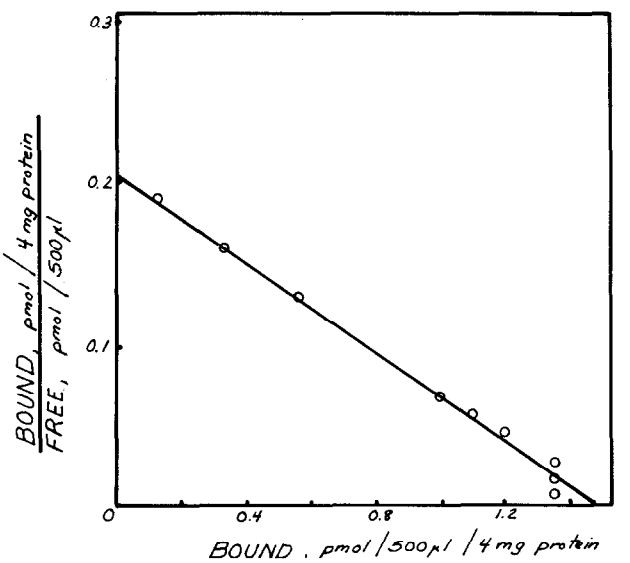

Fig.2. A Scatchard analysis of the data presented in fig.1. The fact that a straight line was obtained indicates that all of the binding sites have the same intrinsic binding constant (commonly referred to as one binding site) with affinity, $K_{d(app)}$ 14.8 nM. Also, maximal binding occurs at 1.45 pmol/4 mg fraction B protein or 363 fmol [^{3}H]diazepam/mg purified forebrain nerve-ending protein.

3.3. *Effect of ions*

The data in Table 2 are somewhat difficult to interpret. The fact that 10 mM EDTA lowered the binding by 20% may indicate an ionic requirement for optimal binding. The ions at the 10 mM concentration may be manifestations of the physicochemical

Table 2
Effect of cations and EDTA on specific [^{3}H]diazepam binding to purified rat synaptosome macromolecules

Addition	Specific binding (% control)	
	Cation concentration	
	10 mM	100 μM
None (control)	100	100
Na^+	83	127
K^+	74	129
Li^+	77	121
Ca^{2+}	93	105
Mg^{2+}	98	103
Mn^{2+}	157	95
Pb^{2+}	148	99
Cd^{2+}	188	103
Hg^{2+}	78	140
EDTA	79	100

Experiments were carried out with 4 mg protein and 5 nM [^{3}H]diazepam by the specific assay method described in the text except that cations or EDTA were added, as indicated. Cation concentrations are final concentrations. All cations were present as the chloride

Table 3
The effects of various enzymes on specific [^{3}H]diazepam binding to purified rat forebrain synaptosomes

Enzyme	Final concentration (μg/assay)	% Control binding
Trypsin	0.4	3.9
	0.08	85.7
	0.04	93.6
Papain	2.0	70.3
	0.4	75.7
α-Chymotrypsin	10.0	3.2
	2.0	8.3
	1.0	7.1
Phospholipase A	1.0	58.3
	0.2	79.9
Neuraminidase	200.0	13.8
	40.0	52.4
	20.0	59.6

Aliquots (500 μl) of Fraction B were incubated with various concentrations of the enzymes (see text). All incubations were carried out with 5 nM [^{3}H]diazepam, using the assay system described in Materials and methods. Numbers are given as percentages of the non-enzyme-treated B fraction binding

properties of the binding interactions but probably have little pharmacologic or physiologic significance. At 100 μM, Na^+, K^+ and Li^+ all significantly raised the [^{3}H]diazepam binding. This may be important since Na^+ and K^+ play a major role in maintaining membrane potentials, and Li^+ is known to have psychotrophic activity. At 100 μM, Hg^{2+} also stimulated binding; the reason for this stimulation is not known.

3.4. *Effects of prior enzyme treatment*

At high concentrations, α-chymotrypsin essentially obliterated the binding of the [^{3}H]diazepam to the purified synaptosomes (table 3). Similarly, high concentrations of trypsin and papain also reduced the binding (table 3). These data implicate a protein involved at the binding site. Similarly, the work with the phospholipase tends to implicate the conjugation of phospholipid at the site. Surprisingly, the treatment with neuraminidase suggests the presence of a sialic acid residue at the binding site (table 3). These data indicate that the binding site may be complex, involving protein, phospholipid and *N*-acetylneuraminic acid.

In summary, we have shown that diazepam binds specifically and tightly to purified rat forebrain synaptosomes and probably acts through a receptor. Furthermore, we have shown that the binding site is complex, probably involving protein, lipid and carbohydrate.

Acknowledgements

We thank Dr I. Feldman for assistance with the kinetic analysis. This work was supported in part by grants from the Muscular Dystrophy Association and grants GM-15190, NS-12461, and CA-19757 from USPHS. H.B.B. is a Scholar of the Leukemia Society of America.

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