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Endogenous Benzodiazepine Ligands in Human Cerebrospinal Fluid

JÜRGEN DECKERT, WILFRIED KUHN AND HORST PRZUNTEK[1]

Neurologische Universitätsklinik, 8700 Würzburg, F.R.G.

DECKERT, J., W. KUHN AND H. PRZUNTEK. *Endogenous benzodiazepine ligands in human cerebrospinal fluid.* PEPTIDES 5(3) 641–644, 1984.—Human cerebrospinal fluid was chromatographed on Bio-Gel P-4. Fractions containing material with molecular weights less than 4000 Dalton were pooled and further fractionated by high pressure liquid chromatography on an UltroPack TSK column G 2000 SW. At least three peaks, which were free of salt and GABA, were shown to displace (3H)-diazepam in the receptor-binding assay. Two of these peaks inhibited diazepam-binding competitively as shown by Lineweaver-Burke and displacement analysis. Their activity could be enhanced by the addition of GABA to the assay mixture. Incubation of these two peaks with various enzymes indicated that at least part of the activity of the second peak is due to a peptide.

Endogenous benzodiazepine ligands Human cerebrospinal fluid High pressure liquid chromatography Competitive binding GABA Peptide

HIGH affinity stereospecific binding sites for the benzodiazepines in the central nervous system were discovered some years ago [19,24]. More recently these benzodiazepine receptors were shown to consist of at least two subtypes [13] which could be separated by different extraction procedures [16]. In the meantime substances other than benzodiazepines have been shown to have an affinity for the benzodiazepine receptor [1, 4, 11].

In animal experiments some of these substances have been shown to be benzodiazepine antagonists [6, 15, 21, 22]. Interest has been focused especially on the search for an endogenous ligand for the benzodiazepine receptor. Among the substances which have been proposed as endogenous ligands are nicotinamide [20], purines [17], indoles [10] and prostaglandins of the A series [2], all weak inhibitors of benzodiazepine-binding whose physiological relevance therefore remains doubtful. A significantly higher affinity for the benzodiazepine receptor was demonstrated for the ethylester of β-carboline-3-carbolic acid, a ligand which seems to be an artefact of the purification procedure [3]. This led Braestrup to postulate that the endogenous ligand is a still unknown derivative of β-carboline-3-carbolic acid. Other possible endogenous ligands for the benzodiazepine receptor which have been discussed include a protein of a molecular weight lower than 100,000 Dalton [5, 12, 18, 25] and a polypeptide with a molecular weight of about 3000 Dalton [7] whose biological activity could be demonstrated in animal experiments [8]. In all of these studies—with the exception of those of Braestrup who worked with human urine—extracts of animal brain or other organs were used. For human cerebrospinal fluid Kuhn, Neuser and Przuntek [14] reported a high molecular weight and several low molecular weight (3H)-diazepam-displacing peaks. In this paper is described the further analysis of the low molecular weight peaks which shows them to consist of several components. The competition of two peaks with diazepam at the binding site is described by Lineweaver-Burke and displacement analysis. Evidence is presented that at least part of the activity of peak 2 is due to a peptide.

METHOD

Material

(3H)-diazepam (specific activity 94 Ci/mmol) and (3H)-flunitrazepam (specific activity 87.9 Ci/mmol) were obtained from NEN. Diazepam was a gift from Hoffman-LaRoche. Chymotrypsin, pronase E and papain were obtained from Sigma.

Receptor Isolation

The procedure was that described in detail by Kuhn *et al.* [14]. The final pellet was homogenized and diluted to a protein concentration of 4 mg/ml. Proteins were measured with the Folin reagent.

Cerebrospinal Fluid

We collected three different pools of CSF from patients with various neurological diseases. CSF of pool C was obtained from patients who were not treated with benzodiazepines, whereas benzodiazepine treatment was not excluded for patients whose CSF was collected in pool A and B. CSF was stored at −20°C.

[1]Requests for reprints should be addressed to H. Przuntek, Neurologische Universitätsklinik, Josef-Schneider-Str. 11, 8700 Würzburg, F.R.G.

TABLE 1

CHARACTERISTICS OF PEAKS HPLC-1 AND HPLC-2

	HPLC-1	HPLC-2
molecular weight (Dalton)	<4000	<4000
peptides (μg/ml)	300	180
absorbance (280/254)	0.32	0.44
activity (units)		
−GABA	0.8	0.9
+GABA	1	1.1
after boiling	1	1.1
after incubation with		
Papain	1	0.85
Pronase E	1	1.1
Chymotrypsin	1	—
after acid hydrolysis	0.75	0.45

In several experiments the (3H)-diazepam-displacing activity of HPLC-1 and HPLC-2 were compared with and without GABA, after boiling, after incubation with various enzymes and after acid hydrolysis.

Each assay was performed four times in duplicate, two times with material from pool A and two times with material from pool B.

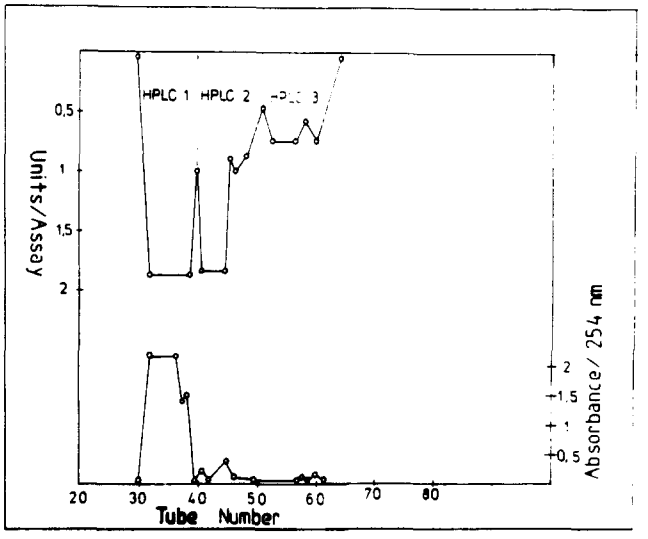

FIG. 1. Chromatographic separation of pooled Bio-Gel P-4 fractions by HPLC on an UltroPac TSK column G 2000 SW (7.5×600 mm). The displacement of 50/25 percent of specific (3H)-diazepam binding in a receptor assay is defined as 1/0.5 unit (u). The displacement of (3H)-diazepam by fractions of CSF was determined three times each for pool A, B and pool C CSF.

Fractionation of CSF

Either 100 ml of pool A and B or 50 ml of pool C was lyophilized, dissolved in 0.1 M acetic acid and centrifuged at 1500 g for 10 minutes. The supernatant was applied to a Bio-Gel P-4 column, previously equilibrated in 0.1 M acetic acid and eluted at 4°C with 0.1 M acetic acid. One hundred fifty, 4 ml fractions were collected. Fractions containing materials with molecular weights below 4000 Dalton—which were free of salt and GABA—were pooled, lyophilized, dissolved in 0.1 M acetic acid, applied to a Millex HA filter (pore size 0.45 μm) and then refractionated by high pressure liquid chromatography under the following conditions:

column: UltroPac TSK column G 2000 SW (7.5×600 mm), previously equilibrated in 0.1 M acetic acid; flow 1.3 ml/min; pressure: 700 PSI; run time: 50 min. Elution was performed at 20°C with 0.1 M acetic acid. One hundred, 1 ml fractions were collected, lyophilized and then each dissolved in 400 μl Tris-HCl 50 mM, pH 7.4. For Lineweaver-Burke analysis, displacement analysis and further characterization of peaks HPLC-1 and HPLC-2 fractions from HPLC runs of CSF pool A and B were lyophilized, dissolved in 400 μl (peak HPLC-1) or 100 μl (peak HPLC-2) Tris-HCl 50 mM, pH 7.4 and then pooled for each peak separately.

Receptor Assay

To determine the (3H)-diazepam-displacing activity of HPLC-fractions the assay mixture contained in a volume of 1 ml 0.14 nM (3H)-diazepam, 75 mM NaCl, 50 mM Tris-HCl, 10 μM GABA, 100 μl membrane pellet and 350 μl CSF of the different fractions. The assay was performed as described by Kuhn *et al.* [14]. Unspecific binding determined by adding 0.2 μM diazepam to the assay was about 5 percent. The displacement by CSF of 50/25 percent of specific (3H)-diazepam binding was defined as 1/0.5 unit (u). For Lineweaver-Burke analysis the assay mixture contained in addition to various (3H)-diazepam concentrations (0.14 nM, 0.07 nM, 0.035 nM, 0.0175 nM) various amounts of HPLC-1 (100 μl or 50 μl), HPLC-2 (25 μl or 12.5 μl) and 5 nM diazepam. Other contents and conditions were as above. For displacement analysis the assay mixture contained in addition to 0.18 nM (3H)-diazepam either various diazepam concentrations ($0.5 \cdot 10^{-7}$ to $0.5 \cdot 10^{-9}$ M) or various amounts of HPLC-1 (400 μl, 200 μl, 100 μl, 50 μl, 25 μl, 12.5 μl) respectively HPLC-2 (100 μl, 50 μl, 25 μl, 12.5 μl, 6.25 μl, 3.12 μl). Other contents and conditions were as described above. For further characterization of peaks HPLC-1 and HPLC-2 the following procedures were chosen:

The displacement of 0.14 nM (3H)-flunitrazepam from the receptor by 100 μl HPLC-1 and by 200 μl HPLC-2 in the presence and absence of 10 μM GABA were compared. The same amounts of HPLC-1 and HPLC-2 (about 30 μg and 36 μg respectively of peptide as determined with the Folin reagent) were incubated independently with 25 μg papain (20 hr, pH 7, 25°C) and with 25 μg pronase E (20 hr, pH 7.4, 37°C). One hundred μl HPLC-1 was also incubated with 25 μg chymotrypsin (20 hr, pH 7.4, 37°C). The enzymes were then removed before carrying out the receptor assay by boiling for 45 min followed by centrifugation at 50,000 g for 20 min. Acid hydrolysis was performed by incubation with 6 M HCl at 95°C for 6 hr. The HCl was removed by distillation. Other contents and conditions of the receptor assay were like the ones for determining the (3H)-diazepam-displacing activity of HPLC fractions.

RESULTS

Fractionation of CSF of pool A, B and C by HPLC shows at least three peaks of (3H)-diazepam-displacing activity associated with substances of molecular weight less than 4000 Dalton. About 75 percent of the total (3H)-diazepam-displacing activity is in peaks HPLC-1 and HPLC-2. Absorbance at 254 nm demonstrates that none of these peaks is homogenous and that at least peaks HPLC-2 and HPLC-3

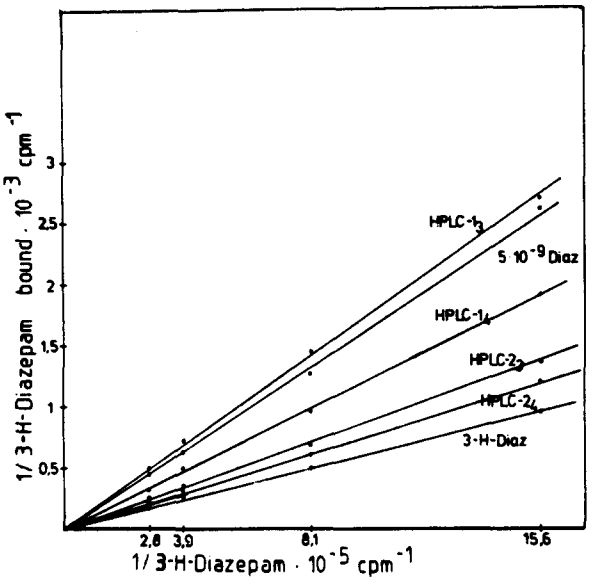

FIG. 2. Lineweaver-Burke analysis of interaction of (3H)-diazepam with the receptor in the presence of HPLC-1 or HPLC-2. To 0.14 nM, 0.07 nM, 0.035 nM and 0.0175 nM (3H)-diazepam was added 100 μl (HPLC-1_3) or 50 μl (HPLC-1_4) HPLC-1 or 25 μl (HPLC-2_3) or 12.5 μl (HPLC-2_4) HPLC-2 or, in a fifth assay, 5 nM diazepam. Each assay was performed at least four times in duplicate with material from pool A.

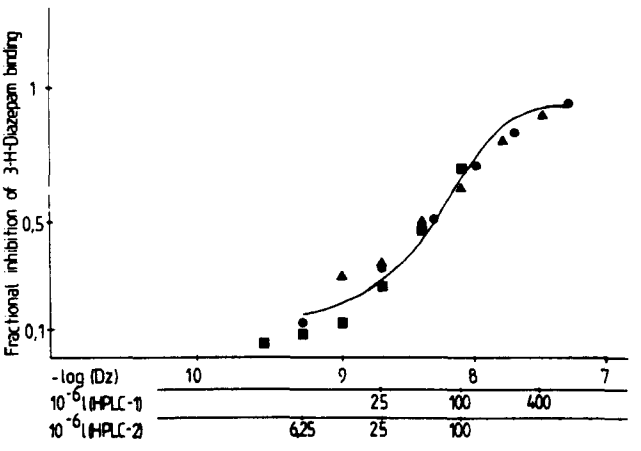

FIG. 3. Displacement analysis of (3H)-diazepam-binding in the presence of either diazepam (●/$0.5 \cdot 10^{-7}$ to $0.5 \cdot 10^{-9}$ M) or HPLC-1 (▲/400 μl to 12.5 μl) or HPLC-2 (■/100 μl to 3.12 μl). Each assay was performed at least twice in duplicate with material from pool A.

may contain more than one component which is active at the receptor binding site (Fig. 1). Lineweaver-Burke analysis (Fig. 2) indicates that the active components of HPLC-1 and HPLC-2 inhibit diazepam-binding to the receptor in a competitive manner. The activity of 100 μl HPLC-1 is approximately equivalent to that of 5 nM diazepam. Displacement analysis (Fig. 3) shows similar slopes of diazepam, HPLC-1 and HPLC-2 displacement curves. This further supports the notion of competitive inhibition as in case of substrate inhibition one should expect a steeper slope [5].

The displacing activity of both peaks was shown to be enhanced by the addition of 10 μM GABA. The ratio of absorbance at 280 nm and 254 nm indicates that peak HPLC-2 contains a slightly higher percentage of peptides than peak HPLC-1. Boiling diminished the activity of neither HPLC-1 nor HPLC-2. Incubation with papain, however, diminished the displacing activity of HPLC-2 about 20 percent whereas none of the enzymes affected the displacing activity of HPLC-1. Acid hydrolysis reduced the activity of HPLC-2 more than that of HPLC-1.

DISCUSSION

We have shown that there are several (3 to 5) low molecular weight components in human cerebrospinal fluid which are active at the benzodiazepine receptor and inhibit benzodiazepine binding in a competitive manner. As the results with two pools of benzodiazepine-containing and one pool of benzodiazepine-free CSF were equivalent, it may be concluded that benzodiazepines do not significantly contribute to the measured activities. These results may be considered as confirmation of results obtained with extracts of animal brain [7,17]. Davis and Cohen e.g., find two small molecular weight fractions active on the benzodiazepine receptor after Bio-Gel P-10 chromatography while Marangos *et al.* report three small molecular weight fractions and a fourth, which also has at least some small molecular weight components, after Sephadex G-10 chromatography.

As no ethanol was used in our extraction procedure, the presence of β-carboline-3-carbolic acid ethylester among the active components may be excluded.

It has been proposed by Skolnick *et al.* [23], Möhler and Richards [21] and Ehlert *et al.* [9] that a substance whose activity at the binding site is enhanced by GABA may be considered to be an agonist. According to this definition among the active components of peaks HPLC-1 and HPLC-2 are agonists at the benzodiazepine receptor.

As the activity of HPLC-2 at the receptor was diminished after incubation with papain, at least part of the activity of peak HPLC-2 may be associated with a peptide. This supports the results of Davis and Cohen [7] who, after coupled anionic and cationic exchange chromatography of the first of their two fractions, obtain a new one whose activity can be destroyed by papain after 16 hr incubation. Our results do not contradict Marangos *et al.* [17] who identify two of their fractions as inosine and hypoxanthine and describe the two others as highly resistant to acid hydrolysis (only the second one loses 30% of its activity) and not destroyable by incubation with various enzymes which, however, do not include papain. Those results seem especially plausible as Guidotti *et al.* [12] find a higher molecular weight "precursor" peptide of 105 amino acids, 19 or which are lysine (papain cleaves after lysine).

In the light of published reports of high molecular weight fractions, in particular of proteins with activity at the receptor site [5, 12, 14, 18, 25], it is tempting to suggest as endogenous ligand a peptide, perhaps with a β-carboline-3-carbolic acid or structurally similar component [3], which is cleaved from a precursor molecule with a higher molecular weight. Further purification of peak HPLC-2 either by ion exchange chromatography or gradient chromatography may thus lead to the isolation of a new peptide neurotransmitter of the human brain.

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