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Endogenous Benzodiazepine Receptor Agonist in Human and Mammalian Plasma*

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With 2 Figures

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Summary

Using ultra-filtration steps and HPLC-separation, a low molecular weight ligand of the benzodiazepine receptor was isolated from plasma of various mammalian species including man. The endogenous ligand acts on benzodiazepine receptors agonistically and apparently has a receptor affinity similar to Diazepam. The ligand is not identical with Diazepam as indicated by HPLC and UV-spectroscopy.

Key words: Benzodiazepine receptor agonist, endogenous ligand, anxiety, mammalian plasma, ultrafiltration.

Introduction

In the last twenty years benzodiazepines (BZDs), the most widely used minor tranquilizers, have played an important part in the treatment of reactive and pathologic anxiety states. The therapeutic effects of BZDs are thought to occur through a common mechanism, which is initiated by their interaction with specific high affinity bind-

* Abbreviations: GABA = γ-aminobutyric acid, EDTA-K_2 = ethylenediaminetetraacetic acid dipotassium salt, Tris-HCl = tris(hydroxymethyl)aminomethane hydrochloride.

ing sites Tallman, Paul, Skolnick and Gallager (1980) which are abundant in mammalian brain Möhler and Okada (1977), Bosman, Case and Di Stefano (1977), Squires and Braestrup (1977), especially in synaptosomal membranes. Strong evidence has been provided that benzodiazepine receptor (BZDR) binding modulates the postsynaptic response of brain GABAergic neurons which is linked to a chloride ionophore Study and Barker (1981), Skolnick and Paul (1982). Probably one third of all central synapses are GABAergic.

Typically, BZDs potentiate GABA mediated inhibition in the central nervous system Haefely (1977). Interactions between BZDRs and GABA, thought to occur at a BZD-GABA supramolecular complex, can also be demonstrated in binding studies both *in vitro* and *in vivo*. While the presence of GABA enhances BZDR binding Karobath and Sperk (1979), the binding of drugs with physiologic effects opposite to those of the BZDs, such as β-carbolines is diminished Möhler and Richards (1981). This GABA effect, therefore, affords a basis for differentiation between agonistic (s. a. BZDs) and inverse agonistic (certain β-carbolines) BZDR ligands. The BZDR binding of antagonists such as Ro 15-1788—which exhibit no intrinsic activity is not influenced by the presence of GABA.

The existence of specific BZDRs suggests, that the mammalian brain may naturally be controlled by endogenous ligands Costa and Guidotti (1985). Due to the lack of suitable bioassays *in vitro,* the inhibition of ^{3}H-BZD binding by putative endogenous BZDR ligands is the method used preferentially for investigation of this hypothesis. Up to now some small molecules with low to moderate affinity to the BZDR were suggested to function as endogenous effectors of the BZDR, including purines Skolnick, Marangos, Goodwin, Edwards and Paul (1978), nicotinamide Möhler, Polc, Cumin, Pieri and Kettler (1979) or N-acetyl-5-methoxy-kynurenamine Marangos (1981).

Despite the low receptor affinity of most of these substances, they were considered to possibly be endogenous agonists at the BZDR because of physiologic effects.

The identification of a potent endogenous ligand, which interacts specifically with the BZDR would have far-reaching implications in neurobiology and possibly on the development of new drugs for the treatment of anxiety, the most common psychic disorder.

Here the isolation from plasma and a partial characterization of an endogenous BZDR agonist (EBA) is described, which exhibits an apparent BZDR affinity similar to that of Diazepam (DZP). This is a marked difference from all ligands suggested so far to possibly function as endogenous agonists.

Materials and Methods

Extraction of EBA

Blood samples were collected by venipuncture of a forearm (humans), an ear vein (rabbits) by decapitation (rat) or by section of a neck artery (pig). Per ml blood 1.5 mg of EDTA-K_2 were added. After acidification to pH 5.0 with 1 N acetic acid plasma was centrifuged at 4000 × g for 10 minutes and ultrafiltrated at 3.0 bar, using a Diaflow filtration device and a PM 30 membrane and further purified by passage through a YM 2 membrane. All membranes and the filtration apparatus were from Amicon (Witten, F.R.G.). The remaining filtrate was evaporated to dryness in vacuo and resuspended in 1/10 of the initial plasma volume of bidestilled water. The concentrate was chromatographed on a reversed phase high performance liquid chromatography (HPLC) column (200 × 4 mm, C-18 Nucleosil, particle size 5 μm from Macherey and Nagel, Düren, F.R.G.) equilibrated with 10 percent acetonitrile, 90 percent H_2O, 0.1 percent trifluroacetic acid. The column was developed with a 0–60 percent linear gradient of 90 percent acetonitrile, 10 percent H_2O, 0.1 percent trifluroacetic acid over 60 minutes. Solvents were degassed by helium. The flow rate was 1 ml per minute, the column pressure 2000–2200 psi. The HPLC equipment was from Waters (Eschborn, F.R.G.) and consisted of the absorbance detector 440 with a 254 nm filter, two HPLC pumps 510 and an automated gradient controller. Absorbance was recorded on a LKB dual channel recorder (Bromma, Sweden). One minute HPLC fractions (1 ml) were collected and brought to dryness under a stream of nitrogen and dissolved in 200 μl of bidestilled water, if not otherwise stated.

Binding Assay

Rat brains were homogenized in 10 volumes of 0.3 M sucrose using a potter Teflon homogenizer (Braun, Melsungen, F.R.G.) and centrifuged at 5000 × g for 10 minutes. The supernatant was recentrifuged at 47,000 × g for 20 minutes. The pellet was resuspended in 20 volumes of Tris-HCl buffer (50 mM, pH 7.4) and incubated for 30 minutes at 37 °C. After centrifugation at 47,000 × g for 20 minutes the pellet was resuspended in 20 volumes of buffer and stored frozen in small samples. Immediately before use of receptor membrane preparation, aliquots were further diluted sixfold with buffer. From the final dilution 400 μl were employed per assay which were run in quadruplicate.

Fifty μl aliquots of samples or DZP standards were tested in a competition BZDR binding assay containing 0.5 nM ^{3}H-FNZ.

In a total volume of 0.5 ml incubation was performed at 6 °C for 15 minutes and terminated by vacuum filtration through GF/C filters from Whatman using a cell harvester (Brandel Biomedical Research, Gaithersburg, Maryland, U.S.A.). In controls, 21 ±1.6 percent of total ^{3}H-FNZ label were bound under these conditions. The interassay variation of the binding studies was less then 10 percent. Membrane bound radiolabelled ligand was

quantified in a Packard scintilation counter, using 4 ml Aquasol cocktails in minivials (Zinsser Frankfurt, F.R.G.).

UV-Spectroscopy

UV-spectra of 2×10^{-7} M Diazepam (DZP) and of an equal amount of EBA, as judged by UV_{254} absorption, either dissolved in absolute methanol at neutral pH and after acidification with acetic acid were run from 180–300 nm, using a Kontron Uvicon 860 spectrometer (Kontron, Zürich, Switzerland).

Conditioning of Rats

Sprague Dawley rats, males 200–250 g (Winkelmann, Borchen, F.R.G.) were housed as usual in a 12 hour day/night cycle, with food ad libitum, but without access to water for two days. Six hours of rehydratization was allowed before sacrifice.

Chemicals

Ethyl-β-carboline-3-carboxylate (CCE) and other harmane derivates were from RBI (Wayland, MA, U.S.A.). ^{3}H-Prazosin (26 Ci/mmol) was from Amersham-Buchler (Braunschweig, F.R.G.). ^{3}H-Flunitrazepam (FNZ) (79.2 Ci/mmol), ^{3}H-Diazepam (DZP) (90 Ci/mmol), ^{3}H-Naloxone (56 Ci/mmol), ^{3}H-SKF 10 047 (48,6 Ci/mmol), ^{3}H-Spiroperidol (29 Ci/mmol) and Aquasol were from NEN (Dreieich, F.R.G.), ^{3}H-Ro 15-1788 (71,4 Ci/mmol), cold DZP and Ro 15-1788 were gifts from Hoffmann-La Roche (Basel, Switzerland). TFA (Merck, Darmstadt, F.R.G.) acetonitrile and water (Baker, Phillipsburg, NJ, U.S.A.) were of HPLC grade. Enzymes and all the other chemicals used, were purchased from Sigma (F.R.G.).

Results

Extracts of human, rabbit, rat and pig plasma showed inhibitory activity of ^{3}H-FNZ binding to synaptosomal rat preparations.

EBA was quantified in relation to DZP utilizing the UV absorbance of both compounds at 254 nm.

The quantities of EBA found in the plasma of different species, expressed as DZP equipotence in the ^{3}H-FNZ binding assay were found to be rather variable (Tables 1 and 2).

The quantity of EBA which gives a peak-height of 3 mm was found to be equivalent to 60 pmol DZP in the ^{3}H-FNZ binding assay. This amount could be isolated from one ml of rat plasma (Fig. 1 A).

Inhibitory potency of whole plasma was to a minor extent dependent on hemolysis. After passage through PM 30 membranes, which have a cut off near 30,000 Dalton, 80.2 ± 10.4 % of genuine inhibitory potency was found in the ultrafiltrate. No activity was retained

Table 1. *EBA concentrations in the plasma of various animal species*

Species	Procedure of blood collection	Plasma volume (ml)	DZP equivalents (pmol/ml plasma)
Rabbit	venipuncture of an ear, samples from 3 animals pooled	21	23
Rabbit	blow on the neck decapitation	64	11
Pig	slaughterhouse	1200	5
Rat	ether anesthesia decapitation 10 conditioned rats	25	60

The potency of EBA to inhibit ^{3}H-FNZ binding was determined as described under "Methods" and is expressed in terms of DZP equivalents.

Table 2. *EBA in the plasma of normal adult humans*

Person	Age (years)	Samples tested	DZP equivalents (pmol/ml)
♀	53	2	1.5 ± 0.5
♀	26	2	6.5 ± 0.5
♀	59	2	0 ± 0
♂	38	2	3 ± 1
♂	39	2	5 ± 2
♂	56	5	4 ± 2.9

See Table 1. The same set of filter membranes was used throughout these studies. DZP equivalents are means of 2–5 determinations ± S.E.M.

by the passage through the YM 2 membrane (82.3 ± 8.9 %) which cuts off nominally at 1000 Dalton.

HPLC analysis showed that the remaining competitive capacity emerging from the column for up to 45 minutes was represented by one single peak which had a retention time of 32.2 ± 0.6 minutes. Under identical conditions DZP had a retention time of 31.0 ± 0.4 minutes (Fig. 1 A-1, 2). Concentrations of inosine and nicotinamide, which have a comparable OD at 254 nm, did not interfere with ^{3}H-FNZ binding up to the μmolar level (data not shown).

In concentrations which yield a UV absorbance in the magnitude of EBA, authentic CCE and DZP showed similar displacements of ^{3}H-FNZ. HPLC fractions collected at the retention time of DZP and

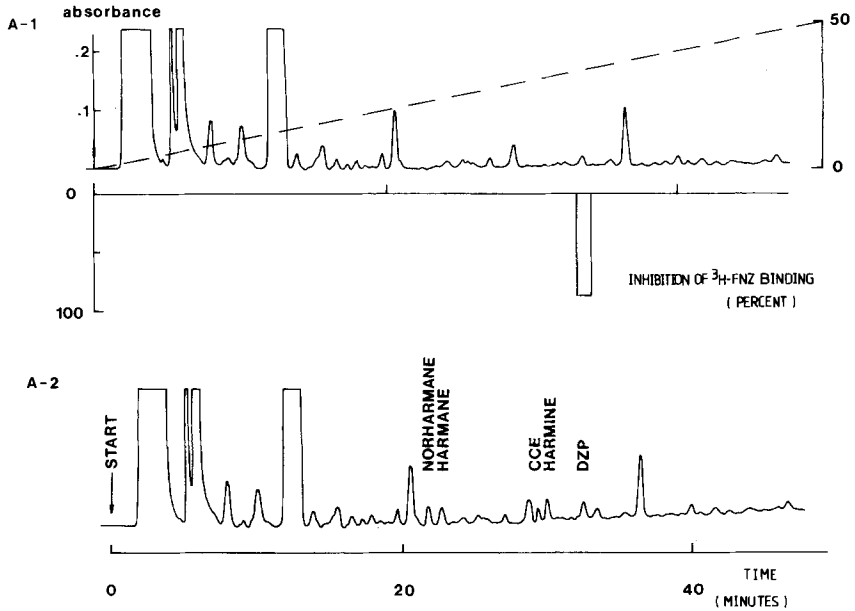

Fig. 1. *A-1* original HPLC separation of concentrated ultra-filtrate from 1 ml of rat plasma; *A-2*, *A-1* with the addition of 100 pmol of norharmane, harmane, harmine and CCE, respectively. Inhibition of ^{3}H-FNZ binding was assayed in 1 ml (1 minute) fractions of the eluate. They were evaporated under nitrogen and redissolved in 50 μl of H_2O

of various β-carbolines, including CCE exhibited no displacing potency in the BZDR assay.

Assuming that the degrees of molar absorbance of DZP and EBA are comparable, the endogenous ligand would show a receptor affinity in the range of DZP (Fig. 2).

The recovery of EBA activity from the HPLC column was found to be about 70 percent; in the 0.1–1 μmolar range, 25–30 percent of DZP was lost by an equal HPLC separation.

Binding to the BZDR is selective because EBA did not compete with ligands of other binding sites tested, such as opiate (^{3}H-SKF 10 047 and ^{3}H-Naloxone), adrenergic (^{3}H-Prazosin) and serotonergic (^{3}H-Spiroperidol) in concentrations 10 times higher than those eliciting half maximal inhibition of ^{3}H-FNZ binding at the BZDR (data not shown).

The displacement of ^{3}H-Ro 15–1788 from BZDR by EBA was increased in the presence of 5 μM GABA. Similarly, GABA influenced the inhibitory action of DZP on the antagonist binding. Opposite effects were found for CCE (Table 3).

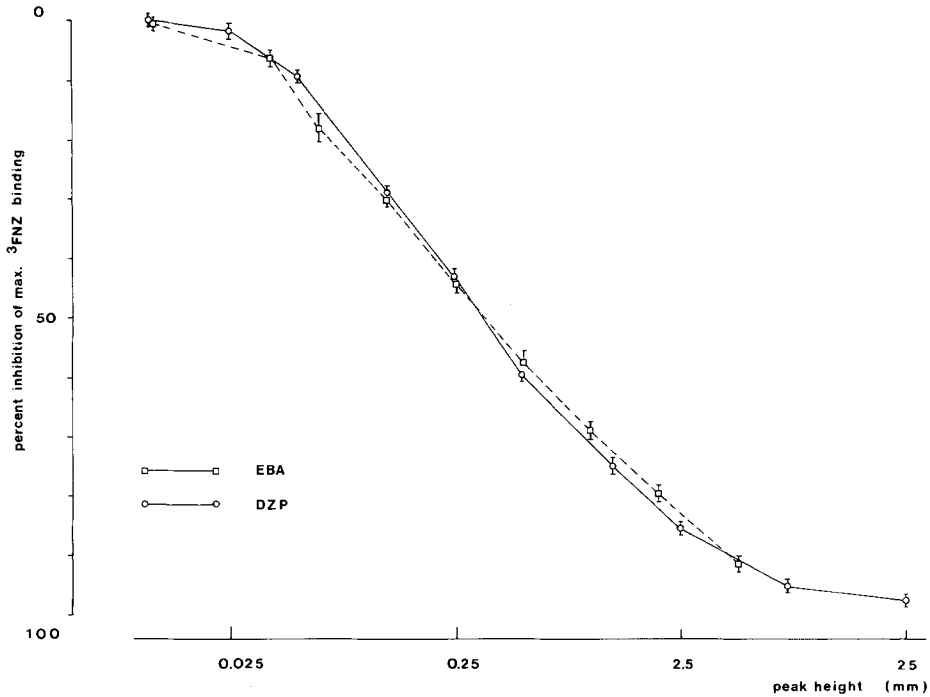

Fig. 2. The quantities of the competitive substances EBA isolated from rat plasma and DZP are defined as a function of the recorded HPLC-absorbance at wavelength 254 nm. An absorbance 0.1 corresponded to a recorder deflection of 20 mm. Fifty μl of 10^{-6} M DZP (50 pmol) corresponded to a peak height of 2.5 mm. Values are means of three determinations. Bars are S.E.M.

Table 3. *Inhibition of ^{3}H-Ro 13-1788 binding to rat brain membranes by EBA is increased by GABA*

Competitor	5×10^{-6} M GABA	without GABA
None	100	100
10^{-8} M DZP	48.5 ± 0.6	55.7 ± 0.2
EBA	39.1 ± 0.4	47.0 ± 0.1
4×10^{-9} M CCE	57.3 ± 1.1	54.4 ± 0.7

Rat brain membranes were incubated in the presence of 5 μM GABA together with 0.56 nM ^{3}H-Ro 15-1788 and 50 μl of the competitive substances in a total volume of 0.5 ml Tris-HCl buffer (50 mM, pH 7.4) at 6 °C for 15 minutes. Values are expressed as percent ^{3}H-Ro 15-1788 binding of controls and are means of 3 quadruplicate experiments $\pm$ S.E.M. The EBA concentration was equivalent to 2.5×10^{-8} M DZP.

Incubation of human and rat EBA for 20 minutes at 95 °C and the incubation for 2 hours at 35 °C in the presence of various proteases including trypsin, chymotrypsin, pepsin and pronase did not result in a diminution of EBA displacement capacity.

At neutral pH, the UV-spectra of DZP and EBA isolated from rat plasma showed maximal absorption at the same wavelength (201 nm). After acidification maxima shifted to 225 nm (DZP) and 232 nm (EBA), respectively. These results indicate that the two substances investigated might be structurely related, but are not identical.

Discussion

Here we describe a simple method for the isolation of an endogenous substance of low molecular weight form plasma displaying a high affinity to the BZDR. Binding of the substance to the BZDR is increased by GABA which indicates an agonistic action at the binding site. EBA quantitatively passes filter membranes cutting off at the molecular weight range of 1000 Dalton. The molecular size of EBA could thus approximate the magnitude of the BZDs. As found in preliminary experiments, the substance is heat stable and resistant to various proteases, which must not necessarily exclude a peptide structure. The shift of the EBA absorbance maximum found after acidification could possibly indicate the existence of a tautomeric structure and/or the protonization of the EBA molecule.

In preconditioned rats physiological concentrations of EBA present in 1 ml of blood (about 0.5 percent of body weight) caused up to half maximal inhibition of ^{3}H-FNZ binding in corresponding 0.5 percent aliquots of rat brain membrane preparations *in vitro*.

The amount of EBA normally found in human plasma was 1 to 10 pmol DZP equivalents per ml.

There must exist central and/or peripheral sources of biosynthesis and release of these endogenous substances. In this respect, adrenals and the pituitary gland could be of upmost importance. In the adrenals of preconditioned rats, EBA was found up to five thousand times more concentrated—based upon organ weight—than in the corresponding plasma. Similar values were found for the pituitary gland (in preparation).

EBA was also found in peripheral tissues of various species and in man. None of the persons tested had taken minor tranquilizers or any other drugs for at least several months. All animals tested had been BZD naive. It appears highly unlikely therefore that exogenous BZDs or other drugs like β-carbolines might have interfered with our

results. DZP exhibits a similar apparent BZDR affinity, but was been distinguished by both UV analysis and HPLC separation.

Among the β-carboline family only CCE and methyl-6,7-dimethoxy-4-ethyl-β-carboxyline-3-carboxylate possess comparable BZDR binding capacity. The HPLC retention time and the different modulation of the BZDR affinity for CCE and EBA in the presence of GABA are clear discriminating parameters.

Recently, the existence of small BZD-like molecules in the soluble fraction of rat brain was demonstrated using monoclonal Ab in immunoaffinity chromatography Sangameswaran and De Blas (1985). At the moment it cannot be ruled out, that these molecules of central origin are related or even identical with the peripheral EBA described here. Some molecular characteristics are indeed corresponding, but in rat central nervous system only small amounts of EBA were found using the filtration technique, possibly because precursors of higher molecular weight remain excluded by this method. High quantities of EBA however can be extracted from hypophysis and the adrenals of the rat (in preparation). These glands might be sources for the peripheral occurrence of EBA.

Monitoring EBA in plasma could provide an important diagnostic tool for discerning anxiety disorders and even psychoses. Levels of the endogenous ligand should be in correlation to intra- and even interindividual variations in anxiety. Of course other parts of the BZDR complex, especially receptor sensitivity and availability as well as GABA levels might contribute to the appearance of both normal and pathologic forms of anxiety.

If the penetration in to the central nervous system and the anti-conflict potency of EBA can be demonstrated, there is a good chance, that *Endotran* (*endo*genous *tran*quilizer), the elusive endogenous ligand, can be identified.

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