Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors

(Abbreviations: GABA, y-aminobutyric acid; DBI, diazepam-binding inhibitor; QNB, quinuclidinyl benzilate)

A brain polypeptide termed diazepam-binding inhibitor (DBI) and thought to be chemically and functionally related to the endogenous effector of the benzodiazepine recognition site was purified to homogeneity. This peptide gives a single band of protein on NaDodSO~4~ and acidic urea gel electrophoresis. A single UV-absorbing peak was obtained by HPLC using three different columns and solvent systems. DBI has a molecular mass of ~11,000 daltons. Carboxyl-terminus analysis shows that tyrosine is the only residue while the amino-terminus was blocked. Cysgen bromide treatment of DBI yields three polypeptide fragments, and the sequences of two of them have been determined for a total of 45 amino acids. DBI is a competitive inhibitor for the binding of $[{}^3H]$diazepam, $[{}^3H]$flunitrazepam, $[{}^3H]$carboline propyl esters, and $[{}^3H]$imipramine binding were inhibited by diazepam and 3H-labeled Ro 15-1788. The $K_d$ for $[{}^3H]$diazepam and $[{}^3H]$carboline binding were 4 and 1 $\mu$M, respectively. Doses of DBI that inhibited $[{}^3H]$diazepam binding by >50% fail to change $[{}^3H]$etorphine, $\gamma$-aminooxy$[{}^3H]$butyric acid, $[{}^3H]$quinuclidinyl benzilate, $[{}^3H]$dihydrolpromolol, $[{}^3H]$adenosine, and $[{}^3H]$imipramine binding tested at their respective $K_d$ values. DBI injected intraventricularly at doses of 5–10 nmol completely reversed the anticonflict action of diazepam on punished drinking and, similar to the anxiety-inducing $\beta$-carbol ine derivative FG 7142 ($\beta$-carboline-3-carboxylic acid methyl ester), facilitated the shock-induced suppression of drinking by lowering the threshold for this response.

Specific high-affinity binding sites for benzodiazepines are located in synaptic membranes prepared from various regions of mammalian central nervous system (1–3). In various species, man included, the occupation of benzodiazepine recognition sites by specific ligands such as diazepam or $\beta$-carbolines induces behavioral changes that oscillate from the relief to the induction of anxiety states characterized by fear of punishment (4–6). For instance, in rats diazepam elicits anticonflict and anxiety-abating responses (4, 5) whereas a number of $\beta$-carboline derivatives elicit proconflict and anxiety-inducing effects (7). In line with reports (8) proposing that ligands of benzodiazepine recognition sites modulate $\gamma$-aminobutyric acid (GABA) receptors, it has been shown that, in vitro, some ligands down regulate and others up regulate GABA recognition sites located in crude synaptic membrane preparations (6). The down regulation has predictive value for the in vivo proconflict action, whereas the up regulation predicts anticonflict action (7).

Thus, it can be proposed that benzodiazepine recognition sites are modulatory sites of GABA receptors and it can be anticipated that the benzodiazepine sites are physiologically occupied by endogenous modulators that, by tuning GABA receptors, elicit a spectrum of behavioral states ranging from anxiety to sedation. Several brain components have been suggested to function as physiological ligands for the benzodiazepine–GABA receptor system (9–16). However, for most of these substances, the evidence that they may act as neuromodulators of GABA receptors is lacking. This report describes the isolation and purification to homogeneity of a rat brain peptide that competitively inhibits benzodiazepine binding to their recognition sites. This peptide was termed DBI (diazepam-binding inhibitor). When injected intraventricularly, this peptide blocks the anticonflict effect of benzodiazepines and facilitates the behavioral suppression induced by punishment. This action of DBI is blocked by previous treatment with Ro 15-1788, a ligand of benzodiazepine recognition sites devoid of intrinsic activity (17). DBI antagonizes the benzodiazepine-elicited increase in the maximal binding value of GABA to its high-affinity recognition site.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (175–200 g) were obtained from Zivic-Miller (Allison Park, PA).

Materials. Sephadex G-100 and G-75 were purchased from Pharmacia. Bio-Gel P-2 and a Bio-Sil ODS-10 reversed-phase HPLC column were obtained from Bio-Rad. The Zorbax CN HPLC column was from DuPont and the Synchromak AX300 anion exchange HPLC column was from Synchrochrome (Lincoln, IN); trypsin (ketone treated) and chymotrypsin were from Worthington; Fronase and papaine were from Sigma; FG 7142 ($\beta$-carboline-3-carboxylic acid methyl ester), $\beta$-carboline-3-carboxylic ethyl ester, and $\beta$-$[{}^3H]$carboline-3-carboxylic propyl ester were a gift from C. Braestrup (Ferrosan, Copenahgen, Denmark); diazepam, Ro 15-1788 (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4] benzodiazepine-3-carboxylate), and $[{}^3H]$labeled Ro 15-1788 were gifts from H. Mohler (Hoffman–LaRoche). Pyroglutamate aminopeptidase was obtained from Boehhringer Mannheim. All the radioactive receptor ligands were purchased from New England Nuclear.

HPLC. Analysis of peptides was carried out by using a Spectrophysics liquid chromatography system equipped with a Spectro-Physics model 8700 or 8000B solvent delivery system. Peptides were detected by monitoring absorbance at 210 nm or by colorimetric reaction using the Lowry method (18).

Abbreviations: GABA, $\gamma$-aminobutyric acid; DBI, diazepam-binding inhibitor; QNB, quinuclidinyl benzilate.

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periments with polycrylamide gels 2.8 mm thick. Urea/acid pH (pH 3.2) gels were prepared according to the procedure of Panyim and Chalkley (19). NaDodSO4 gels were run according to Laemmli and Favre (20) with minor modifications. The NaDodSO4 gel was calibrated using the "low molecular weight" protein calibration kit from Pharmacia. Proteins were stained with Coomassie brilliant blue G.

**Bioassay for DBI-like Activity.** This assay measures the ability of tissue extracts and purified proteins to competitively inhibit the binding of [3H]diazepam or other [3H]-labeled benzodiazepine ligands to Triton X-100 (0.05%)-treated crude synaptic membranes (13). Triton X-100 was washed from the membranes before the assay. The assay (final vol, 1 ml) was carried out in 20 mM potassium phosphate buffer (pH 7.0). Components of the mixture were added to the reaction tubes in the following order: suspension of synaptic membranes (0.3–0.4 mg of protein), tissue extract to be tested for DBI-like activity or control buffer (10–100 μl), and [3H]benzodiazepine at various concentrations (50 μl of buffer). Nonspecific binding (usually 10% of total binding) was determined by adding 5 μM diazepam. After 30 min of incubation at 2–4°C, the reaction was terminated by rapid filtration on GF filters (13). The effect of DBI on [3H]pentobarbital (21), [3H]adenosine (22), [3H]imipramine (23), 3-[3H]quinuclidinyl benzilate (24), [3H]dihydroalprenolol (25), and [3H]CABA (13) binding was studied by previously published methods using a concentration of the ligand in the Kd range.

**Stimulation of [3H]CABA Binding by Diazepam.** The diazepam-induced increase of [3H]CABA binding was studied in frozen-thawed and three-times-washed cortical synaptic membranes using 20 nM [3H]CABA in 20 mM KPO4/50 mM KCl, pH 7. Diazepam was incubated for 10 min at 2–4°C with the membranes prior to addition of the radioactive ligand. The reaction was stopped by centrifugation 10 min later (13).

**DBI Iodination.** DBI was iodinated with 125I using the Bolton–Hunter reagent. Briefly, 10 μg of protein was treated for 1 hr with 250 μCi of Bolton–Hunter reagent (1 Ci = 37 GBq) at 0°C. The reaction was stopped by the addition of 200 μl of 1 M acetic acid, and the sample was applied to a 0.7 × 10 cm Bio-Gel P-2 column equilibrated with 0.1 M acetic acid. The purity of the iodinated material was tested by both NaDodSO4/polyacrylamide gel electrophoresis and reversed-phase HPLC.

**Analysis of Amino Acid Composition of Peptides.** The protein was hydrolyzed with 6 N HCl for 2 hr at 110°C at reduced pressure. Analysis was performed by a Beckman 121MB single-column system with ninhydrin detection (26).

**Peptide Cleavage by CNBr and Separation of Peptide Fragments.** The cleavage of 100 μg of the protein by CNBr was carried out in 70% formic acid. The sequence of the resulting mixture was determined (see below). An additional 150 μg was cleaved with CNBr and the mixture was separated by HPLC on a 4.5 × 30 cm WatersC18 column. The initial solvent was 0.02% trifluoroacetic acid in water and the peptides were eluted with a linear gradient from 100% of 0.02% trifluoroacetic acid in water to 50% acetonitrile in water over 30 min. Peptides were identified by their absorption at 210 nm, collected, and lyophilized. The sequence of the only single peptide (see Discussion), which eluted at 15.6 min, was determined.

**Amino Acid Sequence Determination.** Sequences were determined by using a Beckman model 890C Sequencer (spinning cup), slightly modified, with 4 mg of Polybrene in the cup (27). The phenylthiohydantoin amino acid derivatives were separated and quantitated by HPLC using a Zorbax CN column.

**Pyroglutamate Aminopeptidase Digestion.** The method of Castel et al. (28) was used. After 24 hr of digestion, no NH2-terminal amino acid could be detected in the Sequencer.

**Evaluation of Proconflict and Anticonflict Activities.** These experiments were conducted in rats kept without water for 72 hr prior to the conflict session. Apparatus and other experimental conditions were identical to those reported previously (7). A chronic intraventricular polyethylene cannula was implanted in the left lateral brain ventricle (29) 3 days before the experiment.

To study the “anticonflict” action, the intensity of the current delivered through the drinking tube was 1 mA for a duration of 0.5 sec. To study the “proconflict” action, the shock duration was left constant but the intensity was decreased to 0.25 mA. To study the effect of the tested compound on thirst (unpunished drinking), the shock was omitted. At the time of the test, each rat was allowed to habituate to the chamber without drinking for 15 min. At the end of this period, the animals received an intracerebroventricular injection of DBI or vehicle. After 3 min, the drinking tube was inserted into the cage and the animals were allowed to lick for 3 sec (one "licking period") before the first shock was delivered. A 3-min test period started at the end of the first shock.

**RESULTS**

**Extraction and Purification of DBI.** Typically, 20 rat brains were used for each preparation. After decapitation of the rat, the brain was rapidly (15–30 sec) removed and homogenized in 10 vol of hot (80°C) 1 M acetic acid. The homogenate was centrifuged at 48,000 g for 20 min at 2–4°C, and the supernatant was adjusted to pH 5 and centrifuged under identical conditions. This second supernatant was lyophilized, resuspended in 10 ml of 0.1 M acetic acid and centrifuged again at 48,000 × g, and this supernatant was applied to a 2.5 × 70 cm Sephadex G-100 column previously equilibrated with 0.1 M acetic acid. The column was eluted with 0.1 M acetic acid at a flow rate of 0.2 ml/min, and 5-ml fractions were collected. Each fraction was lyophilized and resuspended in 1 ml of water, and aliquots of 10–100 μl were used for monitoring DBI-like activity and protein content. Proteins with DBI-like activity emerged from this column in the eluate included between the elution positions of ribonuclease A (Mr, 13,700) and 2-mercaptoethanol (Mr, 78). The fractions containing DBI activity were pooled, lyophilized, resuspended in 10 ml of 0.1 M acetic acid, and rechromatographed on a 2.5 × 70 cm Sephadex G-75 column. This column was eluted with 1 M acetic acid at a flow rate of 0.2 ml/min, and fractions of 5 ml were collected. Again, DBI activity emerged with the eluate included between markers of 100 and 14,000 daltons. The fractions containing DBI were lyophilized to dryness, resuspended in 10 ml of 0.1 M acetic acid, and treated with 60% saturated ammonium sulfate. The 48,000 × g supernatant was desalted on a 0.7 × 10 cm Bio-Gel P-2 column previously equilibrated with 0.1 M acetic acid. DBI emerged from this column with the void volume well separated from the bulk of salts.

After lyophilization, the residue was taken up in 1–2 ml of 1 M acetic acid. Aliquots containing 100–200 μg of protein were applied to a Bio-Sil ODS-10 (Bio-Rad) reversed-phase HPLC column previously equilibrated with 0.1 M Na2HPO4/0.1% H3PO4, pH 2.5, and eluted with an acetonitrile gradient of 0% to 60% (Fig. 1A) and then applied to a Bio-Sil ODS-10 reversed-phase HPLC column previously equilibrated with 0.1% trifluoroacetic acid in H2O and eluted with a 0–70% gradient of 0.1% trifluoroacetic acid/acetonitrile (Fig. 1B). DBI-like material eluted from the second HPLC step (Fig. 1B) was examined for purity using different HPLC and acrylamide gel electrophoretic systems.

As shown in Fig. 2, 50 μg of protein gave a single UV-ab-
FIG. 1. HPLC procedure for DBI purification. (A) Reversed-phase HPLC of 250 µg of protein with DBI-like activity. The sample was first purified by chromatography on Sephadex G-100 and G-75, ammonium sulfate precipitation, and Bio-Gel P-2 chromatography (see Table 1). Conditions were column, Bio-Sil ODS-10 (4 x 250 mm); flow rate, 1 ml/min; temperature, 20°C. After sample application, the column was washed for 15 min with the starting buffer (0.1 M NaH2PO4/0.1% M Na2HPO4, pH 2.5). Peptides were eluted with a gradient from 100% starting buffer to 0% acetonitrile under isocratic conditions. (B) Column, Bio-Gel ODS-10 equilibrated with 0.1 M NaH2PO4/0.1% H3PO4, pH 2.5; elution, 60-min linear gradient (----) of 0-60% acetonitrile. Each column was charged with 50 µg of DBI.

Fig. 2. HPLC analysis of purified DBI. DBI obtained from reversed-phase HPLC (Fig. 1B) was tested for purity under three conditions. (A) Column, Synchropak AX300 oxiranes anion exchange resin equilibrated with 0.02 M NaH2PO4, pH 8.3; flow rate 1 ml/min. DBI eluted with the void volume. No other proteins were eluted when the column (at 60 min) was eluted with a linear gradient from 0.02 M NaH2PO4 to 0.2 M NaH2PO4/0.1 M NaClO4, pH 8.3 (-----). (B) Column, Bio-Sil ODS-10 equilibrated with 0.1 M NaH2PO4/0.1% H3PO4/30% acetonitrile under isocratic conditions; flow rate, 1 ml/min. (C) Column, Zorbax CN equilibrated with 0.1 M NaH2PO4/0.1% H3PO4, pH 2.5; elution, 60-min linear gradient (-----) of 0-60% acetonitrile. Each column was charged with 50 µg of DBI.

A 15% acidic urea gel (Fig. 3) produced a single band staining with Coomassie blue.

An average preparation from 20 brains yielded 20-25 µg of DBI. By adding 2 µg of 125I-labeled DBI (specific activity, 5 x 10^6 cpm/ng) to the initial homogenate, we established that the recovery was 1.5-2%.

DBI Characterization. The amino acid composition of DBI is shown in Table 1. The protein contains 105 amino acid residues and basic residues, particularly lysine, are relatively abundant. As expected, DBI biological activity was abolished by incubation with trypsin, chymotrypsin, or papain.

Initial sequence determination of DBI showed that the amino terminus is blocked. The blocking group was not removed by digestion with pyroglutamate aminopeptidase.

Treatment of the protein with CNBr produced two amino

Table 1. Amino acid composition of DBI purified from rat brain

<table>
<thead>
<tr>
<th>Residue</th>
<th>Relative amount, number of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagine/aspartate</td>
<td>10</td>
</tr>
<tr>
<td>Threonine</td>
<td>7</td>
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<tr>
<td>Serine</td>
<td>10</td>
</tr>
<tr>
<td>Glutamate/glutamine</td>
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<td>Proline</td>
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<tr>
<td>Glycine</td>
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<td>Alanine</td>
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<td>Cysteine</td>
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<td>Valine</td>
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<tr>
<td>Methionine</td>
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<td>Leucine</td>
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<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Lysine</td>
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</tr>
<tr>
<td>Arginine</td>
<td>3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
</tr>
</tbody>
</table>

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terminals. Sequence determination of the mixture proceeded for 30 steps with one sequence terminating abruptly at step 14. A second sample was cleaved with CNBr and separated on HPLC. Under the conditions of separation, peptides containing homoserine elute as pairs of peptides 4 min apart, due to partial homoserine lactone formation. The sequence of the only single peptide eluted was determined. It contained 14 amino acids and had a COOH-terminal tyrosine. Since it did not contain homoserine, it must be the carboxy-terminal peptide in the protein. With this information, a partial sequence could then be assigned to the other (middle) peptide (Table 1).

The molecular weight of DBI was estimated to be approximately 10,000 by NaDodSO₄ gel electrophoresis and was calculated on the basis of amino acid composition to be 11,700.

**Binding Studies.** DBI is a competitive inhibitor for the binding of [³H]diazepam, [³H]lunizapane, β-[³H]carboline ethyl ester, and [²H]-labeled Ro 15-1788. The Kᵢ for [³H]diazepam binding was approximately 4 μM and that for β-[³H]carboline ethyl ester, 1 μM. Doses of DBI that inhibited by more than 50% [³H]diazepam and β-[³H]carboline binding failed to inhibit [³H]etorphine, [³H]GABA, [³H]quinulinidimyl benilate, [³H]dihydroalprenolol, [³H]adenosine, and [³H]nimipramine binding when tested at their respective Kᵢ values. Because DBI inhibits the binding of agonists and antagonists of benzodiazepine receptors, we tested whether DBI has binding characteristics similar to those of benzodiazepines or β-carbolines.

In a first group of experiments, we studied the inhibition of [³H]-labeled Ro 15-1788 binding by DBI, β-carboline-3-carboxylic acid ethyl ester, and diazepam in the presence and absence of 0.1 mM GABA. The inhibition of [³H]-labeled Ro 15-1788 binding by diazepam was potentiated by GABA. In contrast, the inhibition by DBI and β-carbolines was not.

In a second group of experiments, the effect of DBI on the diazepam-induced increase in [³H]GABA binding was compared with that of β-carbolines. DBI, like β-carbolines, abolished the increase of [³H]GABA binding induced by diazepam.

**Behavioral Studies.** Intraventricular injection to rats deprived of water for 3 days and implanted with a cerebral ventricle cannula of DBI (50 or 100 μg) failed to alter un punished drinking or shock (1 mA)-suppressed drinking behavior. However, 50–100 μg of DBI injected intraventricularly almost completely reversed the anticonflict action of diazepam on punished drinking (Fig. 4). When the shock delivered through the drinking tube was reduced from 1 to 0.25 mA, intraventricular injection of 50–100 μg of DBI elicited a proconflict effect; that is, it facilitated the shock-induced suppression of drinking by lowering the threshold for this response. This facilitation was blocked by Ro 15-1788 (Fig. 4), and it was similar to that observed for the β-carboline derivative FG 7142.

**DISCUSSION**

Several reports (see ref. 8) have suggested that the benzodiazepine recognition sites are part of GABA receptors and might function as the recognition sites for a second synaptic signal that regulates the gain at which GABA receptors operate. Evidence that brain contains a constituent that displaces [³H]benzodiazepine from its binding sites includes the fact that the Kᵢ for [³H]diazepam binding to GABA-free crude synaptic membranes is reduced by approximately 50% by repeated washing of the membranes with low concentrations of detergent and that the extract obtained produces a dose-related reduction of the Kᵢ for [³H]diazepam binding without affecting the maximal binding capacity (13, 31).

In this report, we identify a peptide of Mᵣ ~11,000 that could be operative in eliciting changes in the Kᵢ of [³H]diazepam binding in brain homogenates.

This peptide was termed DBI, and its extraction was routinely carried out using as a starting material rat brain homogenized in hot (80°C) 1 M acetic acid. Important steps in the purification of DBI are the use of 1 M acetic acid at 80°C and treatment with 60% saturated ammonium sulfate of the material eluted from Sephadex G-75. This treatment precipitates approximately 80% of the proteins, leaving the DBI in solution in the 48,000 × g supernatant. Among the proteins precipitated by the ammonium sulfate treatment is GABA-modulin (30). Removal of GABA-modulin at this stage facilitates the purification of DBI because GABA-modulin is eluted in the HPLC reversed-phase procedure (Fig. 1) very close to DBI and because GABA-modulin also may inhibit [³H]diazepam binding by virtue of its role on the modulation of GABA recognition sites (30).

Brain DBI was purified to homogeneity, as indicated by the presence of a single band of protein on NaDodSO₄ and acidic urea gel electrophoresis and of a single protein peak on HPLC using three different column and solvent systems. In addition, the peptide present in the HPLC peak has DBI activity and contains tyrosine as a single carboxy terminus.

Experiments carried out to establish the amino acid sequence of this purified peptide showed that the NH₂-terminal amino acid is blocked. This blockade was not removed with pyroglutamate aminopeptidase; however, the presence of two methionine residues in the molecule has now allowed the gen-
neurotransmission of three fragments by cyanogen bromide treatment. The entire sequence of the carboxyl-terminal fragment was determined, and a partial sequence of the middle fragment was determined. These sequences do not resemble any known mammalian peptide sequence (32).

An important characteristic of DBI is that it is present at high concentrations (10–25 μM) in rat brain and only traces (less than 1 μM) were found in peripheral organs (liver, kidney, and spleen). DBI appears to interact with benzodiazepine binding sites, competitively inhibiting the specific binding of [3H]benzodiazepine or β-[3H]carboline derivatives; however, it fails to block the binding of a number of ligands to other transmitter recognition sites. DBI functionally interacts with the benzodiazepine recognition site in a specific manner that resembles anxiety-inducing benzodiazepines and steroids (6, 7). It differs from anxiety-abating benzodiazepines and from benzodiazepines that, like Ro 15-1798, bind to the benzodiazepine recognition site with high affinity but are devoid of intrinsic activity (17).

We considered whether a β-carboline structure formed during the DBI purification was associated with DBI. Experiments with trypsin, Pronase, and papain digestion, which resulted in complete loss of DBI activity, indicated that this is unlikely.

However, the similarity in the action of DBI with that of the anxiety-inducing β-carboline FC 7142 suggests that DBI is probably the precursor of a lower molecular weight endogenous effector of the benzodiazepine recognition site. This effector may modulate a specific set of GABA receptors and, when these receptors are down regulated, the onset of anxiety may be facilitated. Further studies of DBI and its active fragments may lead to the identification of endogenous physiologically important ligands for the benzodiazepine receptor. These ligands may provide a fine tuning for the GABAergic transmission, thereby modulating anxiety, excitability, and onset of sleep.

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