

Study of an octadecaneuropeptide derived from diazepam binding inhibitor (DBI): Biological activity and presence in rat brain

(β -carboline-3-carboxylate esters/proconflict response/benzodiazepines/ γ -aminobutyric acid)

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ABSTRACT An endogenous brain neuropeptide with 104 amino acid residues that modulates γ -aminobutyric acid receptor function was termed DBI because it displaces diazepam from its specific brain binding sites. Tryptic digestion of DBI generates an octadecaneuropeptide (ODN) that is more potent than the parent compound in the displacement of specifically bound β -[3 H]carboline-3-carboxylate methyl ester (3 H]BCCM) and in proconflict action (Vogel test in thirsty rats). The proconflict action of ODN is antagonized by the imidobenzodiazepinone Ro 15-1788, which is a specific antagonist of β -carboline and benzodiazepine recognition sites. The ODN amino acid sequence is Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys. The pharmacological properties associated with this sequence were confirmed by comparing the activity of ODN generated from tryptic digestion of DBI with that of ODN obtained by synthesis. Amidation of the terminal lysine of ODN produces a peptide (ODN-NH₂) devoid of pharmacological activity. Three peptides containing the COOH-terminal segment of ODN were synthesized. All these peptides [Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys (octapeptide), Pro-Gly-Leu-Leu-Asp-Leu-Lys (heptapeptide), and Gly-Leu-Leu-Asp-Leu-Lys (hexapeptide)] express the displacing and proconflict actions of ODN. In primary cultures of cerebellar granule cells of rat, DBI, ODN, octapeptide, heptapeptide, and hexapeptide preferentially displace 3 H]BCCM over 3 H]flunitrazepam; moreover, they displace bound 3 H]BCCM completely but 3 H]flunitrazepam only by 50%. These data suggest that ODN includes a specific ligand for the γ -aminobutyric acid receptor regulatory site occupied by β -carbolines. Using rabbit antibodies directed against the NH₂-terminal portion of ODN, we detected ODN-like material in rat brain homogenates. However, whether this material is identical to the ODN generated by tryptic digestion of DBI remains to be established.

Studies on the participation of γ -aminobutyric acid (GABA) in the action of anxiolytic benzodiazepines (BZs) and anxiogenic 3-carboxylate esters of β -carboline (BC) led to the discovery in rat and human brain of diazepam binding inhibitor (DBI), a naturally occurring neuropeptide that displaces BC and BZ from specific high-affinity recognition sites located in brain synaptic membranes (1-3). A partial amino acid sequence of rat DBI has been obtained (2, 3). The pharmacological effect of DBI has been tested in primary cultures of mouse spinal cord neurons on GABA-mediated Cl⁻ currents recorded in patch-clamp conditions (4) and in the Vogel procedure to detect modifications of rat operant behavior in conflict situations (2, 3). In both tests DBI mimicked the action of BC and antagonized the action of BZ. Since BZ/BC recognition sites were shown to be part of the supramolecular organization of postsynaptic GABA_A receptors (5-11), it was proposed that DBI might be the

precursor of the physiological effector that allosterically modulates the probability of Cl⁻ channel opening elicited by the activation of GABA_A receptors. This possibility was supported by the presence in the DBI tryptic digest of an octadecaneuropeptide (ODN) (12) eliciting proconflict responses in the Vogel test and displacing BC from specific binding sites. Since ODN-like peptides can be detected in rat brain homogenates, an attempt was made to determine the minimal amino acid sequence present in ODN that is required to elicit proconflict responses and to displace 3 H]BC. The results reported here suggest that ODN contains a specific domain endowed with BC-like activity.

MATERIALS AND METHODS

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

Materials. Bio-Gel P-6 was obtained from Bio-Rad. Staphylococcal protein A-Sepharose CL-4B was purchased from Pharmacia. Trypsin-TPCK (treated with tosylamido-phenylethyl chloromethyl ketone) was from Worthington. *o*-Phenylenediamine, staphylococcal protein A, and histone type II were from Sigma. Horseradish peroxidase conjugated to antibody specific for rabbit immunoglobulin was from Boehringer Mannheim. Diazepam, flunitrazepam (Flu), clonazepam, and Ro 15-1788 [ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazole-(1,5-*a*)-(1,4)-benzodiazepine-3-carboxylate] were from Hoffmann-La Roche (Basel, Switzerland), CGS 8216 [2-phenylpyrazolo-(4,3*C*)-quinolin-3(5*H*)-one] was from CIBA-GEIGY (Summit, NJ), and all the BC 3-carboxylate ester derivatives were from Ferrosan (Copenhagen, Denmark). The radioactive ligands for the binding assays were purchased from New England Nuclear. Synthetic ODN [QATVGDVNTDRPGLLDLK in the standard one-letter code (13)], its amide ODN-NH₂, and the other DBI peptide fragments (see Table 1) were synthesized by Peninsula Laboratories (San Carlos, CA).

Amino Acid Analysis and NH₂-Terminal Amino Acid Sequence Determination of the Peptides. The peptides (10 nmol) were hydrolyzed for 22 hr under reduced pressure in constant-boiling 6 M HCl at 110°C. Amino acid analysis was carried out with a Hitachi 835 high-speed amino acid analyzer equipped with a data processor. For NH₂-terminal amino acid sequence analysis the peptides (0.2-1 nmol) were loaded on a gas-phase sequencer (14) and submitted to 20-30 cycles of automated Edman degradation. Phenylthiohydantoin derivatized amino acids were identified by HPLC on an IBM cyano column. Details on the procedure have been described (15).

Abbreviations: GABA, γ -aminobutyric acid; BC, β -carboline; BCCM, β -carboline-3-carboxylate methyl ester; BZ, benzodiazepine; DBI, diazepam binding inhibitor; ODN, octadecaneuropeptide; ODN-NH₂, ODN amide; OP, octapeptide; HEP, heptapeptide; HEX, hexapeptide; Flu, flunitrazepam; i.c.v., intracerebroventricularly.

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Extraction and Purification of DBI. The procedure was the one described by Guidotti *et al.* (2). Final purification was achieved by using HPLC with a μ Bondapak C₁₈ reverse-phase column. The purity of the standard DBI preparation was checked by sodium dodecyl sulfate/15% polyacrylamide gel electrophoresis (16) and by amino acid analyses.

HPLC Separation of Peptides Generated by Trypsin Digestion of DBI. DBI was treated with trypsin-TPCK for 24 hr at 30°C in 1 ml of 0.1 M NH₄CO₃ buffer, pH 8.3. The ratio of trypsin to substrate was 1:10 (mol/mol). The peptides were separated by reverse-phase HPLC on a μ Bondapak C₁₈ column (7.8 × 30 mm) by eluting with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Peptides were detected by monitoring absorbance at 210 nm.

Binding Studies. The displacement of [³H]Flu or β -[³H]-carboline-3-carboxylate methyl ester ([³H]BCCM) bound to intact cerebellar granule cells maintained in primary cultures was used to evaluate the displacing potency of various peptides. Primary cultures of cerebellar granule cells were prepared from 8-day-old rats according to the method of Wilkin *et al.* (17), following the modification proposed by Gallo *et al.* (18). These cerebellar neuronal cultures were studied immunohistochemically with a batch of cell markers; 95% of the total cell population consists of neurofilament-positive neuronal elements. About 95% of these neuronal elements are granule cells, and only a small percentage (2–3%) are GABAergic interneurons as evidenced by glutamic acid decarboxylase immunoreactivity. Binding studies were carried out on cultures plated on 35-mm dishes. The dish was washed twice with 2 ml of Locke's solution (154 mM NaCl/5.6 mM KCl/3.6 mM NaHCO₃/2.5 mM CaCl₂/1 mM MgCl₂/5.6 mM glucose/5 mM Hepes, pH 7.4) and then incubated with 1 ml of Locke's solution for the indicated time and temperature in the presence of [³H]BCCM or [³H]Flu with or without the peptide to be tested. The assay was stopped by washing the cells rapidly (three washes in 8 sec with Locke's solution) and resuspending them in 1 ml of 0.1 M NaOH. A 0.5-ml aliquot of this suspension was used for radioactivity measurement and a 0.1-ml aliquot was used for protein determination (19). Nonspecific binding was taken as the radiolabeled ligand bound to cells in the presence of 2 μ M CGS 8216.

Evaluation of Proconflict Activity According to Vogel. These experiments were conducted in rats kept in a water-free environment for 72 hr. We used the punished-conflict drinking procedure developed by Vogel *et al.* (20) with the modification introduced by Corda *et al.* (21) to evaluate proconflict activity of anxiogenic drugs. To study the proconflict activity of various peptides the intensity of the punishing shock was maintained at 0.15 mA, while to test the peptide effect on thirst (unpunished drinking) the shock was omitted. Peptides (10 μ l) or vehicle (10 μ l) was injected intracerebroventricularly (i.c.v.) through polyethylene canulas implanted 3 days before the experiments. The session (3-min duration) started 2 min after the injection of the peptide solution or vehicle. The animals were allowed to lick for 3 sec (one licking period) before the first shock was delivered.

Enzyme-Linked Immunosorbent Assay (ELISA) for the ODN Fragment of DBI. Antisera against ODN were prepared in rabbits by injecting 500 μ g of ODN conjugated with hemocyanin in complete Freund's adjuvant. The antisera used for these experiments were obtained after four booster injections. A screening of the antisera with radioimmunoassay technique using ODN ¹²⁵I labeled with Bolton-Hunter reagent was unsuccessful because this conjugation changes the physicochemical and immunological characteristics of ODN. Therefore, ODN was detected in brain homogenates by the ELISA technique (22). In this test polyvinyl microtiter plates (Immunolone 2, Dynatech, Alexandria, VA) were

coated overnight at 0°C with ODN or other polypeptides or partially purified brain extracts (maximal amount of protein per well was 1 μ g) dissolved in 100 μ l of 0.01 M sodium phosphate buffered saline, pH 8.3 (PBS). The wells were filled with 10% fetal calf serum in PBS and then washed extensively with PBS containing 0.05% Tween 20. Antiserum (1:2000 or 1:4000) in 10% fetal cell serum/PBS was added and incubated for 2 hr at room temperature. The bound antibody was determined with peroxidase-labeled anti-rabbit IgG (diluted 1:1000 in 10% fetal calf serum/PBS) and *o*-phenylenediamine as chromophore. The intensity of the color was determined with a Dynatech densitometer at 490 nm.

Extraction and Purification of Endogenous ODN-Like Immunoreactive Material. Endogenous ODN-like immunoreactive material was extracted from whole rat brain with 10 vol of 1 M acetic acid at 95°C. Aliquots of the homogenate (2.5 ml) were first purified on Bio-Gel P-6 (2 × 60 cm) equilibrated with 1 M acetic acid. The immunoreactive material eluting from the column in the position of ODN was lyophilized and then allowed to react with purified anti-ODN IgG. Approximately 100 μ g of the purified anti-ODN IgG was allowed to react with 10 μ g of protein extract in 2 ml of 0.1 M sodium citrate buffer (pH 7.0) containing 0.05% bovine serum albumin at 0°C for 24 hr. After incubation the reaction mixture was applied to a protein A-Sepharose CL-4B immunoaffinity column (1 × 5 cm) equilibrated with 0.1 M citrate buffer (pH 6.3). The column was washed with 20 ml of the equilibrating buffer and the immunoreactive material was eluted with 0.1 M citrate buffer (pH 3.3). This eluate was applied to a μ Bondapak C₁₈ HPLC column and eluted with an acetonitrile gradient. ODN-like material in the various HPLC fractions was detected with ELISA.

RESULTS

Characterization of DBI Tryptic Fragments. HPLC mapping of peptides generated by tryptic digestion of DBI purified from rat brain revealed seven major tryptic (T) peptide peaks (Fig. 1). The amino acid sequences of peptides T₄, T₅, and T₇ were determined and are reported in Fig. 1. Of these peptides only T₅, when injected i.c.v. into thirsty rats, elicits a proconflict response similar to that described for DBI (12). T₅ is an octadecapeptide (Fig. 1 and Table 1) contained in the CNBr-2 fragment obtained from DBI (for sequence see ref. 3). This octadecaneuropeptide (ODN) was synthesized and its sequence was checked by microsequencing; the peptide was found to be more than 98% pure. The retention properties on HPLC and the pharmacological characteristics of the synthetic ODN were identical to those of T₅ (12).

Presence of ODN-Like Material in Brain Extracts. To study whether DBI is processed in brain to produce measurable amounts of ODN, an antiserum with high affinity for ODN was prepared (Fig. 2). Since this antibody has an equal affinity for ODN and ODN-NH₂ (see Table 1 for sequence) but also reacts with DBI (Fig. 2) its immunodeterminant appears to reside close to the NH₂ terminus of ODN. This inference is supported by the lack of cross-reactivity with 100-fold higher concentrations of various ODN fragments containing five to seven amino acid residues next to the COOH terminus of ODN (see Table 1 for structural details).

Using this antiserum, we detected ODN-like immunoreactivity in acetic acid extracts of rat brain. The ODN-like immunoreactivity eluted from a Bio-Gel P-6 column in two major peaks: a high molecular weight form with an elution profile similar to that of DBI and a smaller molecular weight form that coelutes with ODN (Fig. 3A). This lower molecular weight material was allowed to react with anti-ODN IgG and then adsorb to a protein A affinity chromatography column. The eluate obtained from this column was subjected to reversed-phase HPLC. In this HPLC eluate the ODN anti-

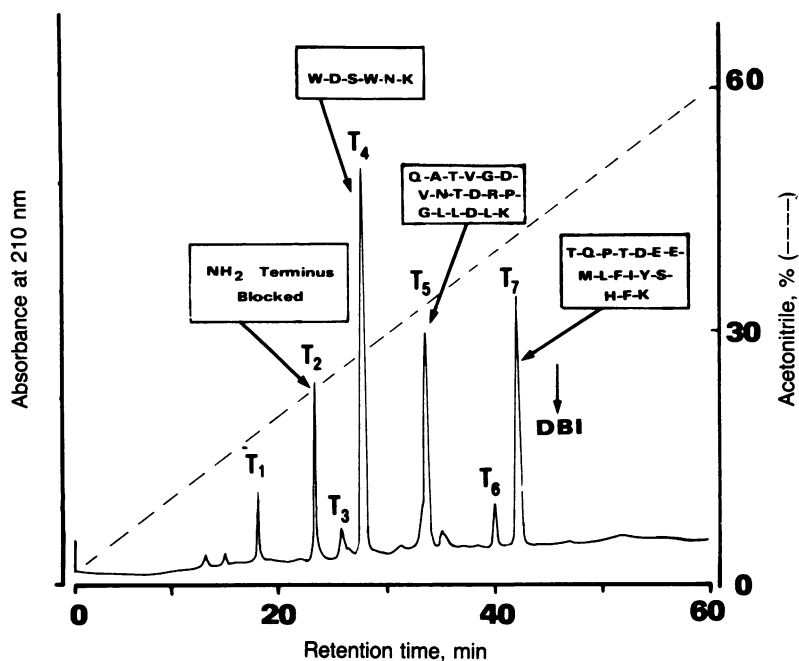


FIG. 1. HPLC separation of peptides generated by tryptic digestion of DBI. DBI (100 nmol) digested by trypsin was applied to a μ Bondapak C₁₈ column. The retention time of the original DBI molecule is marked by an arrow. The tryptic peptide (T) fragments are marked 1 to 7 according to their relative retention times. The amino acid sequences of fragments 4, 5, and 7 are reported in the one-letter notation (13).

serum detected three immunoreactive peaks (Fig. 3B). The second of these peaks coeluted with authentic ODN and in a preliminary analysis it was found to include an amino acid sequence identical to that of ODN. To exclude that this material is produced post mortem, we analyzed the brains of rats sacrificed with a microwave beam focused for 2 sec on the head. Under these conditions also we could detect ODN-like immunoreactivity.

Pharmacological Profile of ODN. *In vitro binding studies.* DBI displaces [³H]Flu and [³H]BCCM from high-affinity binding sites located in crude preparations of brain synaptic membranes (2); however, in this preparation, ODN failed to produce a consistent displacement of these two ligands. The capability of ODN and related peptides to displace [³H]Flu and [³H]BCCM was studied in primary cultures of cerebellar granule cells, which contain all the components of the GABA_A receptor complex (23). In this cell preparation the binding of [³H]Flu and [³H]BCCM is saturable ($B_{\max} = 500$ and 450 fmol/mg of protein, respectively) and of high affinity

(apparent $K_d = 10$ and 3 nM, respectively.) GABA (1–100 μ M increases [³H]Flu and decreases [³H]BCCM binding in a dose-related manner. As shown in Fig. 4, ODN competitively displaces [³H]BCCM with a Hill slope close to 1 and a K_i of 1.5 μ M at about 2–4°C. This K_i increases to 5 μ M at 18–20°C. Also, the displacement of [³H]Flu by ODN appears to be dose related (data not shown), but up to 100 μ M ODN never displaced more than 50% of bound [³H]Flu and the pseudo-Hill slope is smaller than 0.5. ODN-NH₂ fails to displace [³H]BCCM (Fig. 4A, Table 1) up to 50 μ M; in contrast, DBI, OP, HEP, and HEX displace [³H]BCCM, but their potency is less than that of ODN (Table 1). Some DBI fragments not included in the ODN sequence were also tested: the heptapeptide FIYSHFK and the tetrapeptide TYVE failed to displace [³H]BCCM in concentrations up to 50 μ M (Table 1).

In vivo behavioral studies. In thirsty rats subjected to a mild electric shock delivered through the drinking tube, ODN injected i.c.v. elicited a dose-related facilitation of the punishment-elicited suppression of drinking. This behavioral

Table 1. Pharmacological properties of DBI and synthetic peptide fragments of DBI

Generic name	Peptide Amino acid sequence*	M_r	ED ₃₀ for proconflict activity, [†] nmol i.c.v.	K_i for inhibition of [³ H]BCCM binding, [‡] μ M	Tonic-clonic convulsions, [§] nmol i.c.v.
DBI	104 residues (2, 3)	11,000	10	5.2	10
ODN	QATVGDVNTDRPGLLDLK	1,882	2.9	1.5	—
OP	RPGLLDLK	881	11	6	>125
HEP	PGLLDLK	754	48	8	>200
HEX	GLLDLK	655	65	7	>200
ODN-NH ₂	QATVGDVNTDRPGLLDLK-NH ₂	1,881	>100	>50	—
	FIYSHFK	940	>100	>50	—
	TYVE	595	>100	>50	—

OP, octapeptide; HEP, heptapeptide; HEX, hexapeptide.

*Amino acid sequence reported in the one-letter notation (13).

[†]Proconflict activity was determined by injecting 10 μ l of peptide i.c.v. and assessing shock-induced suppression of water drinking in thirsty rats (21). Since the maximal inhibition of drinking behavior reached a base line at approximately 1/3 of the response obtained in the absence of punishment, the ED₃₀ is close to the dose of peptide that inhibits the behavior by 50%. ED₃₀ is calculated from dose-response curves including at least four different doses of the peptides.

[‡]Binding was studied in intact cerebellar granule cells maintained in primary culture. $K_i = IC_{50}/[1 + (L/K_d)]$, in which L (3 nM [³H]BCCM) is the concentration of labeled ligand and K_d (3 nM) is the equilibrium dissociation constant of [³H]BCCM.

[§]Peptides were injected i.c.v. in 10 μ l. DBI, ODN, and ODN-NH₂ failed to induce convulsions at doses up to 100 nmol.

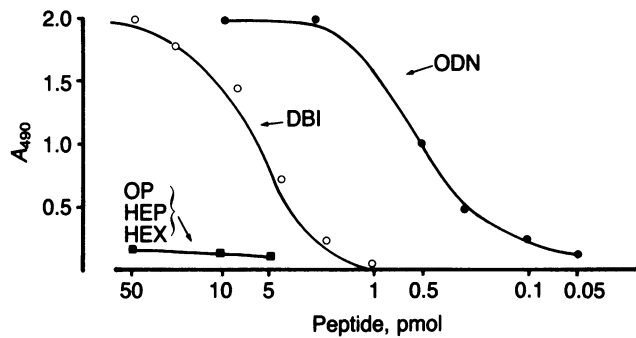


FIG. 2. ELISA immunoreactivity pattern of ODN antiserum. ODN or other peptides (in increasing doses from right to left on the abscissa) were allowed to react with a 1:4000 dilution of rabbit ODN antiserum. The amino acid sequences of these peptides are given in Table 1. The synthetic ODN, the T₅ fragment shown in Fig. 1, which contains the sequence of ODN, and the synthetic ODN-NH₂ reacted with the same potency.

response is indistinguishable from that elicited by i.c.v. injections of DBI (12) or i.p. injections of the anxiogenic FG 7142, the amide of β -carboline-3-carboxylate ethyl ester (2). Since the rat response elicited by ODN denotes an exacerbation of conflict, this action is termed proconflict. The EC₃₀ of the proconflict action of ODN (2.9 nmol) is smaller than that of DBI (Table 1). This dose of ODN fails to alter unpunished drinking, gross behavior, motor activity, or

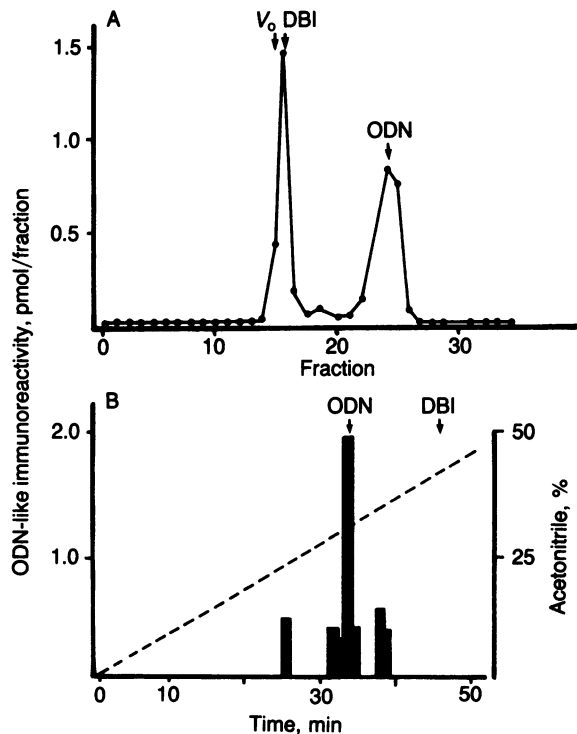


FIG. 3. Immunological identification of ODN-like material in rat brain extracts. (A) ODN-like immunoreactivity (ELISA) in eluates obtained after chromatography of crude acidic acid brain extracts on a Bio-Gel P-6 column. One-milliliter fractions were collected. V₀, void volume. (B) HPLC purification of low molecular weight ODN-like immunoreactive material eluted from Bio-Gel P-6. The material in the position of ODN was lyophilized, allowed to react with anti-ODN IgG, and adsorbed on a protein A affinity chromatography column. The immunoreactive material from the affinity column was applied to a μ Bondapak C₁₈ column and eluted with a linear gradient of acetonitrile. Fractions were assayed for ODN-like material with ELISA.

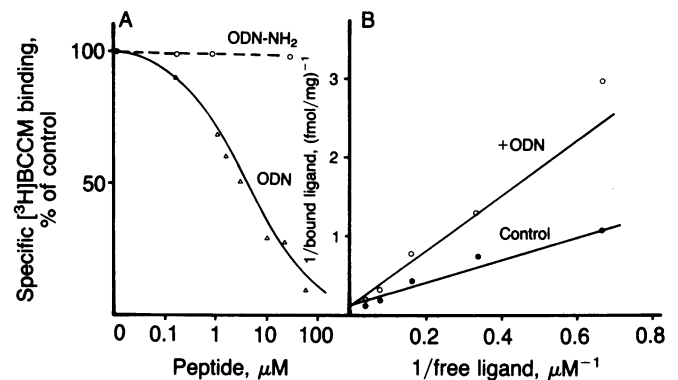


FIG. 4. Inhibition of [³H]BCCM binding to intact cerebellar granule cells by ODN and ODN-NH₂ (A) Displacement of [³H]BCCM binding (3 nM) by increasing concentrations of ODN or ODN-NH₂; 100% specific binding = 200 fmol/mg of protein. (B) Double-reciprocal plot of [³H]BCCM specifically bound versus concentration of BCCM in control cells and in cells treated in 10 μ M ODN. Points are the means of three separate experiments.

nociception (measured by the tail-flick technique). The proconflict effect of ODN is prevented by a pretreatment with Ro 15-1788 (Fig. 5); in contrast, ODN-NH₂ (Table 1) fails to elicit a proconflict response in doses up to 100 nmol i.c.v. Doses of ODN and ODNH₂ up to 100 nmol i.c.v. fail to cause convulsions. Two synthetic DBI fragments not included in the sequence of ODN, FIYSHFK and TYVE (Table 1), failed to change the threshold of punishment in doses up to 100 nmol i.c.v. In contrast, the synthetic ODN fragments OP, HEP, and HEX elicit proconflict effects, albeit their potency is less than that of ODN (see Table 1). The ED₃₀ doses of OP, HEP, and HEX fail to change unpunished behavior; however, higher doses of these peptides (see Table 1) can elicit tonic-clonic convulsions. The convulsive dose (100% of the animals) is approximately 125 nmol i.c.v. for OP and 200 nmol i.c.v. for HEP and HEX. Convulsions start about 3 min after injection and recur for 30 to 60 min; some rats die in convulsions. A pretreatment with Ro 15-1788 (10 or 20 mg/kg i.v.) antagonizes OP, HEP, and HEX convulsions. The convulsions can be attenuated also by diazepam (10 mg/kg i.v.) or pentobarbital (40 mg/kg i.p.) injected after peptide administration. In contrast, Ro 15-1788 (20 mg/kg i.v.) and scopolamine (up to 10 mg/kg s.c.) were ineffective when given to rats with tonic-clonic convulsions elicited by HEP, HEX, or OP.

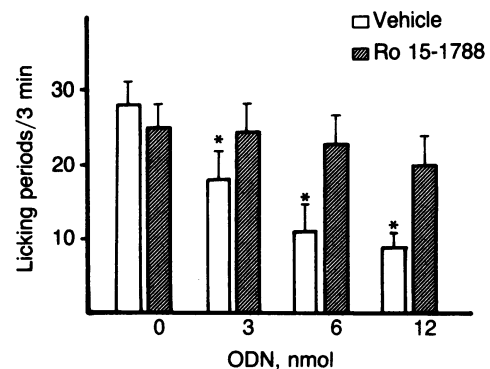


FIG. 5. Ro 15-1788 antagonizes the proconflict affect of ODN. Rats received vehicle alone or Ro 15-1788 at 10 mg/kg i.v. 10 min before the i.c.v. injection of the indicated dose of ODN. Each bar is the mean + SEM for 5–10 rats. Licking period = 3 sec of cumulative drinking, recorded with a drinkometer (21). The drinkometer output and the shock generator wire connected to a relay delivering one shock lasting for 0.1 sec for every licking period. **P* < 0.05 when compared with the respective control.

DISCUSSION

Several brain neuropeptides acting as neuromodulators are synthesized as high molecular weight precursors, stored in vesicles, and then processed into biologically active low molecular weight peptides. The precursor in some cases includes in its sequence several replicas of the same active peptide, and in other cases it includes one or more copies of different neuromodulators with different actions. Here we propose that DBI is the precursor for the putative endogenous ligand(s) of BZ/BC recognition sites. Using tryptic digestion of DBI combined with HPLC separation of the fragments, we isolated an 18-residue peptide (ODN) whose sequence (see Table 1) included a functional domain of DBI. The structure of this functional domain requires the free COOH terminal of lysine, because its amidation abolishes the biological activity of ODN. Preliminary results suggest that ODN could naturally occur in brain as a result of *in vivo* processing of DBI. Using a rabbit antiserum raised against synthetic ODN to perform immunoaffinity chromatography combined with reversed-phase HPLC, we have detected in rat brain at least three major molecular forms of ODN-like immunoreactivity. One of these forms, with an HPLC profile similar to that of authentic ODN, yielded a single sequence 18 residues long, identical to that of ODN. However, we cannot yet rule out the possibility that the endogenous ODN-like material contains some extra amino acid residues at the COOH terminus that failed to appear in the sequence analyses because of the minute amount (20–50 pmol) of peptide analyzed. Moreover, though this ODN-like peptide can be detected in brain we have not purified enough of it to test whether it acts as an endogenous effector for the BC recognition sites. Preliminary studies on the relationship between structure and pharmacological activity of some synthetic ODN fragments (Table 1) show that the last eight to six amino acid residues have a decreased biological activity when compared to ODN (Table 1). Since the ODN antiserum available to us is directed toward the NH₂ terminus of ODN, while the pharmacologically active region of ODN includes the COOH-terminal part of the molecule, a final conclusion on the functional nature of the endogenous ODN-like peptide was not reached.

The study of the displacement of [³H]BCCM and [³H]Flu specifically bound to primary cultures of cerebellar granule cells by ODN, ODN-NH₂, OP, HEP, or HEX revealed that these peptides derived from DBI preferentially displace [³H]BCCM. While ³H-BC is displaced completely, only 50% of [³H]Flu is displaced by these peptides. Several factors can explain this difference; one of them could be the presence in the culture cell preparation of endogenous GABA, which facilitates the displacement of [³H]BCCM and limits that of [³H]Flu. However, the presence of GABA cannot explain why ODN fails to displace more than 50% of the [³H]Flu. Perhaps, similarly to brain synaptic membranes, primary culture granule cells also contain two functional domains in the BZ recognition sites, one for BZ and one for the BC ligands, and the one accessible to Flu is inaccessible to the peptides. Our studies indicate also that crude synaptic membranes prepared from brain cannot be readily used to study the displacement of [³H]BCCM or [³H]Flu by the neuropeptides derived from DBI because of their high peptidase activity.

Since peptide binding studies were plagued by several difficulties, we injected these peptides *i.c.v.* to determine the involvement of BZ/BC binding sites by measuring the changes elicited in rat operant behavior. The studies show

that the conflict action of ODN stems from its interaction with Flu/BC recognition sites because it is antagonized by the imidobenzodiazepinone Ro 15-1788. An important aspect highlighted by the binding and behavioral studies is that the active site of ODN must contain the COOH-terminal sequence. The fragments OP, HEP, and HEX, though weaker than ODN, have a similar but not identical pharmacological profile in the tests we used. OP, HEP, and HEX displace ³H-BCCM and cause proconflict actions similar to ODN and DBI, but, unlike these two latter peptides, they cause convulsions. It will be interesting to raise antibodies against the smaller peptides to establish whether an immunoreactive material similar to OP, HEP, and HEX is detectable in rat brain. An important issue is whether an α -amidating enzyme capable of transforming the α -carboxyl group of the terminal lysine residue of ODN is part of the inactivation process operative in the DBI-ODN family of peptides. Moreover, it remains to be investigated whether DBI is a multifunctional precursor polypeptide capable of generating also a peptide domain that mimics the pharmacological action of the anxiolytic benzodiazepines.

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