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A BRAIN OCTADECANEUROPEPTIDE GENERATED BY TRYPTIC DIGESTION OF DBI (DIAZEPAM BINDING INHIBITOR) FUNCTIONS AS A PROCONFLICT LIGAND OF BENZODIAZEPINE RECOGNITION SITES

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Summary

An octadecaneuropeptide (ODN) produced by the tryptic digestion of DBI was purified and sequenced and its activity on the Vogel test determined. In vitro ODN displaces ^{3}H-diazepam from specific brain recognition sites and injected intraventricularly in thirsty rats facilitates the onset of behavioral inhibition elicited by punishment. The α-amide derivative of ODN is devoid of either action. Evidence is presented suggesting that DBI sequence includes at least two replicas of ODN or one replica of ODN and a fragment with similar if not identical amino acid sequence but identical biological activity.

A natural neuropeptide termed DBI (diazepam binding inhibitor) which displaces diazepam from its specific brain recognition sites, was isolated and purified from rat and human brain (Guidotti, Forchetti, Corda, Konkel, Bennett and Costa, 1983). DBI might function as the precursor of an endogenous ligand of benzodiazepine recognition sites generating proconflict responses in thirsty rats similarly to beta carboline-3-carboxylate derivatives (Guidotti et al., 1983; Costa, Corda and Guidotti, 1983; Corda, Ferrari, Guidotti, Konkel and Costa, 1984). DBI is a 105 amino acid neuropeptide containing several Arg and Lys residues (Guidotti et al., 1983), immunohistochemically it can be shown to be located in neurons and to be highly localized in the molecular layer of cerebellum, the pyramidal cell layer of the hippocampus and in several telediencephalic nuclei (Alho, H., this laboratory, personal communication). Preliminary electrophysiological studies indicate that DBI, like the benzodiazepines is per se incapable of opening Cl^- channels. But when it is added with GABA decreases the duration of the Cl^- channel opening bursts elicited by this putative amino acid neurotransmitter (Bormann, J., Gottingen, Max Planck Foundation, personal communication). Thirsty rats receiving DBI intracerebroventricularly suppress drinking when they receive mild electrical shocks, which fail to change drinking when given in absence of DBI treatment (Guidotti et al., 1983). Moreover, in thirsty rats the attenuation of GABAergic transmission potentiates the proconflict action of DBI and of beta carboline-3-carboxylate derivatives (Costa et al., 1983; Ferrero et al., in preparation). These data suggest that DBI is the precursor of the putative ligand of the benzodiazepine recognition site and is endowed with proconflict action in rats and presumably with anxiogenic action in man. To identify the active fragment of DBI we proceded to do a partial tryptic digestion of DBI and to study biochemically and pharmacologically the action of the tryptic fragments thus generated.

METHODS

DBI was purified from rat brain homogenates prepared with 1 N acetic acid at 95°C. The supernatant was processed as previously described (Guidotti et al., 1983) and final purification was obtained with HPLC using a uBondapak TM C_{18} (7.8 mm x 30 mm, Waters, Milford, MA) reverse phase column. DBI was eluted from this column by a linear gradient of 0.1% TFA-acetonitrile well separated from other contaminating peptides and was routinely tested for purity determining its migration on SDS-PAGE and amino acid composition (Guidotti et al., 1983). Such standard preparation of DBI was used as starting material to study biochemically, pharmacologically and immunologically the products of trypsin digestion. DBI (100 nmol) was treated with trypsin TPCK, Worthington (10 nmol) for 24 hr at 30° in 1 ml of 0.1 M NH_4HCO_3, pH 8.2. The reaction was terminated by adding 1 ml of 0.1% TFA in H_2O and the digest was applied to a μBondapak TM C_{18} preparative column (7.8 mm x 30 mm) equilibrated with 0.1% TFA in H_2O. The separation of peptides was achieved by eluting with a linear gradient of 0.1% TFA in acetonitrile (from 0 to 60% in 60 min, flow rate 2 ml/min) (see Fig. 1). The emerging absorption peaks (210 nm) were collected separately and analyzed for their immunoreactivity with a rabbit antiserum direct against DBI, for their proconflict action, for their capacity to displace ^{3}H-diazepam from recognition sites on brain synaptic membranes and for their amino acid composition and sequence.

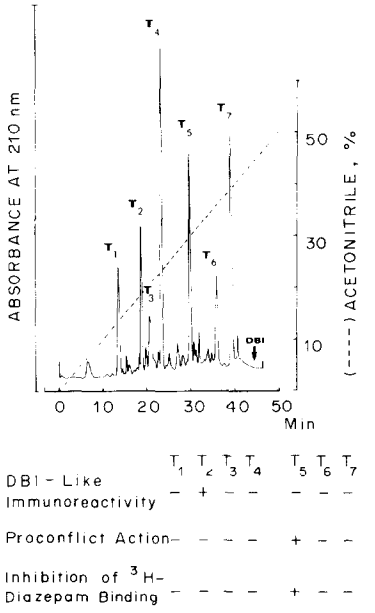

Fig. 1

HPLC separation and biological characterization of peptides generated by trypsin digestion of DBI. DBI (100 nmole) digested by trypsin (see Method section) was applied to a μBondapak C_{18} column (see Method). The retention time of the original DBI molecule is marked by an arrow. The tryptic peptide fragments (T) are marked from 1 to 7 according to their relative retention time. DBI-like immunoreactivity, proconflict action and ^{3}H-diazepam binding inhibition of the various tryptic fragments of DBI were measured as described in the Method section. Only peptide T_5 displaces ^{3}H-diazepam from specific binding sites and facilitates punishment-induced suppression of drinking in thirsty rats.

DBI-like immunoreactivity was determined using DBI antiserum generated in rabbits repeatedly injected with purified DBI and Freund adjuvant. This antiserum is directed toward the NH_2-terminal portion of DBI (Costa, Ferrari, Ferrero and Guidotti, 1984) and shows no cross reactivity with other endogenous basic proteins (histone, large or small myelin basic proteins, GABA-modulin) and several neuropeptides: VIP, enkephalin (met^5-, leu^5, met^5-arg^6, octapeptide and peptide E) endorphin, dynorphin, substance P, phe-met-arg-phe-like peptide, peptide P, somatostatin, CRF, ACTH (intact molecule and various fragments), alpha-, beta- and gamma-MSH, neurotensin and cholecystokinin at concentrations of 10^{-6}M. RIA was performed by adjusting the sensitivity of the antiserum (1/10,000 dilution) to detect 10 fmole of DBI using ^{125}I-labeled DBI (S.A. 10 Ci/mmol) as ligand. An aliquot of each tryptic fragment obtained from the equivalent of 10 μg of DBI was used in the assay.

The pharmacological activity of the various fragments of DBI was determined in rats by the Vogel test with the modalities used to determine a proconflict action (Corda, Blaker, Mendelson, Guidotti and Costa, 1983). The experiments were carried out in rats with chronic implanted intraventricular cannulae and kept without water for 72 hrs prior to the conflict session. Three minutes before this test, an aliquot of each fragment, equivalent to 50 μg of peptide, was injected intracerebroventricularly (i.c.v.). Control animals were injected with an equivalent volume (10 μl) of HPLC eluate collected during a control gradient.

Inhibition of ^{3}H-diazepam binding was carried out in a final volume of 1 ml in 20 mM KPO_4 (pH 7.0) using a preparation of rat brain synaptic membranes containing 0.2-0.3 mg protein, 10 mM ^{3}H-diazepam and 50 μg of each peptide fragment (conditions are the same as those described by Guidotti et al., 1983).

Amino acid sequence analysis was carried out by automated Edman degradation identifying the phenylthiohydantoin-derivatized amino acids with HPLC on a IBM cyano column. Details on identification of phenylthiohydantoin-derivatized amino acids and standard chromatograms have been described (Hunkapiller and Hood, 1981).

RESULTS

HPLC mapping of peptides generated from DBI by tryptic digestion revealed seven major peptide peaks (Fig. 1). The retention time of these proteolytic fragments is shorter than that of the original DBI (retention 45 min) which appears to be completely digested by trypsin (Fig. 1). Further digestion of the cleaved material by additional trypsin for 12 more hrs failed to change significantly the peptide profile

of the DBI digest. The seven major trypsin fragments of DBI were tested for immunoreactivity, for inhibitory effects on ^{3}H-diazepam binding to crude synaptic membranes prepared from brain homogenates and for their proconflict action on shock-induced suppression of drinking in thirsty rats.

As shown in Figure 1 only the T_2 fragment maintains immunoreactive properties; trypsin fragment T_5 lacks the ability to react with the antiserum directed against DBI but possesses the ability of inhibiting ^{3}H-diazepam binding and has proconflict aciton in the Vogel test modified to detect proconflict actions (Corda et al., 1983). All the other fragments are inactive (Fig. 1). T_5 fragment has been subjected to sequence analysis. The peptide appears more than 95% pure and in three separate experiments the sequence determination proceded for 18 steps terminating abruptly with a Lys residue. Therefore it was determined that T_5 fragment obtained by trypsin digestion of DBI is an octadecaneuropeptide with the following amino acid sequence:

Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys

This sequence is included between position 68 and 87 of CNBr 2 fragment of DBI (for sequence of CNBr 2 of DBI, see Costa et al., 1983). An octadecaneuropeptide (ODN) with a sequence identical to that established for the T_5 fragment of DBI has been synthesized (Peninsula Laboratory). The synthetic peptide (whose purity has been monitored by HPLC and amino acid sequence analysis), like the ODN obtained by digestion of DBI, elicits proconflict action when injected intraventricularly in thirsty rats subjected to Vogel test. A dose response curve obtained by comparing the potency of ODN and that of DBI reveals that on the molar basis ODN is more potent than DBI (Table). Since many bioactive

Table. Proconflict properties of DBI, octadecaneuropeptide (ODN) and octadecaneuropeptide-amide (ODN-NH_2) on shock-induced[a] suppression of drinking in thirsty rats[b]

Dose (nmole i.c.v.)	Licking Periods in 3 Min[c] DBI	ODN	ODN-NH_2
0	28±2.4	28±2.4	28±2.4
0.75	25±1.8	21±1.4	--
1.5	--	19±1.8*	--
3	23±1.9	15±1.7*	--
5	20±1.2*	12±1.5*	30±2.5

a) Current intensity, 0.25 mA.
b) Rats of 175 g were deprived of water for 72 hrs prior to test; the peptides were injected intraventricularly (i.c.v.) 3 min before the test. The conditions of the test are the one described by Corda et al. (1983). Each value is the mean of 5-6 animals±S.E.M. *Denotes statistical significance ($P < 0.05$).
c) One licking period is equal to 3 sec of continuous licking.

neuropeptides isolated from brain are biologically active in an α-amidated form, and since the Lys in DBI is followed by Gly (Costa et al., 1983) we obtained an α-amidated synthetic ODN which has the lysine carboxyl terminal group modified. As shown in the Table, the ODN-amide fails to lower the threshold for punishment-induced inhibition of drinking.

DISCUSSION

Rat and human brain contain DBI, a neuropeptide which when injected in rats facilitates punishment-induced suppression of operant behavior in a conflict test paradigm with a profile similar to that of beta carboline-3-carboxylate derivatives (Guidotti et al., 1983; Costa et al., 1983). To decide whether the biological activity of DBI resides in the entire peptide or if DBI may function as a precursor for a smaller peptide which contains the active domain of the molecule we began to examine the proconflict action of proteolytic fragments of DBI. We have already reported (Corda et al., 1984; Costa et al., 1984) that of three fragments generated by CNBr cleavage of DBI only the N-terminal fragment maintains the biological activity and immunoreactivity of DBI. Here we report that by trypsin digestion of DBI it is possible to obtain an ODN fragment which loses the capacity to immunoreact with the DBI antibody but maintains the ability to inhibit diazepam binding and it is more active in lowering the punishment threshold for suppression of drinking than DBI itself. The ODN is rich in aspartic acid residue, has been sequenced and found to be contained in the structure of CNBr 2 fragment of DBI between residue 68 and 87 (for sequence of CNBr 2 of DBI, see Costa et al., 1983).

The biological activity of ODN is absent in the lysine-amidate ODN derivative (Table) and in the CNBr 2 fragment of DBI (Corda et al., 1984). This suggest that the biological activity of ODN is dependent upon the presence of free active determinants perhaps at the lysine carboxyl terminal of the molecule. One of the intriguing features about the characterization of active fragments of DBI is that after CNBr cleavage of DBI the amino terminal fragment (CNBr 1 fragment) contains biological activity (Corda et al., 1984). To establish whether the biological activity CNBr 1 fragment of DBI resides in the

ODN fragment or in a complete separate molecular structure, CNBr 1 fragment was digested with trypsin and then applied to HPLC. The preponderant trypsin peptide fragment obtained has retention time and amino acid sequence identical to that of the ODN contained in the T_5 tryptic fragment of DBI. This observation confirms that the biological activity of DBI is confined to relatively small and characteristic part of the sequence and suggest that there are at least two ODN replicas in DBI. This is a frequent feature in polyproteins functioning as precursors of neuropeptides. Usually the active sites of neuropolyprotein precursors are flanked by pairs of basic amino acids which are cleavage sites for specific endopeptidases generating the small active neuropeptides which are the putative endogenous neuromodulators. The experiments with trypsin digestion of DBI suggest that in the chemical structure of DBI there are specific signals for the action of proteolytic enzymes and that DBI is the precursor of ODN. At this time it cannot be excluded that the natural effector(s) of the benzodiazepine recognition site(s) are smaller than ODN.

REFERENCES

Corda, M. G., Blaker, W. D., Mendelson, W. B., Gudotti, A. and Costa, E. (1983). Beta-Carbolines enhance shock-induced suppression of drinking in rats. Proc. Natl. Acad. Sci. USA 80: 2072-2076.

Corda, M. G., Ferrari, M., Guidotti, A., Konkel, D. and Costa, E. (1984). Isolation, purification and partial sequence of a neuropeptide (diazepam binding inhibitor) precursor of an anxiogenic putative ligand for benzodiazepine recognition site. Neurosci. Lett. 47: 319-324.

Costa, E., Corda, M. G. and Guidotti, A. (1983). On a brain polypeptide functioning as a putative effector for the recognition sites of benzodiazepine and beta-carboline derivatives. Neuropharmacology 22: 1481-1492.

Costa, E., Ferrari, M., Ferrero, P. and Guidotti, A. (1984). Multiple signals in GABAergic transmission: Pharmacological consequences. Neuropharmacology 23: 989-991.

Guidotti, A., Forchetti, C. M., Corda, M. G., Konkel, D., Bennett, C. D. and Costa, E. (1983). Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. Proc. Natl. Acad. Sci. USA 80: 3531-3533.

Hunkapiller, M. W. and Hood, L. E. (1981). Analyses of phenylthiohydantoins by ultrasensitive gradient high performance liquid chromatography. Method Enzymol. 91: 486-511.