BIOCHEMICAL LETTERS

Diazepam-binding inhibitor: a neuropeptide and/or an acyl-CoA ester binding protein?

Diazepam-binding inhibitor (DBI) (Guidotti *et al.*, 1983) also called endozepine (Shoyab *et al.*, 1986) is an endogenous 10 kDa polypeptide that was initially purified from mammalian brain based on its ability to displace a specific ligand from the benzodiazepine recognition site on the γ -aminobutyric acid (GABA_A) receptor (Guidotti *et al.*, 1983; Shoyab *et al.*, 1986) and on mitochondrial membranes (Costa *et al.*, 1987; Guidotti *et al.*, 1989). The amino acid sequence of DBI has been determined by chemical and cDNA analysis from brains of rats (Mocchetti *et al.*, 1986; Ferrero *et al.*, 1986a), humans (Gray *et al.*, 1986; Ferrero *et al.*, 1986b; Marquardt *et al.*, 1986; Webb *et al.*, 1987), pigs (Chen *et al.*, 1988), and cows (Marquardt *et al.*, 1986; Webb *et al.*, 1987).

Acyl-CoA binding protein (ACBP) was initially purified from mammalian liver based on its ability to modify the pattern of fatty acids synthesized by goat mammary gland fatty acid synthetase (Mogensen *et al.*, 1987). The sequences of bovine and rat liver ACBP have been determined (Mikkelsen *et al.*, 1987; Knudsen *et al.*, 1989). Bovine and rat ACBP have sequences identical to those of bovine and rat brain DBI respectively (Knudsen *et al.*, 1989). Furthermore, time-of-flight plasma-desorption mass spectrometry on intact human liver ACBP gives an M_r which is identical with the M_r for human brain DBI calculated from its sequence (unpublished results). It can therefore be concluded that ACBP and DBI are one and the same protein (Knudsen *et al.*, 1989).

ACBP binds acyl-CoA esters of medium- and long-chain fatty acids (C₈–C₁₈) with high affinity (K_d 0.1–0.2 μ M) (Mikkelsen & Knudsen, 1987; Knudsen *et al.*, 1989). This fact raises the following question: is ACBP/DBI a neurotransmitter or an acyl-CoA carrier protein or both? ACBP/DBI is mainly a cytosolic protein and is present in all tissues tested. The highest concentration is found in liver (3–6 μ g/mg of soluble protein; Knudsen *et al.*, 1989). The concentration in bovine liver is about three times the concentration found in bovine brain (Mikkelsen *et al.*, 1987). mRNA for DBI and immunoreactivity have also been detected in numerous peripheral tissues including testes, heart, kidney, liver, intestine, adrenals, gut and striated muscle (Gray *et al.*, 1986; Mocchetti *et al.*, 1986; Alho *et al.*, 1988).

We therefore decided to reinvestigate the DBI activity of highly purified rat and bovine ACBP/DBI. The assay was carried out in two independent laboratories at Sct. Hans Hospital, Roskilde, and at Odense University, on four different brain membrane preparations from rat cortex *in vitro* using ACBP/DBI concentrations up to 20 μ M.

Specific binding of [³H]diazepam (85.0 Ci/mmol; NEN) at 1.7 nM was measured in four different membrane preparations from rat cortical tissue *in vitro*. The mem-

brane preparations were (a) fresh tissue three times washed in Tris/citrate buffer (50 mM, pH 7.1); (b) membranes preincubated with ACBP/DBI for 60 min at 0–4 °C before addition of [³H]diazepam; (c) membranes treated with Triton X-100 (0.05%, v/v) followed by three times washing and (d) membranes three times washed, twice frozen/thawed and three times washed. Assays of 1 mg of original tissue/0.5 ml were incubated at 0–4 °C for 40 min before filtration through Whatman CG/C filters. The filters were washed with 2 × 10 ml of ice-cold Tris/citrate buffer. Non-specific binding was obtained by adding midazolam (10^{−6} M) to separate samples. In some experiments GABA (10^{−4} M final concentration) was added to assays before diazepam addition. ACBP/DBI was dissolved and diluted in Tris/citrate buffer (50 mM, pH 7.1) just prior to use.

Bovine and rat ACBP/DBI were unable to show any significant DBI activity toward [³H]diazepam binding. Typical results for rat brain are seen in Table 1. A similar result was obtained with cow ACBP/DBI, and also if [³H]flunitrazepam was used instead of [³H]diazepam [in membrane preparation (a)].

ACBP/DBI (0.1, 0.5 and 5.0 μ M) showed no inhibition of [³H]diazepam specific binding in membrane preparation (c₃) and (d) (results not shown). ACBP/DBI (5 μ M) showed weak inhibition (17%) of [³H]PK11195 specific binding in membrane preparation (a) (results not shown) { [³H]PK11195 (90.5 Ci/mmol) at 0.62 nM; 2 mg original tissue/0.5 ml; incubation 40 min at 0–4 °C}. Nonspecific binding was obtained by adding Ro 5-4864 (10^{−6} M) to separate samples.

There is a large body of evidence which questions the role of ACBP/DBI as a neurotransmitter or neurotransmitter precursor. The major points are: (i) the low or even lack of diazepam binding inhibitor activity of ACBP/DBI (present data); (ii) the high concentration of ACBP/DBI in peripheral tissue, especially liver, compared to the concentration in brain; (iii) analysis of cDNA clones of rat DBI shows that the sequence does

Table 1. Diazepam binding inhibitory activity of rat liver ACBP/DBI

Expt.	Rat ACBP/DBI (μ M)	Specific binding as % of control (without ACBP/DBI added)	
		– GABA	+ GABA (10 ^{−4} M)
(a)	0.1	91	167
	0.5	100	171
	5.0	98	165
(b)	0.1	91	n.d.
	1.0	95	n.d.
	5.0	101	n.d.
	20.0	94	n.d.

not include a *N*-terminal signal sequence as one would expect for a secreted neuropeptide (Mocchetti *et al.*, 1986). These points, combined with the fact that ACBP/DBI can function as a high-affinity acyl-CoA binding protein, indicate for us that ACBP/DBI is not a neurotransmitter but an acyl-CoA carrier and storage protein.

It cannot be excluded that a fragment of DBI (e.g. CDN) acts as an endogenous modulator on the brain GABA_A/benzodiazepine receptor chloride channel complex.

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Diazepam-binding inhibitor: response to Knudsen & Nielsen

As remarked in Knudsen & Nielsen (1990), DBI was first isolated from rat brain (Guidotti *et al.*, 1983), and then from bovine brain (Shoyab *et al.*, 1986), by monitoring its ability to inhibit benzodiazepine ligand binding. However, several other *in vitro* and *in vivo* tests es-

tablished the action of DBI on GABA_A receptor function. For example, rat brain DBI, though itself devoid of action, reduces GABA-activated Cl⁻ currents in cultures of spinal cord neurons prepared from mouse embryos (Bormann, 1988). This attenuation resembles that elicited by beta-carbolines (i.e., negative allosteric modulators of GABA_A receptors) because it is blocked by flumazenil. Furthermore, DBI, when injected intracerebroventricularly into rats, not only completely reverses diazepam's anticonflict action but also induces a proconflict effect very similar to that induced by the beta-carboline derivative FG 7142 (Guidotti *et al.*, 1983), which in man induces panic attacks (Dorow *et al.*, 1983). Clinical studies show increases in the content of DBI in cerebrospinal fluid of patients who suffer from severe depression (Barbaccia *et al.*, 1986) or hepatic encephalopathy (Rothstein *et al.*, 1989). Since in either pathology an alteration of GABAergic transmission has been postulated, these findings also point to a role of DBI in GABA_A receptor regulation.

Exogenously applied DBI (Guidotti *et al.*, 1983; MacDonald *et al.*, 1986) is rapidly metabolized following exposure to endo- and exo-peptidases (Guidotti *et al.*, 1983; Ferrero *et al.*, 1986). Peptidase susceptibility in many instances, therefore, minimizes the biological activity of DBI or that of its processing products and may explain the difficulties encountered by Knudsen & Nielsen in demonstrating displacement of [³H]diazepam and [³H]PK-11195 bound to crude (presumably protease-rich) brain membranes.

To avoid inconsistencies and to establish an experimental protocol suitable for studying the potency and efficacy of DBI and its processing products, we used intact primary cultures of rat cerebellar granule neurons which contain functional GABA_A receptors while their culture media cannot degrade DBI rapidly. As shown in Table 1, DBI (DBI-1–86) and ODN (DBI-33–50) displaced [³H]flumazenil binding. However, ODN could not displace [³H]Ro-5-4864 (4'-chlorodiazepam) bound to highly purified mitochondria obtained from rat adrenal cortex or from cultured glial homogenates. In contrast, DBI, DBI-17–50 (TTN), and DBI-22–50 displaced [³H]4'-chlorodiazepam effectively.

ODN and TTN are two processing products of DBI present in rat brain (Slobodyansky *et al.*, 1989). We believe ODN functions as a neuromodulator at GABAergic synapses because: (i) it is located in synaptic vesicles (Alho *et al.*, 1989); (ii) it possesses an α -helical configuration (Berkovich *et al.*, 1989) and it is released from nerve terminals following nerve depolarization (Ferrarese *et al.*, 1987); (iii) it down-regulates GABA_A receptors when tested *in vitro* or *in vivo* (Ferrarese *et al.*, 1987; Bender & Hertz, 1986; Slobodyansky *et al.*, 1989; Kavalier & Hirst, 1986); (iv) its brain content increases in benzodiazepine tolerance (Miyata *et al.*, 1987). Also, TTN is biologically active yet differs from ODN because it binds preferentially to a benzodiazepine receptor (peripheral type) operative in the regulation of the biosynthesis of neurosteroids, which in turn may modulate GABA_A receptors (Muhkin *et al.*, 1990). These data strongly suggest that brain DBI is not directly transduced into an intracellular signal, but may function as the precursor of smaller neuropeptides acting as endogenous allosteric modulators of GABA_A receptor. DBI is also present in proliferative astroglia and in peripheral tissues. In the peripheral tissues, DBI is unevenly distributed