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Protracted treatment with diazepam increases the turnover of putative endogenous ligands for the benzodiazepine/β-carboline recognition site

(diazepam-binding inhibitor/octadecaneuropeptide/chronic diazepam tolerance/γ-aminobutyric acid receptors/benzodiazepine receptors)

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ABSTRACT **DBI (diazepam-binding inhibitor) is a putative neuromodulatory peptide isolated from rat brain that acts on γ-aminobutyric acid–benzodiazepine–Cl^- ionophore receptor complex inducing β-carboline-like effects. We used a cDNA probe complementary to DBI mRNA and a specific antibody for rat DBI to study in rat brain how the dynamic state of DBI can be affected after protracted (three times a day for 10 days) treatment with diazepam and chlordiazepoxide by oral gavage. Both the content of DBI and DBI mRNA increased in the cerebellum and cerebral cortex but failed to change in the hippocampus and striatum of rats receiving this protracted benzodiazepine treatment. Acute treatment with diazepam did not affect the dynamic state of brain DBI. An antibody was raised against a biologically active octadecaneuropeptide (Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) derived from the tryptic digestion of DBI. The combined HPLC/RIA analysis of rat cerebellar extracts carried out with this antibody showed that multiple molecular forms of the octadecaneuropeptide-like reactivity are present and all of them are increased in rats receiving repeated daily injections of diazepam. It is inferred that tolerance to benzodiazepines is associated with an increase in the turnover rate of DBI, which may be responsible for the γ-aminobutyric acid receptor desensitization that occurs after protracted benzodiazepine administration.**

Protracted daily doses of benzodiazepines yield tolerance to the acute effects of these drugs (1–3), induce physical dependence (2, 4), and evoke withdrawal reactions in humans and animals when the benzodiazepine treatment is discontinued abruptly (3, 5). The tolerance to benzodiazepines might result from γ-aminobutyric acid (GABA) receptor desensitization (6, 7); however, our present understanding of the GABA/benzodiazepine receptor function as a gating mechanism for a specific Cl^- ionophore (8–10) does not allow a comprehension of the molecular basis for this GABA-receptor desensitization.

We (11) and others (12) have demonstrated the existence in brain of a 10-kDa polypeptide termed DBI (diazepam-binding inhibitor) (11). DBI is a negative allosteric modulator of the Cl^--channel opening mediated by GABA in primary cultures of mouse spinal cord neurons (13), and it elicits a proconflict response in the behavioral paradigm described by Vogel when injected intraventricularly into rats (11). Brain DBI is a N terminus-blocked neuropeptide whose amino acid sequence has been partially determined by tryptic fragment analysis (14). This has allowed the preparation of synthetic oligonucleotide probes to isolate cDNA clones from libraries of human and rat hypothalami (15, 16). With these cDNA probes the complete sequence of DBI has been obtained (15, 16). The large molecular weight of DBI has suggested that it might function as a precursor of smaller biologically active neuropeptides. To begin studies of DBI processing, an antiserum was raised in rabbits directed against a biologically active octadecaneuropeptide (Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys designated ODN) derived from the tryptic digestion of DBI (14). With this antiserum, ODN-like immunoreactive peptides have been detected in rat brain extracts (14). Immunohistochemical studies with antisera directed against ODN and DBI have allowed the detection in neurons of materials that react with both antisera (17). Moreover, specialized glial cells (Bergman cells) contain DBI-like immunoreactive material (18) but not ODN-like immunoreactivity (IR). DBI is unevenly distributed in various brain structures (18), and it can be released from primary neuronal cultures by depolarization in a Ca^{2+}-dependent manner (19). DBI is located in synaptic vesicles (19) and coexists in a large percentage of neurons with glutamic acid decarboxylase (17). However, some neurons that are devoid of glutamic acid decarboxylase contain DBI (17, 18). Four to five genes contain a DNA sequence that can be hybridized with cDNA probes complementary to specific DBI mRNA (15, 16).

To verify whether the content and the turnover rate of brain DBI changes during benzodiazepine tolerance, we studied the content of specific DBI mRNA and DBI- and ODN-like IR in various brain structures of rats receiving dose regimens of diazepam and chlordiazepoxide that are known to cause tolerance (2, 3).

MATERIALS AND METHODS

Benzodiazepine Treatment Schedules. Male Sprague–Dawley rats (10–12 wk old) were purchased from Zivic–Miller (Pittsburgh), 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] was from Hoffman–La Roche. Diazepam and chlordiazepoxide (Hoffman–LaRoche) were suspended in water with vigorous mixing and administered intragastrically by oral gavage; control rats received only water. The treatment schedule was a slight modification of that used by Ryan and Boisse (2, 3) and included three doses per day. Each of the three diazepam doses was 10 mg/kg of body weight for the first 3 days, 13.3 mg/kg for the next 3 days, and 16.7 mg/kg for the last 4 days; each of the three chlordiazepoxide doses was 75 mg/kg for the first 3 days, 100 mg/kg for the next 3 days, and 125 mg/kg for the last 4 days. To assay specific mRNA, the rats were decapitated, and various brain regions were dissected by the method of Glowinski and Iversen (20) and stored at −70°C.

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Abbreviations: GABA, γ-aminobutyric acid; DBI, diazepam-binding inhibitor; ODN, octadecaneuropeptide; IR, immunoreactivity.

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To measure DBI and ODN content, brains were fixed by focused microwave irradiation in less than 1 sec with a microwave instrument model NJE2603-10 KW (New Japan Radio, Tokyo). This procedure became mandatory after we obtained evidence that, in rats killed by decapitation, DBI undergoes a time-dependent proteolytic degradation.

Preparation of a DBI Probe Complementary to the mRNA for Rat DBI. The plasmid pIMc61 contains ≈450 bases of the rat DBI cDNA sequence cloned into the *Eco*RI sites of pUC18 (15). The 450-base insert was cut out by digestion with *Eco*RI (Pharmacia), separated on a 3.5% acrylamide gel to isolate the DBI complementary sequence, and electroeluted from the gel with an electroelution apparatus (IBI, New Haven, CT). The probe was nick-translated by using [α-^{32}P]dNTP (New England Nuclear) (800 Ci/mmol; 1 Ci = 37 GBq) to a specific activity of ≈5×10^8 cpm/μg and was purified on a Sephadex G-50 column by the method of Maniatis *et al.* (21).

DBI mRNA Hybridization Analysis. The brain structures were homogenized in 8 vol of guanidine thiocyanate (Bethesda Research Laboratories) (22). Poly(A)$^+$ RNA was prepared by two passages over an oligo(dT)-cellulose column (23). Poly(A)$^+$ RNA was applied to a 1.1% agarose/6% formaldehyde gel for electrophoretic separation and was transferred to nitrocellulose paper as described by Thomas (24). Hybridization with the nick-translated cDNA probe was carried out as described by Thomas (24). The nitrocellulose blot was washed in 0.3 M NaCl/3 mM Na citrate/0.1% $NaDodSO_4$ at room temperature and in 0.015 M NaCl/0.15 mM Na citrate/0.1% $NaDodSO_4$ at 55°C and was exposed to Kodak X-Omat film for 24–72 hr at −70°C with Kodak intensifying screens. The amount of poly(A)$^+$ RNA loaded on the formaldehyde gel was measured by absorbance at 260 nm. The tissue sample content of DBI mRNA was expressed relative to its total poly(A)$^+$ RNA content corrected for the poly(A)$^+$ RNA loss during extraction and purification. Densitometric scans of the negative pictures of the gel were performed (15) with a laser densitometer (LKB). The blots were hybridized with DBI and p1B15 (25) probes. The p1B15 mRNA codes for a structural protein; therefore, its brain content is very stable. The correlation between the densitometric area of DBI mRNA and that of p1B15 mRNA and the amount of poly(A)$^+$ RNA measured by absorbance at 260 nm remained relatively similar (Fig. 1). This correlation uniformity was obtained by running on the gel 5–20 μg of poly(A)$^+$ RNA. Hence, the content of DBI mRNA was expressed in arbitrary units defined in terms of the ratio of the densitometry values of the two hybridized specific mRNAs obtained in the same blot:

$$\text{unit} = \frac{\text{DBI mRNA area}}{\text{p1B15 mRNA area}}.$$

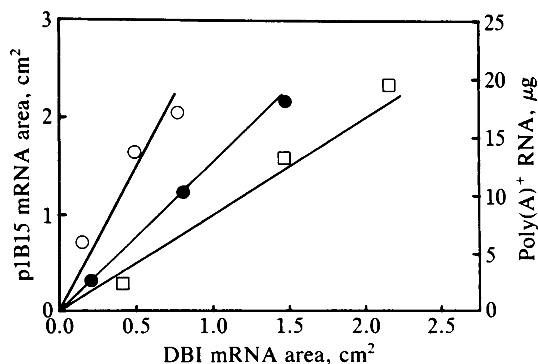

FIG. 1. Correlation between the densitometric area of DBI mRNA, that of p1B15 mRNA, and the poly(A)$^+$ RNA content as measured by absorbance at 260 nm. ○, Cerebral cortex; ●, hippocampus; and □, cerebellum. The RNA blot was hybridized with the DBI cDNA probe and was rehybridized with the p1B15 cDNA probe. Values of the densitometric scan of both autoradiograms were plotted. Poly(A)$^+$ RNA was applied to the gel in 5-, 10-, and 20-μg amounts.

RIA for DBI and ODN. Various brain areas (30 mg wet weight) were homogenized in 5 ml of 1 M acetic acid and centrifuged at 40,000 × *g* for 20 min. Aliquots of the supernatant were lyophilized and assayed for the DBI content by using a previously described RIA method with a well-characterized rabbit antiserum (18). Protein content was determined in aliquots of the same homogenates (26).

The RIA for ODN was performed with a rabbit antiserum directed against synthetic rat ODN (14) and, as a tracer, a synthetic 19-tyrosine extension of ODN (ODN-Tyr) iodinated with $Na^{125}I$ by mild oxidation with *N*-chlorobenzenesulfonamide adsorbed on polystyrene beads (Iodo-Beads, Pierce). The iodinated peptide was immediately purified on a Sep-Pak C_{18} column (Waters Associates). ODN antiserum (1:4000 dilution) detected as little as 0.1 pmol of synthetic ODN when using 0.1 pmol of ^{125}I-labeled ODN-Tyr as ligand. The cross-reactivity of this antiserum with DBI and with fragments that contain the sequence of the N or C terminus of ODN has been reported (14). In tissue extracts, because of DBI cross-reactivity the ODN RIA was performed in the effluent of a reverse-phase μBondapak C_{18} column (30 cm × 7.5 mm, Waters Associates). To establish recovery of ODN through the HPLC column, a trace amount of radioactive ODN-Tyr (2000 cpm; 6.7 fmol) was added to each tissue sample. The HPLC fractions were lyophilized and resuspended in 50 μl of H_2O and 100 μl of ^{125}I-labeled ODN-Tyr (30,000 cpm/0.1 pmol) diluted in H_2O. Subsequently, 100 μl of the ODN antiserum diluted 1:4000 in 0.05 M phosphate buffer (pH 7.4) containing 5% (wt/vol) bovine serum albumin was added. The RIA samples were incubated for 16 hr or longer at 4°C. Then 300 μl of protein A (2.5 mg/ml of 0.05 M Tris/2 mM $MgCl_2$, pH 8.0) was incubated with the reaction mixture at 4°C for 2 hr. After centrifugation at 5000 × *g*, the solid residues were counted.

RESULTS

Distribution of DBI-Like IR and DBI mRNA in Normal Rat Brain. Fig. 2 shows that DBI [^{32}P]cDNA hybridized with ≈600-base mRNA prepared from different brain areas but failed to hybridize with an equivalent amount of poly(A)$^+$ RNA prepared from adrenal chromaffin cells. The DBI mRNA units were highest in the poly(A)$^+$ RNA prepared from cerebellum and brain stem and lowest in that prepared from striatum (Table 1); moreover, cerebellum and brain stem had the highest DBI-like IR content, whereas striatum had the lowest (Table 1). The correlation coefficient (r) between the ranking orders of DBI-like IR and DBI mRNA contents of various brain areas was 0.87 ($P < 0.01$).

DBI-Like IR and DBI mRNA Content in the Brain of Rats Receiving Benzodiazepines. Rats were given intragastrically, three times a day for 10 days, increasing doses of diazepam or chlordiazepoxide. This dose regimen was designed to elicit immediately after administration an equivalent degree of motor function impairment. The abrupt interruption of such treatment resulted within 24 hr in a syndrome of hyperexcitation including tremor, twitches, arched back, piloerection, and, in some cases, vocalization upon handling. Similar behavioral changes were obtained when the withdrawal syndrome was precipitated by administration of Ro 15-1788 (10 mg/kg i.v.) 2 hr after the last administration of diazepam. Fig. 3 shows in a typical experiment that the densitometric areas of DBI mRNA in the cerebral cortex and cerebellum of rats receiving repeated daily doses of diazepam were greater

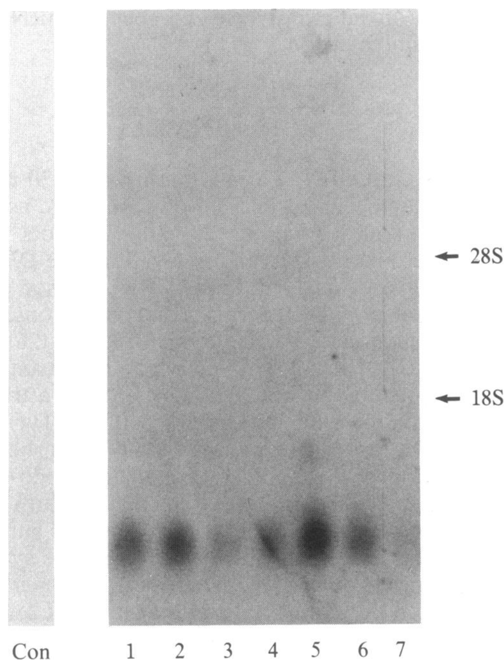

FIG. 2. RNA blot analysis of various rat brain regions with the DBI [^{32}P]cDNA probe. Poly(A)$^+$ RNA was hybridized with the nick-translated DBI cDNA insert probe. RNA from the primary culture of cow adrenal chromaffin cells is shown as the negative control; however, this sample was positive for the p1B15 cDNA probe (data not shown). Lanes: Con, control; 1, cerebral cortex; 2, cerebellum; 3, hypothalamus; 4, hippocampus; 5, brain stem; 6, midbrain; 7, striatum.

than that of rats receiving only the vehicle; the densitometric area of pB1B15 mRNA was almost identical in the two groups of rats. The amount of DBI-like IR in the cortex and cerebellum of rats receiving diazepam was higher than that of rats receiving vehicle (Fig. 4). This increase in DBI-like IR is due to an increase of authentic DBI because reverse-phase HPLC analysis of cerebellar extracts showed a single peak of DBI IR emerging at the position of standard DBI (see Fig. 6). In five groups of rats receiving the repeated daily treatment with diazepam, DBI-like IR content increased in cerebral cortex (33%) and in cerebellum (50%) but failed to increase in hippocampus and striatum (Fig. 4). In the same rats, DBI mRNA increased in cortex (60%) and cerebellum (22%) but failed to change in hippocampus and striatum (Fig. 5). Similar results were obtained after protracted treatment with chlordiazepoxide (data not shown). There was no difference in DBI-like IR content between untreated rats and rats receiving vehicle. The measurements reported in Figs. 4 and 5 were performed 12 hr after the last diazepam administration, but similar changes were obtained also when the measurements were performed 2 hr after the last treatment. In contrast, when the rats received only one or two injections of 10 mg of diazepam per kg i.p., the DBI content in cerebral cortex, cerebellum, hippocampus, and striatum remained unchanged.

Table 1. DBI-like immunoreactive material and DBI mRNA in different structures of rat brain

Brain structures	DBI-like IR, pmol/mg of protein ± SEM (n = 5–10)	DBI mRNA, unit ± SEM (n = 4 or 5)
Cerebral cortex	45 ± 3.4	0.54 ± 0.05
Cerebellum	130 ± 4.9	1.00*
Hippocampus	60 ± 5.3	0.57 ± 0.09
Striatum	43 ± 2.8	0.28 ± 0.03
Brain stem	130 ± 6.7	1.10 ± 0.21
Midbrain	73 ± 5.2	0.87 ± 0.12
Hypothalamus	99 ± 4.7	0.66 ± 0.03

*In cerebellum, the ratio of the densitometric area of DBI mRNA divided by the densitometric area of p1B15 mRNA was assigned the arbitrary value of 1.

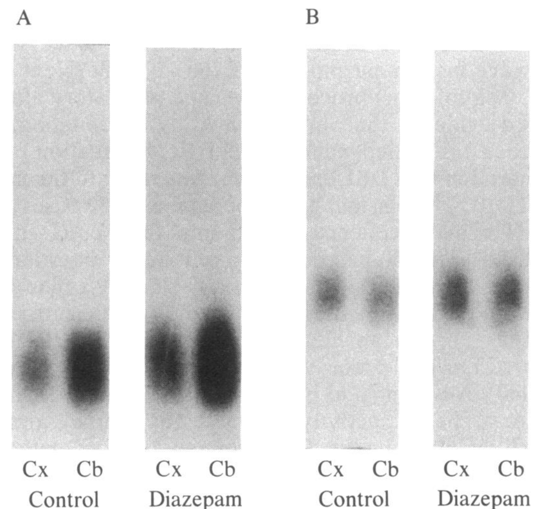

FIG. 3. RNA blot analysis of cerebral cortex (lanes Cx) and cerebellum (lanes Cb) from control (vehicle-treated) and chronic diazepam-treated rats. RNA was hybridized with the DBI [^{32}P]cDNA probe (A) and the p1B15 [^{32}P]cDNA probe (B).

ODN-Like IR Content in the Cerebellum of Rats Treated with Diazepam. When the ODN-like IR present in cerebellar extracts was measured in various fractions eluted from the reverse-phase HPLC column, it partitioned into four major peaks (Fig. 6). One peak emerged with a retention time identical to that of DBI; this ODN-like IR peak probably is DBI and reflects the DBI cross-reactivity with the ODN antibody. The peak eluted at fraction 32 presumably represents ODN because this fraction was eluted with radioactive ODN-Tyr. The other two ODN-like IR peaks (fractions 28 and 30) perhaps contain peptide fragments that include

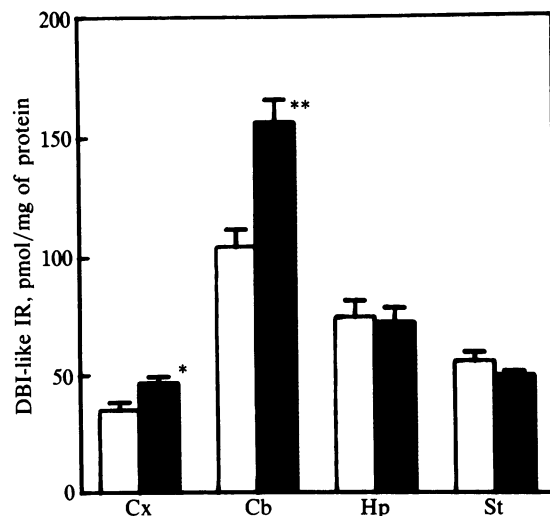

FIG. 4. DBI-like IR content in brain areas after chronic diazepam treatment. DBI-like IR of each brain area was assayed by RIA after microwave fixation. Bars: □, vehicle-treated rats; ■, diazepam-treated rats; Cx, cerebral cortex; Cb, cerebellum; Hp, hippocampus; St, striatum. Each value is the mean of 15–20 animals ± SEM. A significant difference between the vehicle- and diazepam-treated rats was calculated by Student's t test. *, $P < 0.01$; **, $P < 0.001$.

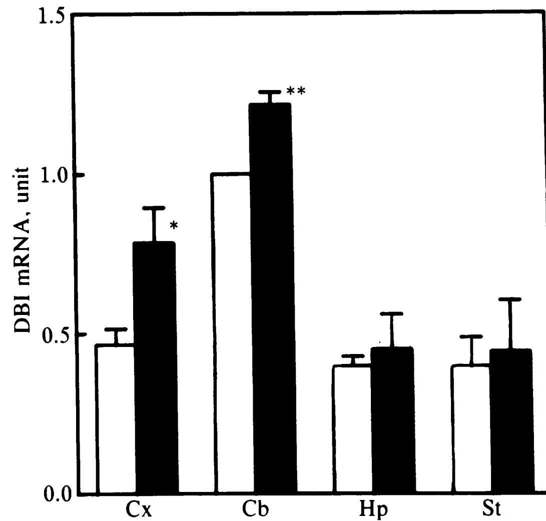

FIG. 5. DBI mRNA content in brain areas after chronic diazepam treatment. DBI mRNA of each brain area was measured by the hybridization technique. Bars: □, vehicle-treated rats; ■, diazepam-treated rats; Cx, cerebral cortex; Cb, cerebellum; Hp, hippocampus; St, striatum. Each value is the mean of 4 or 5 samples ± SEM. A significant difference between the control rats and diazepam-treated rats was calculated by Student's t test. *, $P < 0.05$; **, $P < 0.001$.

epitopes reacting with ODN antiserum. The ODN-like IR content of fractions 28–32 was increased in rats made tolerant to diazepam (Fig. 6). An increase of IR was particularly evident in the HPLC eluate corresponding to fraction number 28 (Fig. 6). With the average content of ODN-like IR expressed as ODN equivalents, the cerebellum of tolerant rats contained several times the amount of ODN-like IR found in rats receiving the vehicle only, and the extent of this increase was greater than that of DBI-like IR content (Table 2).

DISCUSSION

Tolerance to the effects of daily doses of benzodiazepines repeated for extended time periods is associated with a down-regulation in the function of the GABA–benzodiazepine–Cl^- ionophore complex (6, 7). However, during tolerance the characteristics of the benzodiazepine/β-carboline recognition sites remain unchanged, whereas the characteristics of the GABA recognition sites appear to change (6, 7). Perhaps GABA-recognition-site desensitization is associated and depends on the phosphorylation of some receptor constituents (27, 28). This posttranslational covalent modification might shift the GABA recognition sites to a low-capacity state with low intrinsic activity (28).

When Ro 15-1788 (a high-affinity, low-intrinsic-activity benzodiazepine recognition site ligand) is administered to tolerant rats that received the last benzodiazepine dose 16–24 hr before the experiment, it rapidly reverts GABA subsensitivity (29) as if it were displacing an endogenous benzodiazepine/β-carboline ligand presumably mediating the posttranslational covalent modification of the GABA receptor.

The present experiments were designed to test whether a change in the dynamic state of DBI, the putative endogenous ligand for benzodiazepine binding sites, may mediate benzodiazepine tolerance. To this end, we have assessed the dynamic state of DBI (the precursor) and ODN (a putative biologically active fragment) in brain structures of rats receiving increasing doses of diazepam or chlordiazepoxide three times a day for 10 days.

Table 2. DBI-like and ODN-like IR in cerebellar extracts after chronic diazepam treatment

Rats	IR, pmol/mg of protein	
	DBI-like	ODN-like
Control	99 ± 6.3	14.8 ± 1.8
Diazepam-treated	130 ± 6.8*	36.9 ± 4.0*†
% increase	31	149

Cerebellar extracts were chromatographed on a reverse-phase HPLC column (see Fig. 6). Each fraction was assayed for DBI-like and ODN-like IR. The value of ODN-like IR was expressed as an ODN equivalent and referred to the major peaks that emerged at 28–32% acetonitrile. Each value is the mean of four rats ± SEM.
*$P < 0.05$.
†$P < 0.01$.

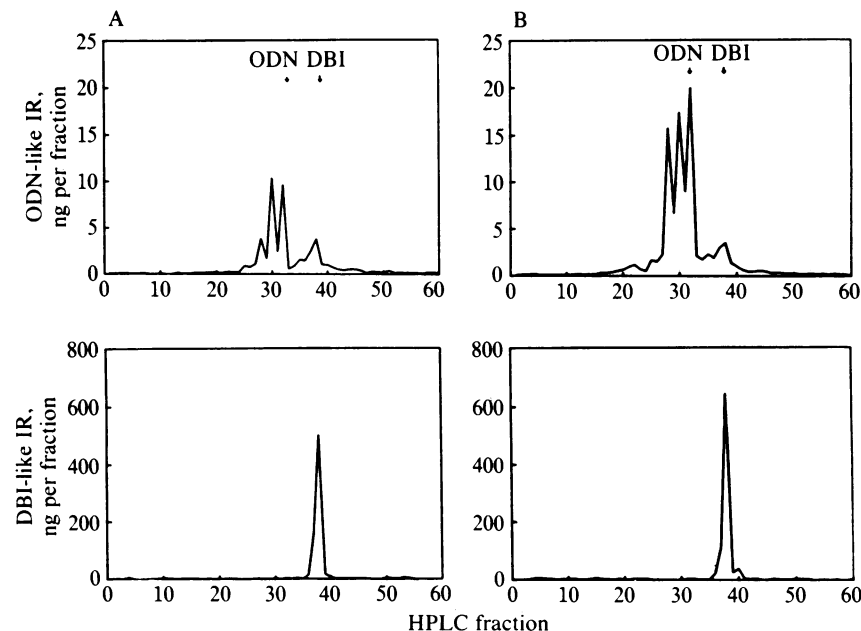

FIG. 6. Reverse-phase HPLC profile of ODN-like and DBI-like IRs in rat cerebellum after microwave fixation. Acetic acid (1 M) cerebellar extract (equivalent to 30 mg of wet tissue) was chromatographed on a μBondapak C_{18} (30 cm × 7.5 mm) HPLC column equilibrated with 0.1% trifluoroacetic acid. The column was developed with a 0–60% linear gradient of 0.1% trifluoroacetic acid in acetonitrile over 60 min at a flow rate of 2 ml/min. Aliquots (100 μl) of each fraction were assayed for DBI-like IR, and the remainder was assayed for ODN-like IR. (*A*) Vehicle-treated rats. (*B*) Chronic diazepam-treated rats. Recovery of ODN and DBI in this column was ≈50%.

We found that such a treatment increases DBI-like IR content selectively in the cerebral cortex and cerebellum, two brain areas with the highest density of benzodiazepine/β-carboline recognition sites (30). Chromatographic analysis of this DBI-like immunoreactive material indicates that probably the main immunoreactive material accumulating in cerebellum and cerebral cortex that is monitored by the DBI antiserum is authentic DBI. This contention is supported by the specificity of the antiserum whose antigenic binding site is directed against the N terminus of DBI and, therefore, recognizes only one form of immunoreactive material migrating as authentic DBI on polyacrylamide gel electrophoresis/blot-hybridization analysis (18).

An increase in DBI content can be attributed either to an increase in DBI synthesis or to a decrease in DBI utilization or processing rates. To distinguish between these two possibilities, we have measured the amount of DBI-specific mRNA as an indirect index of the peptide processing rate. Using a recently isolated cDNA clone specific for DBI mRNA (15) and the cDNA clone specific for p1B15 (25) as a probe to correct for poly(A)$^+$ RNA recovery, we have assessed in arbitrary units the DBI mRNA content in various brain structures and found that, in the two brain structures of tolerant rats, where DBI-like IR increases there is a parallel increase in DBI mRNA. These results suggest that the increase of DBI in tolerant rats might be triggered by an increase of the specific transcription rates of DBI mRNA and/or may be the result of mRNA stabilization with unchanged transcription rates. The hypothesis that an increase in synthesis associated with an increase in processing of DBI is operative in diazepam tolerance is supported by the finding that also the ODN-like IR content increases in cerebellum. Since ODN is a DBI fragment endowed with biological activity (14), these results support the view that during diazepam tolerance there is an increase in DBI synthesis and processing.

Because one or two doses of diazepam or a protracted diazepam dosage schedule that fails to elicit tolerance changes neither the DBI nor the ODN content of cortex and cerebellum, we can infer that the increase in the indices of the DBI turnover that we have measured is related to benzodiazepine tolerance. This increase in endogenous ligands of benzodiazepine recognition sites does not appear related to diazepam withdrawal because there is no difference in DBI content in rats killed during withdrawal or in tolerant rats killed before the withdrawal syndrome has begun. A change in the transsynaptic regulation of brain neuropeptide content and biosynthesis appears to be operative in increasing striatal enkephalin turnover rate following daily haloperidol administration (31). By analogy, the selective increase in ODN, DBI-like IR, and DBI mRNA in cortex and cerebellum could be triggered by diazepam-induced modification of transsynaptic modulation of DBI biosynthesis by afferent stimuli to both brain structures. This effect of benzodiazepine could be operative in causing tolerance to benzodiazepines and the concomitant desensitization of GABA receptors.

1. Greenblatt, D. J. & Shader, R. I. (1978) *Drug Metab. Rev.* **8**, 13–28.
2. Ryan, G. P. & Boisse, N. R. (1983) *J. Pharmacol. Exp. Ther.* **226**, 100–107.
3. Ryan, G. P. & Boisse, N. R. (1984) *J. Pharmacol. Exp. Ther.* **231**, 464–471.
4. McNicholas, L. F., Martin, W. R. & Cherian, S. (1983) *J. Pharmacol. Exp. Ther.* **226**, 783–789.
5. Pevnick, J. S., Jasinski, D. R. & Haertzen, C. A. (1978) *Arch. Gen. Psychiatry* **35**, 995–998.
6. Gallager, D., Lakoski, J., Gonsalves, S. & Rauch, S. (1984) *Nature (London)* **308**, 74–77.
7. Gallager, D., Rauch, S. & Malcolm, A. (1984) *J. Pharmacol.* **98**, 159–160.
8. Haring, P., Stahli, C., Schoch, P., Takacs, B., Staehelin, T. & Mohler, H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4837–4841.
9. Study, R. E. & Barker, J. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7180–7184.
10. Vicini, S., Meinville, J. M. & Costa, E. (1986) *Clin. Neuropharmacol.* **9**, Suppl. 4, 395–397.
11. Guidotti, A., Forchetti, C. M., Corda, M. G., Konkel, D., Bennet, D. N. & Costa, E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3531–3535.
12. Marquardt, H., Todaro, G. J. & Shoyab, M. (1986) *J. Biol. Chem.* **261**, 9727–9731.
13. Bormann, J., Ferrero, P., Guidotti, A. & Costa, E. (1985) *Regul. Pept. Suppl.* **4**, 33–38.
14. Ferrero, P., Santi, M. R., Conti-Tronconi, B., Costa, E. & Guidotti, A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 827–831.
15. Mocchetti, I., Einstein, R. & Brosius, J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7221–7225.
16. Gray, P. W., Glaister, D., Seeburg, P. H., Guidotti, A. & Costa, E. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7547–7551.
17. Costa, E., Alho, H., Santi, R., Ferrero, P. & Guidotti, A. (1986) in *Progress in Brain Research*, eds. Hokfelt, T., Fuxe, K. & Pernow, B. (Elsevier, New York), Vol. 68, pp. 343–356.
18. Alho, H., Costa, E., Ferrero, P., Fujimoto, M., Cosenza-Morphy, D. & Guidotti, A. (1985) *Science* **229**, 179–182.
19. Ferrarese, C., Vaccarino, F., Alho, H., Mellstrom, B., Costa, E. & Guidotti, A. (1987) *J. Neurochem.*, in press.
20. Glowinski, J. & Iversen, L. L. (1966) *J. Neurochem.* **13**, 655–669.
21. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
23. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
24. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
25. Milner, R. J. & Sutcliffe, J. G. (1983) *Nucleic Acids Res.* **11**, 5497–5520.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
27. Gallager, D. W., Sweetnam, P. M., Chafee, W. V. & Tallman, J. R. (1986) *Neurosci. Abstr.* **12**, 665 (abstr. 182.5).
28. Guidotti, A., Ferrero, P., Fujimoto, M., Santi, R. M. & Costa, E. (1986) in *Advances in Biological Psychopharmacology*, eds. Biggio, G. & Costa, E. (Raven, New York), Vol. 41, pp. 137–147.
29. Gonsalves, S. F. & Gallager, D. W. (1985) *Eur. J. Pharmacol.* **110**, 163–170.
30. Braestrup, C., Schmiechen, R., Neff, G., Nielsen, M. & Petersen, E. N. (1982) *Science* **216**, 1241–1243.
31. Tang, F., Costa, E. & Schwartz, J. P. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3841–3844.