10. W. Hunkeler, H. Möhler, P. Pieri, et al., Nature, 290, 514 (191).
11. J. P. Lapin, Pharmacol. Biochem. Behav., 14, 589 (1981).
12. J. Patel, P. J. Marangos, and F. Goodwin, Eur. J. Pharmacol., 72, 419 (1981).
13. J. H. Skerritt, S. C. Chow, G. A. Johnston, et al., Neurosci. Lett., 34, 63 (1982)
14. P. P. Skolnick, P. K. Marangos, M. Goodwin, et al., Life Sci., 23, 1473 (1978).
15. V. V. Zakusov, R. U. Ostrovskaya, V. V. Markovich, et al., Arch. Int. Pharmacodyn., 214, 188 (1975).

ENDOGENOUS INHIBITORS OF SPECIFIC BENZODIAZEPINE BINDING IN THE BOVINE CEREBRAL CORTEX

A. V. Kladnitskii and A. G. Mukhin

UDC 612.825.014.467:615.31:547.891.2

KEY WORDS: benzodiazepine receptors; endogenous inhibitors.

In the modern view the pharmacologic effects of benzodiazepines are realized through their interaction in the CNS with specific binding sites, known as benzodiazepine receptors [2, 10]. However, there is as yet no reliable proof of the existence of endogenous ligands for these binding sites, and consequently their physiological role is not yet clear. An intensive search for such ligands is now in progress [1, 3-5, 7-9, 11-13].

The writers have attempted to solve this problem by demonstrating the presence of low-molecular-weight inhibitors of specific binding of [^{3}H]diazepam in a homogenate of whole bovine cerebral cortex.

EXPERIMENTAL METHOD

Fresh bovine cerebral cortex was homogenized in 5 volumes of 0.01 HCl in a Waring blender at room temperature. After heating to 80°C for 30 min followed by cooling to 20°C the homogenate was centrifuged for 20 min at 1000g. The supernatant was neutralized with 0.1 M NaOH to pH 7.0 and filtered successively through Filtrak-88 (East Germany), GF/B (Whatman, England), and Twin-90 (Millipore, USA) filters. The filtrate was subjected to ultrafiltration through a filter consisting of hollow H10P5 fibers (nominal filtration limit 5000 daltons) on a DC-10 apparatus (Amicon, The Netherlands). The ultrafiltrate (about 40[illegible] liters from 12 g of original tissue) was frozen and lyophilized.

From 8 to 16 g of the freeze-dried products was dissolved in 20-25 ml of buffer A (24 mM NH_4HCO_3 pH 8.0), filtered through a GF/B filter, and applied to a column (4.4 × 80 cm) with Sephadex G-10, equilibrated with the same buffer. Elution was carried out with buffer A at the rate of 75 ml/h at 4°C, volume of fractions 12.5 ml. After determination of inhibitory activity, the fractions were pooled and freeze-dried.

Binding of [^{3}H]diazepam was carried out at 0.8-1.0°C for 45 min. The composition of the incubation medium was: 0.4 ml of a suspension of synaptic membranes, 0.1 ml of [^{3}H]diazepam (71 Ci/mmole, Amersham Corporation, England), giving a final concentration of 0.5 nM in the sample, and 0.5 ml buffer B (25 mM Tris-HCl, pH 7.4). The bound ligand was separated by filtration through GF/B filters. All solutions of the incubation mixture were made up in buffer B. To obtain the coarse fraction of synaptic membranes, the gray matter of the bovine cerebral cortex was homogenized in 10 volumes of 0.32 M sucrose by means of a Super 30 homogenizer (Virtis, USA); the residue obtained by centrifugation at 1000g for 15 min was resuspended in 10 volumes of buffer B and centrifuges at 30,000g for 30 min; the procedure was repeated 3 times and the residue resuspended in 5 volumes of buffer B, poured out in aliquots, and kept at −70°C. Before the experiment the membranes were diluted 8 times with buffer B and

Laboratory of Biochemistry, All-Union Mental Health Research Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR A. V. Snezhnevskii.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 10, pp. 439-441, October, 1984. Original article submitted October 14, 1983.

 0007-4888/84/9810-1366$08.50

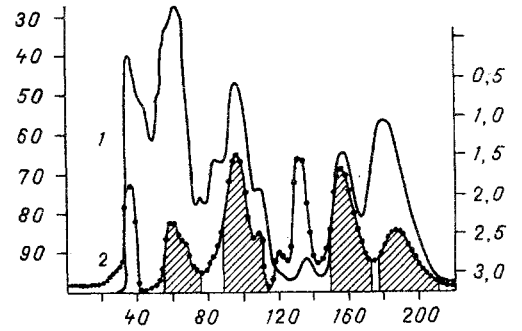

Fig. 1. Typical results of chromatography of acid extract on column with Sephadex G-10 (chromatography repeated 9 times). Abscissa, Nos. of fractions; ordinate, transmittance (in %) at 254 nm (left) and value of specific binding (in 10^3 cpm) of [^{3}H]diazepam (right). 1) Transmittance at 254 nm, 2) effect of material of fractions on specific binding of [^{3}H]diazepam. Fractions 56-76) peak B, 90-115) peak C, 150-175) peak D, 178-210) peak E.

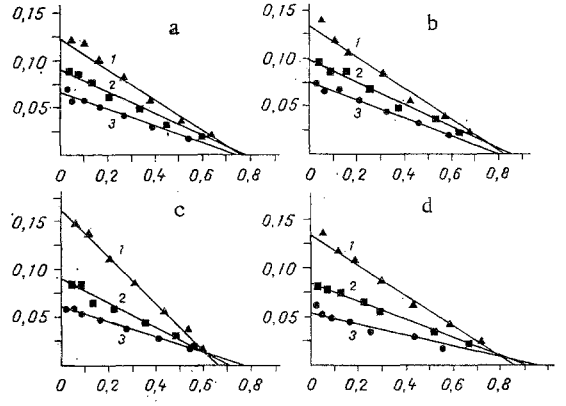

Fig. 2. Analysis of inhibitory acitivity of material from peaks B (a), C (b), D (c), and E (d) by Scatchard plots. Abscissa, specific binding of [^{3}H]diazepam (in nM); ordinate, ratio of specific binding (in nM) to equilibrium concentration of [^{3}H]diazepam (in nM). 1) Binding without inhibitor; a) concentration of inhibitor 14.3 mg/ml (2) and 28.6 mg/ml (3); b) 0.378 mg/ml (2) and 0.757 mg/ml (3); c) 0.075 mg/ml (2) and 0.15 mg/ml (3); d) 0.073 mg/ml (2) and 0.146 mg/ml (3).

homogenized in a Potter's (Teflon–Glass) homogenizer. Nonspecific binding was determined in the presence of nonradioactive diazepam in a concentration of 5 μM.

To determine inhibition of specific binding of [^{3}H]diazepam, instead of 0.5 ml of buffer A the test fraction was added to the sample in a volume of 0.5 ml. When freeze-dried specimens were used they were dissolved in buffer B. The character of inhibition was determined by the use of Scatchard plots within a concentration range of [^{3}H]diazepam from 0.5 to 32 nM.

Treatment of the material of the fractions with pronase was carried out by incubating samples taken in a concentration equal to twice that which inhibits binding of [^{3}H]diazepam by 50%, with the enzyme (0.7 IU/ml) for 30 min at 40°C. Activity of the enzyme was verified relative to proteolysis of a ^{125}I-labeled protein substrate.

EXPERIMENTAL RESULTS

Gel-chromatography of the ultrafiltrate of an acid extract of bovine cerebral cortical tissue (Fig. 1) revealed eight distinct peaks of absorption in UV light, seven of which contained substances inhibiting specific binding of [^{3}H]diazepam. Becuase of the technical characteristics of the N1OP5 cartridge used for ultrafiltration, the molecular weight of the isolated substances can be taken not to exceed 5000 daltons.

Since the volume required for elution of the material was 2.3 times greater than the volume of the column, it follows that under the conditions chosen and, in particular, when an

eluting buffer with low ionic strength was used, what took place was a combination of gel and adsorption chromatography [6]. Accordingly, accurate determination of the molecular weight of the test substances on the basis of the results of such chromatography was impossible. Meanwhile increasing the ionic strength of the eluting buffer in order to abolish adsorption was not indicated, because in that case it would be impossible to determine inhibitory activity actually in the samples of eluate.

Examination of the chromatographic profile (Fig. 1) reveals close coincidence of the principal peaks of inhibitory activity with peaks of absorption of the eluate in UV light. This coincidence is most probably evidence of the insufficiently high degree of purification of inhibitors at this stage of isolation.

The results of determination of the type of inhibition of [^{3}H]diazepam binding by the material contained in the four principal peaks of inhibitory activity are given in Fig. 2. It will be clear from Fig. 2 that the type of inhibition was close to competitive in all cases studied. This fact suggests that the substances contained in the peaks can be regarded as potential ligands of benzodiazepine receptors.

Determination of the properties of the isolated inhibitors showed that activity of inhibitors contained in peaks B, C, D, and E still remained after treatment with pronase, evidence supporting the view that they are nonpeptide in nature. Further purification and identification of the inhibitors described above are currently in progress.

LITERATURE CITED

1. T. Asano and S. Spector, Proc. Natl. Acad. Sci. USA, 76, 977 (1979).
2. C. Braestrup and R. F. Squires, Br. J. Psychiat., 133, 249 (1978).
3. C. Braestrup, M. Nielsen, and C. E. Olsen, Proc. Natl. Acad. Sci. USA, 77, 2288 (1980).
4. G. D. Collello, D. M. Hockenberg, H. B. Bosmann, et al., Proc. Natl. Acad. Sci. USA, 75, 6319 (1978).
5. L. G. Davis and R. K. Cohen, Biochem. Biophys. Res. Commun., 92, 141 (1980).
6. V. Di Gregorio and M. Sinibaldi, J. Chromatog., 129, 407 (1976).
7. A. Guidotti, G. Toffano, and E. Costa, Nature, 275, 553 (1978).
8. A. Guidotti, C. M. Forchetti, M. G. Corda, et al., Proc. Natl. Acad. Sci. USA, 80, 3531 (1983).
9. M. Karobath, G. Sperk, and G. Schonbeek, Eur. J. Pharmacol., 49, 323 (1978).
10. H. Möhler and T. Okada, Br. J. Psychiat., 133, 261 (1978).
11. H. Möhler, P. Polc, R. Cumin, et al., Nature, 278, 563 (1979).
12. P. Skolnick, P. J. Marangos, F. K. Goodwin, et al., Life Sci., 23, 1473 (1978).
13. P. Skolnick and F. K. Goodwin, Psychopharmacol. Bull., 16, 9 (1980).