DETECTION AND PROPERTIES OF BENZODIAZEPINE RECEPTORS OF GLIAL AND NEURONAL FRACTIONS OF THE HUMAN CEREBRAL CORTEX

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The mechanism of action of the benzodiazepines has not yet been fully explained. At the same time it can be taken as established that the action of drugs of this series is mediated through interaction with benzodiazepine receptors [4, 10]. Since the presence of specific binding sites for diazepam has been demonstrated [5-8, 12] both with the glial fraction isolated from the animal brain and also with glial cells in tissue culture, it can be postulated that the glia plays a definite role in the realization of the pharmacological effect of these drugs. However, the results of the investigations cited above are contradictory. For example, receptors for diazepam have not been found in a culture of bovine astroglial cells [3]. Yet workers who have found receptors for this ligand in glial cell cultures have demonstrated that their properties differ significantly from those of receptors in a fraction isolated from animal brain [8, 12]. This problem thus requires further study.

It was accordingly decided to attempt to discover benzodiazepine receptors and to study their properties in neuronal and glial fractions from the human cerebral cortex.

EXPERIMENTAL METHOD

To separate neurons and glia, brains were taken from persons dying from chronic ischemic heart disease or acute cardiac failure between the ages of 45 and 70 years. The time between death and taking of the material did not exceed 24 h. Tissue samples were frozen after isolation and kept at −70°C for not more than 3 months. The frontal cortex was chosen for the work. The oligodendroglia was isolated by the method suggested by Poletaev et al. [1], which was modified for the study of postmortem material. Tissue from the frontal cortex (10 g) was added to a small volume of original medium (1000 ml water, 150 g glucose, 10 g Ficoll, 8.5 g NaCl, 1.5 g KH_2PO_4, 0.2 g NaOH, and 1 g human serum albumin, pH 7.0) and ground with a stiff brush in a mortar, after which the volume of the cell suspension was made up to 100 ml with original buffer. The cell suspension was centrifuged twice at 127g for 5 min each time, after which the supernatant, containing capillaries and cell fragments, was discarded and the residue diluted with original buffer. The resulting suspension was filtered through steel sieves with different pore diameters: 1000 μ once, 140 μ twice, and 70 μ twice.

Sucrose solutions used as density gradients were made up in the original medium, and repeated centrifugation was carried out [1]. The cell filtrate was layered on gradients a, b, and c and centrifuged for 20 min at 2300g (4000 rpm). This and subsequent centrifugations were carried out on a Beckman L5-75 centrifuge (Austria), using an SW-27 bucket rotor. The top layer, containing an unpurified fraction of glia, was then layered on gradients d and e and centrifuged at 2300g (4000 rpm) for 30 min. The fraction located in the interphase of gradients d and e consisted of oligodendrogliocytes. The purity of the fraction was estimated both in phase-contrast and in light microscopes after staining of the cells with 1% methylene blue solution made up in physiological saline. The degree of contamination of the glial fraction with neuronal components did not exceed 10%.

To isolate neurons, the bottom layer in the interphase of gradients b and c, containing unpurified neurons, was layered on gradient e and centrifuged at 2870g (5000 rpm) for 25 min. Cells falling into the residue

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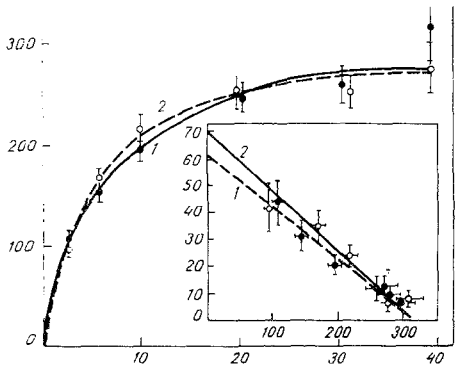

Fig. 1. Dependence of specific binding of [^{3}H]diazepam with glial (1) and neuronal (2) fractions of human cerebral cortex on concentration of ligand. Abscissa, concentration of free [^{3}H]diazepam (in nM); ordinate, specific binding (in femtomoles/mg protein). Here and in Figs. 2 and 3 each point is mean value of three independent measurements; each curve shows result of determination of binding with fraction isolated from brain tissue from a single person. Inset: same results expressed as a Scatchard plot. Abscissa, specific binding of [^{3}H]diazepam (in femtomoles/mg protein); ordinate, ratio of specific binding (in femtomoles/mg protein) to concentration of free [^{3}H]diazepam (in nM).

were pyramidal neurons, mainly surrounded by fragments of neuropil. The degree of contamination of this fraction with glial components was about 20%.

Binding of [^{3}H]diazepam was determined by incubating the suspension of neurons and glia (protein content in the sample 0.5 mg) with [^{3}H]diazepam (87 Ci/mmole, from Amersham Corporation, England) in a volume of 0.5 ml at 0°C for 60 min. After incubation the samples were quickly filtered in vacuo through GF/B filters (from Whatman, England), after which the filters were washed 3 times with cold Tris-HCl buffer, pH 7.4 (5 ml each time), poured into flasks containing Bray's scintillator, and radioactivity determined in a scintillation counter. Nonspecific binding was determined by adding a 1000-fold excess of unlabeled diazepam to the sample. Specific binding was determined as the difference between binding of samples not containing and containing an excess of unlabeled ligand [11].

EXPERIMENTAL RESULTS

Determination of specific binding of [^{3}H]diazepam with the isolated fractions of human brain tissue showed (Fig. 1) that both fractions, glial and neuronal, have quite a considerable number of specific binding sites for this ligand. As Fig. 1 shows, in both cases the process continues until saturation, indicating that the number of binding sites is limited. In both fractions saturation was virtually reached in the region of concentration of 20 nM.

Analysis of a Scatchard plot (Fig. 1, inset) revealed the presence of binding sites of only one type with dissociation constants (K_{diss}) of 5.2 and 4.5 nM and with a maximal number of binding sites (BS_{max}) of 305 and 310 femtomoles/mg protein for glia and neurons respectively. During determination of K_{diss} for diazepam in five different cases, with the aim of detecting differences between individuals, the relative constancy of this parameter was revealed: 4.6 ± 0.6 and 4.6 ± 0.5 respectively for the glial and neuronal fractions. These values agree with those for K_{diss} given in the literature: 4 nM for human brain homogenate, 2 nM for rat brain [4], and also 5 nM for the astroglial function obtained from bovine brain [7]. Values of BS_{max} were 264 ± 31 and 287 ± 42 femtomoles/mg protein for neurons and glia respectively. These figures are close to values obtained for astroglia of the squirrel's cerebral cortex, namely 142 femtomoles/mg protein [6].

A study of the kinetics of diazepam binding with neuronal and glial fractions showed (Fig. 2) that the process reaches saturation in both cases after 15 min at 0°C. The half-saturation period in these cases was 2 min.

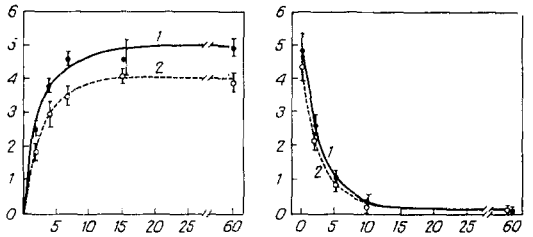

Fig. 2. Kinetics of specific binding of [^{3}H]diazepam (2 nM) with glial (1) and neuronal (2) fractions of human brain. Abscissa, time (in min); ordinate, specific binding, in cpm × 1000. Incubation temperature 0°C.

Fig. 3. Kinetics of dissociation of receptor–ligand complex (concentration of [^{3}H]diazepam 2 nM) from glial (1) and neuronal (2) fractions after addition of excess of nonradioactive diazepam (1 μM). Legend as to Fig. 2.

The kinetics of dissociation of the receptor–ligand complex was studied after addition of a 1000-fold excess of unlabeled ligand (1 μM diazepam) to the medium, i.e., by displacement of the radioactive ligand (Fig. 3). The value of BS_{max} as early as 15 min after the beginning of the displacement procedure was only 2% of its initial level. The half-dissociation time of the complex was 2 min. Dissociation of the complex indicates reversibility of the specific binding of diazepam.

Analysis of kinetic curves for the purpose of evaluating K_{diss} [9] showed its value to be about 3 nM, i.e., close to that found by means of a Scatchard plot.

The study of the human frontal cortex thus demonstrated the presence of specific binding sites in the fraction of oligodendroglia, comparable in order of magnitude with binding in the neuronal fraction.

Comparison of the properties of benzodiazepine receptors (equilibrium K_{diss}, kinetics and reversibility of binding) of the glial and neuronal fractions demonstrates the identity of the receptors revealed by this ligand.

Discovery of benzodiazepine receptors on glial cells isolated from the human brain makes the further study of the role of glia in the mechanism of action of benzodiazepines an urgent task.

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