IDENTIFICATION, ISOLATION, AND PHYSICOCHEMICAL PROPERTIES OF NEUROSPECIFIC α_2-GLOBULIN

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According to current concepts, properties specific for nerve tissue cells are mainly determined by the structure and functions of neurospecific proteins (NSP), which are unique tools by means of which genetic information is realized [3, 5, 6, 13].

Virtually all cytoplasmic NSP have been identified with the aid of polyspecific antisera, obtained by immunizing animals with a saline extract of brain tissue [3, 6, 14, 15]. There are now good grounds for postulating that brain tissue does not contain cytoplasmic NSP, in a concentration above 200 μg/g total protein, and with the immunogeneticity that would be required in order to obtain high-quality antisera to them by means of the usual approaches [2].

The aim of this investigation was to look for new cytoplasmic water-soluble NSP by the use of protein fractions of extract from human brain tissue, semipurified and concentrated 500-600-fold for immunization.

EXPERIMENTAL METHOD

The first series of polyspecific antisera were obtained to a semipurified protein preparation produced by the following method: brain tissue (material was sampled during the first 6 h after death) was thoroughly washed in physiological saline to remove blood, homogenized, and extracted with 0.05 M Na-phosphate buffer, pH 7.6 (working buffer), containing Triton X-100 and Tween-80 in a concentration of 1 g/liter. After freezing and thawing 3 times, the homogenate was centrifuged at 20,000g for 20 min, the supernatant was separated and NaCl was added to it to a final concentration of 0.2 M, after which it was subjected to ion-exchange chromatography on a column with DEAE-cellulose ("Whatman," England), equilibrated with working buffer containing NaCl in a concentration of 0.2 M, with a flow rate of 40 ml/h. The fraction eluted within the NaCl concentration range of 0.3 to 1 M was withdrawn and, after transfer into a system of acetate buffer 0.05 M, pH 7.8, and with the addition of $CaCl_2$, $MnCl_2$, and $MgCl_2$ in concentrations of 1 mM, it was subjected to affinity chromatography on a column with Con A-separose 4B ("Pharmacia Fine Chemicals," Sweden), equilibrated with Na-acetate buffer, pH 7.8, containing the above-mentioned salts in a concentration of 1 mM, and with a flow rate of 15 ml/h. For elution from the carrier a 0.2 M solution of α-D-manno-pyranoside was used. The resulting fraction was concentrated by freeze-drying and used to immunize chinchilla rabbits weighing 3-4 kg, using the traditional schedule [2].

Gel-filtration was carried out in a column packed with Sephadex G-200 ("Pharmacia"), equilibrated with working buffer, with a flow rate of 5 ml/h. The molecular mass was determined with the aid of standards obtained from "Serva" (West Germany).

Hydrophobic chromatography was carried out in a column packed with phenylsepharose (LKB, Sweden) and equilibrated with working buffer, with a flow rate of 40 ml/h. Proteins were eluted from the carrier in a linear concentration gradient of ammonium sulfate from 1 to 0 M, created by means of an "Ultragrad" gradient mixer (LKB).

The yield of protein fractions was recorded by means of a "Uvicord S" flow photometer (LKB) followed by immunochemical verification.

The amino-acid composition was studied after hydrolysis of the preparation with 6 N HCl for 12, 24, and 32 h at 105°C on a "Durrum DS-500" amino-acid analyzer (USA). Monosaccharides were determined by the phenol-sulfuric acid and orcine method [8]; before analysis the preparation was hydrolyzed with 2 M trifluoroacetic acid at 100°C for 6 h. The test antigen was compared with NSP known previously by immunodiffusion by Ouchterlony's method with certain modifications [4]. Preparations

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TABLE 1. Method of Obtaining α_2-GB

Saline extract from human brain tissue containing 0.2 M NaCl
+
Ion-exchange chromatography on column with DEAE-52-cellulose, equilibrated with working buffer containing 0.2 M NaCl
Protein fraction eluted from the carrier within the NaCl concentration range from 0.3 to 1M withdrawn and dialyzed against 0.05M Na-acetate buffer, pH 7.6, after which $CaCl_2$, $MgCl_2$, and $MnCl_2$ were added in concentrations of 1 mM
+
Affinity chromatography on column packed with con A-sepharose and equilibrated with 0.05 M Na-acetate buffer, pH 7.6, containing $CaCl_2$, $MnCl_2$, and $MgCl_2$ in a concentration of 1 mM.
Protein fraction eluted from carrier by 0.2 M solution of α-D-mannopiranoside withdrawn and concentrated by freeze-drying
+
Gel-filtration on Sephadex G-200
Protein fraction with molecular mass of 90 ± 10 kD withdrawn
+
Hydrophobic chromatography on phenyl-sepharose
Fraction eluted within ammonium sulfate concentration range from 0.45 to 0.17 M withdrawn and dialyzed against 0.05 M carbonate buffer, pH 9.8
+
Isochromatic focusing on PBE gel ("Pharmacia Fine Chemicals," Sweden)
Elution carried out with polybuffers 96, and 74 also glycine-HCl buffer, 0.05 M, pH 2.2. In the course of elution a protein fraction with isoelectric point within the pH range from 4.25 to 4.10 was withdrawn, alkalified with dry Tris, and dialyzed against working buffer.

of α_2-glycoprotein [15], GFAP [10], and proteins 10-40-4 and 14-3-2 [1] were obtained by methods suggested previously; a specimen of the preparation S-100 was generously provided by V. Yu. Alakhov (Mental Health Research Center, Ministry of Health of the USSR). The purity of the preparation and also the electrophoretic mobility of the test protein were estimated by crossed immunoelectrophoresis [11], polyacrylamide gel (PAG) disk electrophoresis [7], and NH_2-terminal analysis.

EXPERIMENTAL RESULTS

Assuming that the saline extract of brain tissue contains hitherto unidentified NSP, in a concentration insufficient to obtain antisera by immunizing animals with a saline extract of human brain tissue, we used concentrated semipurified preparations for immunization. For these purposes we used the protein fraction obtained by successive ion-exchange and affinity chromatography from a saline extract of human brain tissue. The polyspecific antiserum obtained by immunization, exhausted with dry plasma and by freeze-dried tissue extracts from the lung, spleen, liver, kidney, and testis, were investigated by methods of crossed immunoelectrophoresis and immunodiffusion. With the aid of this antiserum we were able to detect three antigens in the preparation used for immunization, including GFAP and two hitherto unidentified NSP with the electrophoretic mobility of α_1- and α_2-globulins. Later in our research, we used only antigen with the electrophoretic mobility of α_2-globulins, present in a larger quantity, and which we subsequently described as specific brain α_2-globulin (α_2-GB).

Under the control of this antiserum we developed a method of purifying the α_2-GB (Table 1), which enabled us to obtain a homogeneous preparation of α_2-GB, in which no impurities could be detected by disk-electrophoresis analysis followed by immunodevelopment (Fig. 1). The use of the α_2-GB preparation thus obtained to immunize rabbits yielded a monospecific antiserum to the test antigen.

Obtaining a monospecific antiserum and homogeneous preparation of α_2-GB enabled us to study some physicochemical properties of this NSP (Table 2), and also to undertake a chemical analysis of the preparation (Table 3). When the data given in these tables are analyzed, it must be noted that the test antigen, possessing electrophoretic mobility of α_2-globulins, has a molecular mass of 90 ± 10 kD and an isoelectric point of 4.1-5.4. Disk-electrophoresis of α_2-GB, treated with β-mercaptoethanol

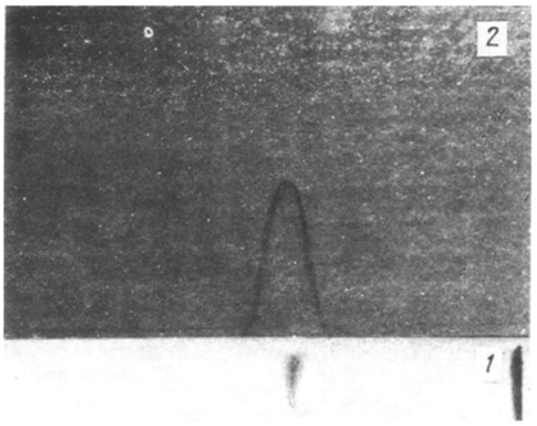

Fig. 1. Results of disk-electrophoretic analysis of purified α_2-GB preparation in 7.5% PAG followed by immunodevelopment. 1) Disk-electrophoresis of purified α_2-GB preparation in 7.5% PAG; 2) immunoelectrophoresis and immunodevelopment in 1% agarose gel containing 10% monospecific antiserum obtained by immunization of rabbits with a purified α_2-GB preparation.

TABLE 2. Physicochemical Properties of α_2-GB

Physicochemical properties of α_2-GB	α_2-GB
Precipitation with ammonium sulfate	Precipitated at 70-40 % saturation
Precipitation with ethanol	50% Ethanol precipitates 25% of antigen
Precipitation with butanol	10% Butanol denatures the antigen
Precipitation with TCA	10% TCA precipitates the antigen
Adsorptive properties:	
a) Alumina	Adsorbed
b) Hydroxyapatite	35-40% of antigen adsorbed
c) Barium sulfate	Not adsorbed
d) Magnesium oxide	Not adsorbed
Interaction with ions of metals	
a) Zinc	Not bound
b) Copper	15% of antigen bound
c) Cobalt	Not bound
Thermostability	Withstands incubation at 80°C for 30 min
Interaction with lectins	
a) Con A	80-90% of antigen bound
b) lentil-lectin	50-60% of antigen bound
c) Weat germ	Not bound
Relative molecular mass	90 ± 10 kD
a) Measured by gel-filtration	90 ± 10 kD
b) Measured by disk-electrophoresis	Peak 1 – 53.0 ± 1.8 kD Peak 2 – 31.0 ± 1.1 kD
Relative electrophoretic mobility	0.86 ± 0.01
Isoelectric point	4.10-4.25
Interaction with phenyl-sepharose	100% of antigen bound

TABLE 3. Results of General Chemical Analysis of α_2-GB

Chemical composition	α_2-GB, mg/100 mg antigen	Method of determination
Protein	78,92	[12]
Monosaccharides	10,22	[8]
Sialic acids	1,65	
Hexosamines	1,84	[9]
Sulfate	+	IR-spectroscopy

Amino-acid composition of α_2-GB

Amino acid	% of amino acid relative to 100 amino acids	Amino acid	Per cent of amino acid relative to 100 amino acids
CYS (O_3H)	2,15	LYS	6,18
HIS	3,36	ARG	4,79
ASP	10,82	GLU	11,34
TYR	4,22	SER	5,78
PRO	1,35	GLY	10,04
ALA	8,61	VAL	7,75
MET	2,07	LEU	1,96
ILEU	5,23	THR	6,22
PHE	4,17	TRY	decomposed under conditions of acid hydrolysis, and so not determined

Carbohydrate composition of α_2-GB

Monosaccharide	mg/100 mg of substance	Ratio by weight
Glucose	3,43	5,53
Mannose	2,12	3,41
Galactose	1,66	2,67
Fucose	0,82	1,32
Glucosamine	1,36	2,19
Galactosamine	0,62	1,00

in PAG with dodecylsulfate, revealed two protein zones with molecular masses of 53.0 ± 1.8 and 31.0 ± 1.1 kD. This fact may be evidence of the subunitary structure of α_2-GB, consisting of two subunits evidently connected by a disulfide bond. Among the other properties of α_2-GB must be noted its high affinity for con A, so that it may be assumed that carbohydrate chains containing D-glucose and D-mannose are present in its structure. Specific binding with phenyl-sepharose is evidence that hydrophobic domains are present in the native structure of the antigen molecule.

After analysis of the results of the general chemical analysis it can be concluded that α_2-GB is a sulfated sialoglycoprotein, the main components of which are a protein (78.9%) and monosaccharides (10.22%). The amino-acid composition is dominated by monoaminocarboxylic and alliphatic amino acids, which explains the acid properties of the antigen and the strong electrically negative charge of its molecule, suggesting an amide type of bond with the carbohydrate component. NH_2-terminal analysis of α_2-GB revealed two terminal amino groups in its structure, belonging to glutamic acid and glycine, confirming the results of disk electrophoresis, which indicated a two-subunitary structure of this antigen.

Having obtained a purified preparation of α_2-GB and monospecific antisera to it we undertook an immunochemical comparison of this antigen with cytoplasmic NSP known previously. After analysis of the results of immunodiffusion analysis it was concluded that α_2-GB is totally unidentified with cytoplasmic NSP such as GFAP, specific α_2-glycoprotein [15], and proteins 10-40-4, 14-3-2, and S-100, and it is evidently a hitherto unidentified human brain tissue antigen.

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SPECIFIC BINDING OF ^{3}H-GABA BY SYNAPTIC MEMBRANES OF THE RAT HYPOTHALAMUS AND HIPPOCAMPUS AFTER ADRENALECTOMY AND HYDROCORTISONE AND ACTH ADMINISTRATION

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The concept of heterogeneity of GABA receptors has now been successfully elaborated [2], their structure elucidated [4], and many aspects of the function of the oligomeric GABA/benzodiazepine/Cl^--channel complex have been studied both under normal conditions [5] and in pathological states of the brain [3], and the role of GABA receptors in the mechanism of action of drugs has been investigated [8]. Meanwhile, the question of the effect of hormones and, in particular, those of the hypothalamo-hypophyseo-adrenal system, on the state of GABA receptors remains largely unexplained, although it is well known that any change in the corticosteroid level in the body is accompanied to a greater or lesser degree by the changes in the functional state of brain structures [1], which are directly linked with disturbances of ionic permeability of nerve cell membranes.

The aim of this investigation was to study the effect of adrenalectomy, and also of single or repeated injections of hydrocortisone and ACTH on specific binding of ^{3}H-GABA by synaptic membranes of the rat hypothalamus and hippocampus.

EXPERIMENTAL METHOD

Experiments were carried out on male Wistar rats weighing 150-200 g. The adrenals were removed under ether anesthesia 8 days before sacrifice of the animals. Hydrocortisone ("Gedeon Richter," Hungary) in a dose of 5 mg/kg, and ACTH and ACTH-zinc-phosphate (Kaunas Endocrine Preparations Factory), in a dose of 2.5 U/100 g, was injected intramuscularly in a single dose or daily for 7 days. The rats were decapitated 4 h after the single injection and 24 h after the last of the series of injections of the preparations. Rats undergoing a mock operation, and receiving injections of the corresponding volume of physiological saline, served as the control.

The synaptic membrane fraction was obtained from the coarse mitochondrial fraction (18,000*g*, 20 min). The residue of the fraction was suspended in 20 volumes of bidistilled water, frozen at –20°C for 18-20 h, thawed, and centrifuged (45,000*g*,

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