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Molecular Weight and Subunit Composition of the Sensitive to GABA-Ergic Ligands Cl^-/HCO_3^--Stimulated Mg^{2+}-ATPase from Plasma Membrane of Rat Brain

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Abstract—The molecular weight and subunit composition of Cl^-,HCO_3^-- and picrotoxin-stimulated Mg^{2+}-ATPase from rat brain plasma membrane solubilized in sodium deoxycholate were studied by gel filtration chromatography. The enzyme activity eluted from a Sephacryl S-300 column in a single peak associated with a protein of molecular weight ~300 kD and a Stokes radius of 5.4 nm. The enzyme-enriched fraction, concentrated and denatured by SDS, migrated through a Sephacryl S-200 column as three peaks with molecular weights of approximately 57, 53, and 45 kD. SDS-PAGE also showed three major protein bands with molecular weights of about 57, 53, and 48 kD. The molecular weight and subunit composition of the Cl^- and HCO_3^--stimulated Mg^{2+}-ATPase from neuronal membrane of rat brain are similar with the molecular properties of $GABA_A$-benzodiazepine receptor complex from mammalian brain but are different from those of P-type transport ATPases.

Key words: rat, brain, plasma membranes, Cl^-,HCO_3^--ATPase, γ-amino-*n*-butyric acid (GABA), picrotoxin, gel chromatography, molecular weight, subunit composition

Chloride stimulated ATPase (Cl^--ATPase) from the plasma membranes of cells of different origin is considered by the number of researchers as an ATP-dependent Cl^--pump that transports chloride against an electrochemical gradient [1]. A Cl^--ATPase from rat brain neuronal membranes (Cl^--pump) involved in the transport of chloride from inside the neuron to the extracellular milieu has been investigated [2]. In preliminary studies with fish and later rat brain, we have also revealed Cl^--stimulated Mg^{2+}-ATPase containing "basal" Mg^{2+}-ATPase activity stimulated by chloride [3-5]. More detailed study of this enzyme has shown its ability to undergo stimulation not only by chloride, but to a large extent by both chloride and bicarbonate simultaneously (Cl^-,HCO_3^--ATPase) [6, 7]. The enzyme is specifically inhibited by bicuculline and picrotoxin (this effect is accompanied by significant stimulation of "basal" Mg^{2+}-ATPase activity) [3], thus indicating its functional and structural conjugation with inhibitory receptors and possible role in the functioning of $GABA_A$-receptors. The Cl^-,HCO_3^--ATPase is highly sensitive to SH-reagents, *o*-vanadate, and oligomycin, which is typical for P-type transport ATPases [8, 9]. The identification of the enzyme requires, in particular, the study of its molecular weight and subunit composition. Therefore, the aim of this work was purification and determination of subunit composition of Cl^-,HCO_3^--ATPase from rat brain membranes by using gel chromatography and SDS-PAGE.

MATERIALS AND METHODS

Rats weighing 150-200 g were used in this work. After decapitation the brain was extracted and homogenized at ratio 1 : 8 in 10 mM Hepes-Tris buffer, pH 7.4, containing 0.125 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then centrifuged in a Beckman ultracentrifuge (USA) in a bucket rotor (SW-28) for 20 min at 10,000*g* and 4°C. The supernatant was centrifuged for 1 h at 100,000*g* and 4°C; and the pellet was resuspended and used as plasma membrane enriched fraction.

To obtain a soluble enzyme preparation, plasma membranes were incubated with 1% sodium deoxycholate at room temperature for 20 min, and then centrifuged at 100,000*g* for 30 min.

Gel chromatography. The molecular weight of the enzyme was determined by gel filtration on a column

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(1.6 × 59 cm) packed with Sephacryl S-300 and equilibrated with 10 mM Hepes-Tris buffer, pH 7.4, containing 0.25% sodium deoxycholate, 0.125 mM EDTA, and 0.1 mM PMSF. The column was loaded with 2 ml of soluble enzyme preparation. Fractions (1 ml volume) were collected at elution rate of 21.0 ml/h per cm^2 at 4°C, and Mg^{2+}-ATPase activities stimulated by Cl^-/HCO_3^- and picrotoxin were determined. The column was calibrated using standard protein markers: apoferritin (4 mg, Stokes radius *a* is 5.8 nm), catalase (3.5 mg, *a* 5.2 nm), γ-globulin (3.0 mg, *a* 4.5 nm), BSA (3.5 mg, *a* 5.8 nm), myoglobin (3.0 mg), and cytochrome *c* (3.0 mg, *a* 1.7 nm). The void volume (V_o) of the column was measured using blue dextran 2000 (0.7 mg). Stokes radius was calculated according to the standard method [10].

Fractions enriched with the enzyme were pooled, concentrated to 1 ml using ultrafiltration through Centricon YM-100 filters (Amicon, USA), and then used for subunit composition studies by gel chromatography and SDS-PAGE. To obtain separate subunits, concentrated Cl^-,HCO_3^--ATPase was denatured by 5% SDS and 4% β-mercaptoethanol. A column (1.6 × 59 cm) containing Sephacryl S-200 was equilibrated with buffer containing 10 mM Hepes-Tris buffer, pH 7.4, 0.1% SDS, and 0.125 mM EDTA, and after the loading of protein sample (5 mg), elution was performed at flow rate 13.0 ml/h per cm^2 and room temperature. The column was calibrated using the following protein standards (4 mg each): BSA, ovalbumin, chymotrypsinogen A, and myoglobin.

SDS-PAGE. Protein preparations were mixed with equal volumes of buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 8% β-mercaptoethanol, and 10% sucrose and boiled for 3 min. Electrophoresis was performed in 12% polyacrylamide gel according to Laemmli at 35 mA current [11]. Electrophoregrams were stained with 0.1% Coomassie blue. Protein molecular weights were determined by the conventional method, comparing their electrophoretic shift mobility with that of protein markers (transferrin, BSA, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome *c*).

Measurement of enzyme activity. Concentrated protein preparations were incubated in 0.5 ml of the medium containing 10 mM Hepes-Tris buffer, pH 7.4, 0.75 mM $MgSO_4$, 0.75 mM Tris-ATP, 0.125 mM EDTA, and a mixture 10 mM NaCl plus 2 mM $NaHCO_3$ or 0.1 mM picrotoxin. The reaction was started by addition of protein (10-20 μg) and stopped by addition of 0.5 ml of 10% trichloroacetic acid (TCA) to the incubation medium. Specific ATPase activity was determined by the increment in inorganic phosphorus (P_i) in 0.5 ml of incubation medium during 30 min at 37°C and expressed as μmol P_i/h per mg protein. Phosphorus content was measured according to Chen et al. [12], and total protein according to Bradford [13]. "Basal" Mg^{2+}-ATPase activity was calculated as the difference between enzyme activities in the presence and absence of 0.75 mM $MgSO_4$. Activity of Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase (Cl^-,HCO_3^--ATPase) was estimated by the difference between Mg^{2+}-ATPase activities in the presence and absence of 10 mM NaCl plus 2 mM $NaHCO_3$. Activity of picrotoxin stimulated Mg^{2+}-ATPase was calculated as the difference between Mg^{2+}-ATPase activities in the presence and absence of 0.1 mM picrotoxin.

The following reagents were used in this work: Tris, Hepes, picrotoxin, bicuculline, GABA, SDS, sodium deoxycholate, a set of protein standards (apoferritin, γ-globulin, BSA, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome *c*) (ICN, USA); reagents for electrophoresis (Bio-Rad, USA); Sephacryl (Amersham, Great Britain); catalase, EDTA, β-mercaptoethanol (Sigma, USA); ATP (Reanal, Hungary). Other chemicals were of domestic production.

RESULTS

Using gel chromatography on an S-300 Sephacryl column, we studied the elution profile of Cl^-,HCO_3^-- and picrotoxin-stimulated Mg^{2+}-ATPase activities from rat brain plasma membranes solubilized by sodium deoxycholate. The Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity eluted from the column in a single peak corresponding to eluent volume V_e = 57 ml (Fig. 1). Picrotoxin-stimulated Mg^{2+}-ATPase activity is also eluted in a single peak at V_e = 57 ml (Fig. 1). These data suggest that Cl^-,HCO_3^-- and picrotoxin-stimulated Mg^{2+}-ATPase activities belong to the same enzyme. To confirm this assumption, we also investigated the influence of ligands of $GABA_A$-receptors on the activity of eluted Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase, since earlier we have shown that agonists and antagonists of inhibitory receptors activate "basal" Mg^{2+}-ATPase in the absence of mediator and prevent its activation by Cl^- [3]. It was established that GABA (0.1 mM), picrotoxin (0.1 mM), and bicuculline (0.05 mM)

Influence of $GABA_A$-receptor ligands on Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity eluted from a Sephacryl S-300 column after gel chromatography

Ligand	Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity, μmol P_i/h per mg protein
Without additions	1.8 ± 0.20
GABA, 0.1 mM	1.0 ± 0.12
Picrotoxin, 0.1 mM	0.9 ± 0.07
Bicuculline, 0.05 mM	1.1 ± 0.10

Note: The table shows arithmetic mean and standard deviation ($M \pm m$), $n = 3$.

decrease Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity by 40-50% (table), indicating that the investigated ATPase activities belong to the same protein, sensitive to $GABA_A$-ergic ligands Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase. Based on the calibration curve constructed using the marker proteins, we demonstrated that molecular weight of the studied enzyme is ~310 kD. The stokes radius for the enzyme, determined from the corresponding calibration curve and its gel filtration parameters, is 5.4 nm.

Fractions enriched with Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity and concentrated after protein separation on the column with Sephacryl S-300 were used for determination of enzyme subunit composition. It was shown by gel chromatography that the enzyme, denatured by SDS and β-mercaptoethanol, is eluted from the Sephacryl S-200 column in three peaks (Fig. 2). Using a calibration curve constructed for protein markers, we determined the molecular weights of the subunits corresponding to those peaks and found that they are about 57, 53, and 45 kD. Moreover, using SDS-PAGE in 12% polyacrylamide gel it was revealed that the initial protein preparation after SDS treatment results in more than 30 proteins on an electrophoregram, whereas after gel chromatography of enriched Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase fractions three major bands with molecular weights of about 57, 53, and 48 kD as well as an insignificant amount of high and low molecular weight proteins are detected (Fig. 3).

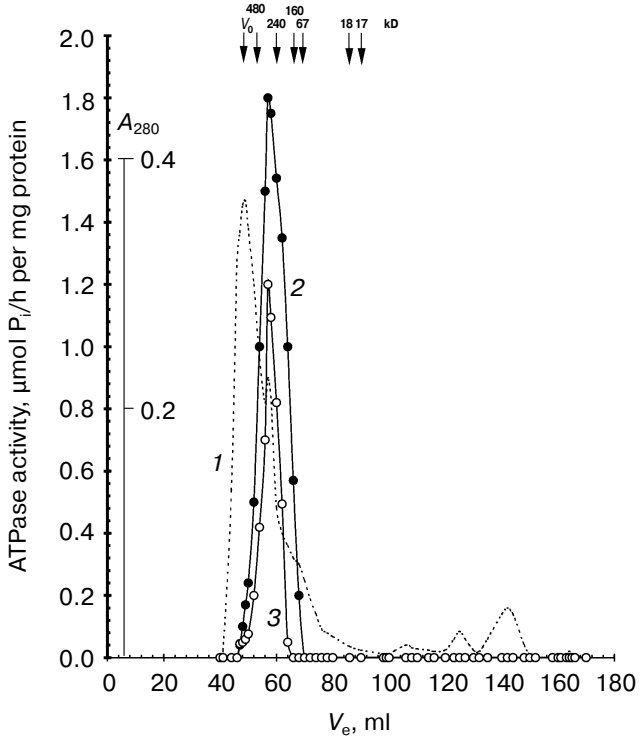

Fig. 1. Elution profile of soluble protein fraction (absorption at 280 nm, A_{280}) (*1*) and activities of Cl^-,HCO_3^-- (*2*) and picrotoxin-stimulated (*3*) Mg^{2+}-ATPases from rat brain plasma membrane solubilized in 1% sodium deoxycholate on a Sephacryl S-300 column. Protein standards (from left to right): dextran blue 2000, apoferritin, catalase, γ-globulin, BSA, myoglobin, cytochrome *c*.

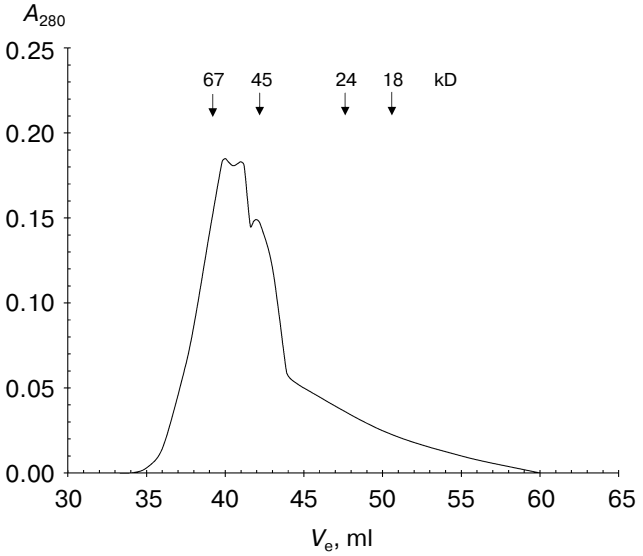

Fig. 2. Elution profile of SDS denatured protein fraction enriched with Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity on the Sephacryl S-200 column. Protein standards: BSA, ovalbumin, chymotrypsinogen A, and myoglobin.

DISCUSSION

Our experiments with gel chromatography showed that $GABA_A$-ergic ligand sensitive Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase of rat brain was eluted from the Sephacryl S-300 column as one peak with molecular weight of ~310 kD. It was shown earlier on determining molecular weight of integral membrane proteins by gel chromatography that a hydrodynamic particle in sodium deoxycholate solution appear as the protein–detergent complex, where detergent amount bound to the purified receptor may reach 1-3% of the protein mass [14]. Considering this factor, the molecular weight of Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase is ~300 kD.

At present, several ion-transporting ATPases with similar molecular properties and ion translocation mechanisms are known; they are classified as a single family of P-type (or E_1/E_2-type) ATPases. The most studied enzymes are Na^+,K^+-ATPase from plasma membrane of various cells, and Ca^{2+}-ATPase from sarcoplasmic reticulum [15]. The majority of enzymes from this group are represented by a single polypeptide with molecular weight of ~100 kD, the exception being Na^+,K^+-ATPase from various tissues containing one more smaller subunit with molecular weight of 60 kD [16]. The Cl^--ATPase (Cl^--pump) from plasma membranes of shellfish intestinal

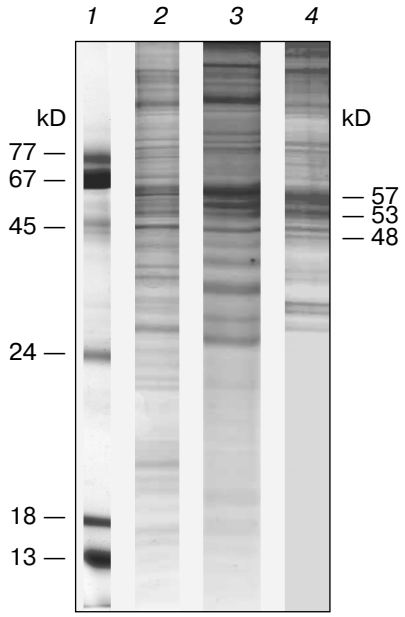

Fig. 3. Electrophoregrams of protein preparations during different stages of gel chromatography (their molecular weights in kD are shown on the left and right): *1*) protein markers (transferrin, BSA, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome *c*); *2*) soluble protein fraction from plasma membrane solubilized in 1% sodium deoxycholate; *3*) fraction enriched with Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity after gel chromatography on the Sephacryl S-300 column; *4*) fraction after gel chromatography on the Sephacryl S-200 column.

epithelium has a molecular weight of 110 kD [17], whereas Cl^--ATPase (Cl^--pump) from rat brain neuronal membranes has molecular weight of 520-580 kD [18]. It is evident that the molecular weight of the studied Cl^-,HCO_3^--ATPase from rat brain plasma membrane is significantly different from that described in the literature for transport P-type ATPases and ATP-dependent Cl^--pumps transporting chloride against an electrochemical gradient. However, our results are in good agreement with data obtained for inhibitory receptors. For example, during gel filtration chromatography of the native $GABA_A$-receptor from bovine brain pre-treated with Triton X-100 and solubilized by sodium deoxycholate, the protein was eluted from Sephacryl CL-6B column as a single peak with molecular weight of ~230 kD. The Stokes radius for this enzyme was 7.3 nm [19]. In the same investigation, $GABA_A$-receptor solubilized in 3-[(3-cholamidopropyl)-dimethylammonium]-1-propylsulfate (CHAPS) also migrated as a single peak with molecular weight of ~290 kD, the Stokes radius being 6.8 nm. In other publications it was shown that the molecular weight of $GABA_A$-benzodiazepine receptor complex for different regions of rat brain can be 220-300 kD, and the Stokes radius ~5 nm [20]. Moreover, using gel chromatography with Sephacryl S-200 column and SDS-PAGE in our work, it was shown that studied Cl^-,HCO_3^--ATPase consists of three subunits with molecular weights of 57, 53, and 45-48 kD. It is known from the literature data that $GABA_A$-benzodiazepine Cl^--channel from rat brain is a heterooligomeric protein (most likely a pentamer) including at least three different types of subunits, β, α, and γ with molecular weights of ~57, 53, and 48 kD, respectively [20, 21]. It is also known that Cl^--ATPase (Cl^--pump) from rat brain neuronal membranes contains four subunits with molecular weights of 62, 60, 55, and 51 kD [18]. The protein in our investigation is also similar to $GABA_A$-receptors from rat brain by its subunit composition, but is different from that of P-type transport ATPases.

The data on inhibition of Cl^-,HCO_3^--stimulated Mg^{2+}-ATPase activity eluted from the Sephacryl S-300 column suggest structural conjugation between the ATP-hydrolyzing sites and binding centers for $GABA_A$-receptor ligands. In a number of studies, it has been shown that the function of $GABA_A$-receptors is dependent on the intracellular content of Mg^{2+}-ATP complex [22-24]. Thus, in the investigation of Mg^{2+}-ATP influence on $GABA_A$-receptor from guinea pig hippocampal neurons it was assumed that this ligand maintains the function of inhibitory receptor through its phosphorylation, and the implication that ATPase is necessary for the regulation of Mg^{2+}-ATP concentration [22]. Further electrophysiological and biochemical investigations of $GABA_A$-receptors from rat brain have confirmed that there are two ATP binding sites on the intracellular surface of inhibitory receptors, and their function is controlled by protein phosphatases and protein kinases [23, 24]. However, ATPase that hydrolyzes ATP and participates in maintenance of inhibitory receptor function was not found. The possible candidate for this role may be the studied Cl^-,HCO_3^--stimulated, sensitive to $GABA_A$-ergic ligand, Mg^{2+}-ATPase from neuronal membranes, which is, according to our preliminary results [25] and this work, functionally and structurally conjugated to a protein system that specifically links the activators and blockers of $GABA_A$-benzodiazepine Cl^--channels.

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