Effect of HCO_3^- Ions on the ATP-Dependent $GABA_A$ Receptor-Coupled Cl^- Channel in Rat Brain Plasma Membranes

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 152, No. 7, pp. 43-47, July, 2011
Original article submitted March 21, 2010

We studied the effect of Cl^- (10-75 mM) and HCO_3^- ions (10-25 mM) on the ATP-dependent $GABA_A$ receptor-coupled Cl^- channel (Cl^--ATPase) in rat brain plasma membranes. The total enzyme activity was detected in the presence of both anions at a Cl^-/HCO_3^- ratio of 5:1 (Cl^-,HCO_3^--ATPase). Specific inhibitors of P-type transport ATPases (N-ethylmaleimide, *o*-vanadate, and oligomycin) suppressed Cl^-,HCO_3^--ATPase, while the Cl^-- and HCO_3^--ATPase activities were low sensitive to these ligands. Bicuculline abolished the activating effect of Cl^- and HCO_3^- ions on the enzyme. HCO_3^- ions had no effect on the ATP-dependent Cl^- transport into proteoliposomes (with the involvement of reconstituted ATPase). In experiment with Cl^--preloaded liposomes, addition of HCO_3^- ions to the incubation medium caused the reversion of Cl^- transport (ion efflux from liposomes). Our results suggest that HCO_3^- ions play an important role in the modification of properties of the ATP-dependent $GABA_A$ receptor-coupled Cl^- channel and $GABA_A$ receptor-induced Cl^-/HCO_3^- exchange. These ions are probably involved in $GABA_A$ receptor-induced Cl^-/HCO_3^- exchange in neuronal membranes.

Key Words: *rat brain plasma membranes; ATPase; chlorine; bicarbonate*

Cl^--activated ATPase (Cl^--ATPase) of plasma membranes in various cells (*e.g.*, neuronal cells) is an ATP-dependent Cl^- channel, which has a role in Cl^- transport against the electrochemical gradient [9]. Previously, we identified Cl^--ATPase in rat brain plasma membranes. It is functionally and structurally coupled to the $GABA_A$/benzodiazepine receptor complex [1,2]. Further studies showed that this enzyme can be activated not only by Cl^-, but also by HCO_3^-. Enzyme activity with Cl^- and HCO_3^- at low concentrations (8 and 2 mM, respectively) was much higher than in the presence of each anion [3]. The data suggest that this enzyme has a role in $GABA_A$ receptor induced Cl^-/HCO_3^- exchange [15]. To confirm this hypothesis, it is necessary to evaluate the effect of combined treatment with Cl^- and HCO_3^- ions in physiological concentrations on enzyme activity. Previous studies showed that Cl^- concentrations inside and outside the neuronal cell are 6 and 120 mM, respectively. These parameters for HCO_3^- ions are 16 and 26 mM, respectively [10,14].

This work was designed to study the effect of Cl^- and HCO_3^- at the 5:1 ratio on Cl^--ATPase activity in rat brain plasma membranes. Moreover, we evaluated the role of these ions in ATP-dependent Cl^- transport across the membranes of artificial proteoliposomes.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 200-210 g. After decapitation of animals, the cerebral cortex was isolated, homogenized in 10 mM HEPES-Tris buffer (pH 7.2, 1:8 ratio) containing 0.125 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride, and centrifuged in a Beckman ultracentrifuge (SW-28 bucket rotor) at 10,000*g* and 4°C for 20 min. The supernatant was centrifuged at 100,000*g* and 4°C for 1 h. The microsomal fraction (pellet) was frozen at -20°C and used for further measurements of Cl^--ATPase activity.

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0007-4888/11/1521-0038

The enzyme preparation (~20 μg) was added to 0.5 ml incubation medium containing 10 mM HEPES-Tris buffer (pH 7.2), 0.5-1.5 mM $MgSO_4$, 1.5 mM Tris-ATP, 10 mM NaCl, and 2 mM $NaHCO_3$ to measure enzyme activity. The ligand was preincubated with the protein at room temperature for 15 min to evaluate the effect of bicuculline (25 μM). Specific activity of ATPase was estimated from an increase in the content of inorganic phosphorus (P_i) in 0.5 ml incubation medium at 30°C for 30 min. The reaction was stopped by addition of 1.8 ml 30% H_2SO_4 to the incubation medium. Phosphorus concentration in samples was measured by the method of Chen and expressed in μmol P_i/h/mg protein [1,3]. Each measurement was performed with 4 samples.

To obtain soluble form of enzyme, plasma membranes were incubated with 1% sodium deoxycholate at room temperature for 20 min and centrifuged at 100,000*g* and 4°C for 30 min. Cl^-,HCO_3^--ATPase was isolated by the method of preparative gel filtration and reconstituted into proteoliposomes [2]. Proteoliposomes were resuspended in 0.7 ml 30 mM HEPES-Tris buffer (pH 7.2) containing 0.125 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. Proteoliposomes were loaded with a fluorescent probe 6-methoxy-N-ethylquinolinium iodide (MEQ; highly sensitive to Cl^- ions) [8] by the method of freezing/defrosting [3]. Each measurement was performed with 4 samples.

Cl^- transport into proteoliposomes was induced by addition of 2 mM Tris-ATP or $GABA_A$ receptor ligands to the incubation medium. The medium consisted of 30 mM HEPES-Tris buffer (pH 7.2), 30 mM NaCl, 2 mM $MgSO_4$, and proteoliposomes (70 μg). Incubation was conducted for 4 min. Cl^- transport was evaluated from variations in fluorescence on a Perkin Elmer MPF44A fluorometer equipped with a temperature-controlled cuvette at 30°C. The excitation and emission wavelengths were 350 and 480 nm, respectively [2]. Fluorescence was calculated as follows [8]:

$$\Delta F=(1-F/F_0)\times 100,$$

where F_0 is fluorescence of the control sample in the absence of ligands; and F is fluorescence of the sample after addition of ligands (ATP and bicuculline).

The significance of differences was evaluated by Student's t test at $p<0.05$.

RESULTS

Basal Mg^{2+}-ATPase activity in rat brain plasma membranes is 7.0 μmol P_i/h/mg protein. The enzyme is activated by Cl^- ions. The dependence of enzyme activity on Cl^- concentration (1-150 mM) is described by a bell-shaped curve. The highest activity of this enzyme (9.0 μmol P_i/h/mg protein) was observed at a Cl^- concentration of 25-50 mM (Fig. 1, *a*). The dependence of Mg^{2+}-ATPase activity on HCO_3^- concentration (1-20 mM) is described by a hyperbolic curve (Fig. 1, *b*). The highest activity of this enzyme was observed at a HCO_3^- concentration of 14-20 mM. The effect of HCO_3^- on Cl^--ATPase was evaluated at a Cl^-/HCO_3^- ratio of 5:1. This ratio is typical of anion permeability through the $GABA_A$ receptor ion channel [12]. HCO_3^- ions did not modulate the dependence of Cl^--ATPase activity on anion concentration. This dependence was also described by a bell-shaped curve (Fig. 1, *c*). Maximum enzyme activity was revealed in the same range of concentrations (25-50 mM Cl^-). However, this parameter increased from 9.0 to 13.9 μmol P_i/h/mg protein.

Our results indicate that total ATPase activity is observed in the presence of two anions, Cl^- and HCO_3^- (Cl^-,HCO_3^--ATPase). This is typical of P-type transport ATPases (Na^+,K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase, *etc*.) [11]. To confirm the belonging of Cl^-,HCO_3^--Mg^{2+}-ATPase to P-type transport ATPases, further experiments were performed with the following agents that specifically inhibit this type of ATPases: *N*-ethylmaleimide (NEM, SH-reagent); *o*-vanadate (inhibitor of the transient-state phosphate bond), and oligomycin (inhibitor of phosphorylation). The test ligands in low concentrations (~10 μM) were shown to inhibit Cl^-, HCO_3^--Mg^{2+}-ATPase. Cl^--ATPase and HCO_3^--ATPase

TABLE 1. Effect of HCO_3^- Ions on Fluorescence of Proteoliposomes with the Reconstituted Enzyme from Rat Brain in the Presence of 2 mM ATP over 5 min (*M*±*m*)

Proteoliposomes	ΔF, % inhibition	
	30 mM Cl^-	30 mM Cl^-+6 mM HCO_3^-
Not containing Cl^- ions	20±2	26±3
Containing Cl^- ions	33±4*	15±2*
Containing Cl^- ions and 25 μM bicuculline	30±3*	28±4

Note. *$p<0.05$ compared to proteoliposomes not containing Cl^- ions.

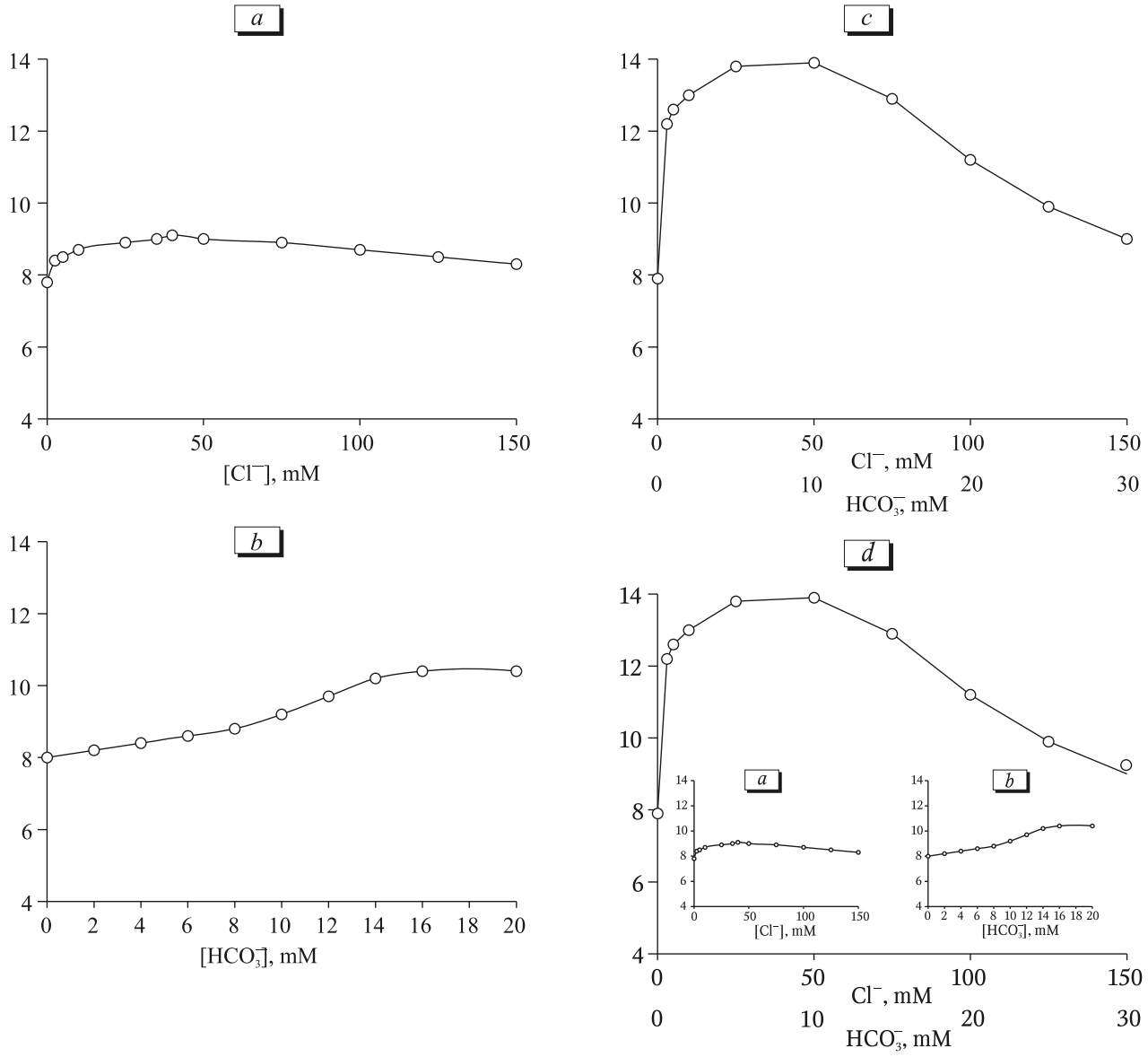

Fig. 1. Dependence of basal Mg^{2+}-ATPase activity from rat brain plasma membranes on the concentration of Cl^- (*a*), HCO_3^- (*b*), and Cl^- +HCO_3^- (*c*). Ordinate: Mg^{2+}-ATPase activity, P_i/h/mg protein.

activities were suppressed only in the presence of *o*-vanadate or oligomycin in high concentrations (~100 μM). They were insensitive to NEM.

We previously hypothesized that this enzyme is coupled to $GABA_A$ receptors [2]. Therefore, it was important to evaluate the effect of bicuculline (competitive antagonist of $GABA_A$ receptors) on enzyme activity. Experiments were performed with the substrate Mg^{2+}-ATP in concentrations of 0.25-3.00 mM. Cl^-+HCO_3^-, and bicuculline (25 μM) were shown to activate Mg^{2+}-ATPase. The dependence of enzyme activity on Mg^{2+}-ATP concentration was described by a hyperbolic curve (Fig. 2). Combined treatment with Cl^-+HCO_3^- and bicuculline did not potentiate the effect of these ligands. Hence, the influence of Cl^-+HCO_3^- ions on this enzyme is not manifested in the presence of a competitive $GABA_A$ receptor antagonist.

The next series was performed to confirm the involvement of HCO_3^- in ATP-dependent Cl^- transport. Artificial proteoliposomes were loaded with a fluorescent probe MEQ (highly sensitive to Cl^- ions). ATP-induced Cl^- transport across the membranes of proteoliposomes was studied at Mg^{2+}-ATP concentrations of 0.5-3.0 mM. Addition of 1.0-1.5 mM Mg^{2+}-ATP to the incubation medium was followed by a decrease in fluorescence. The effect was most pronounced at a substrate concentration of 2-3 mM (Fig. 3). We hypothesized that the enzyme plays a role in Cl^-/HCO_3^- exchange. Hence, we studied the influence of HCO_3^- on Cl^- transport in the absence or presence

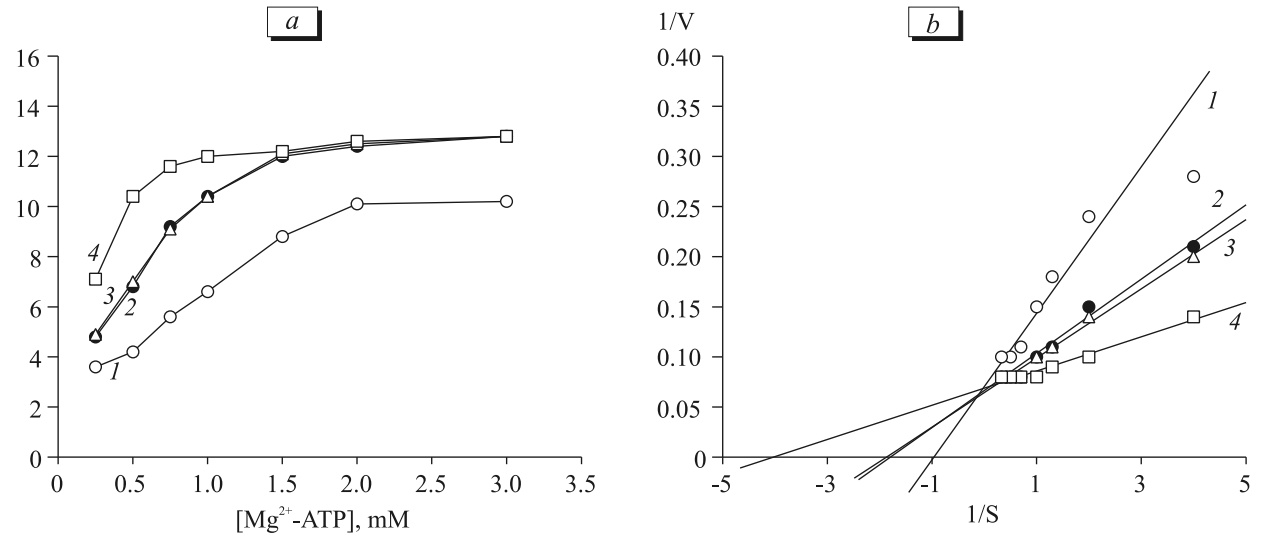

Fig. 2. Dependence of basal Mg^{2+}-ATPase activity on substrate concentration in the absence (*1*) and presence of 25 μM bicuculline (*2*), 100 mM Cl^- and 20 mM HCO_3^- (*3*), and 25 μM bicuculline and 100 mM Cl^-+20 mM HCO_3^- (*4*). (*a*) Ordinate: Mg^{2+}-ATPase activity, P_i/h/mg protein. (*b*) The data are presented as a Lineweaver–Burk plot.

of Cl^- in liposomes. Cl^- transport into proteoliposomes was induced by addition of 2 mM ATP to the incubation medium containing 30 mM HEPES-Tris buffer (pH 7.2), 30 mM NaCl, 2 mM $MgSO_4$, and proteoliposomes (80 μg). HCO_3^- ions didn't modulate Cl^- transport into proteoliposomes not loaded with Cl^-. In experiments with Cl^--loaded proteoliposomes, addition of HCO_3^- to the incubation medium was followed by an increase in fluorescence. These data illustrate the efflux of Cl^- ions from liposomes (Table 1). The effect of HCO_3^- ions on ATP-dependent Cl^- transport was not observed in the presence of 25 μM bicuculline.

Our results indicate that anion-activated Mg^{2+}-ATPase exhibit the properties of Cl^--ATPase and

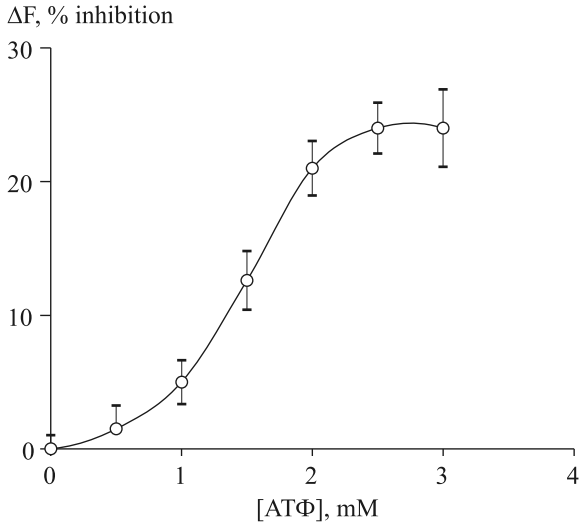

Fig. 3. Effect of ATP concentration on fluorescence of proteolysosomes with the reconstituted enzyme from rat brain.

HCO_3^--ATPase. Similarly to P-type transport ATPases, the total activity of this enzyme is observed in the presence of two ions [11]. This conclusion was derived from high sensitivity of Cl^-,HCO_3^--ATPase to *o*-vanadate, SH-reagents, and oligomycin. Published data on other biochemical properties of the enzyme illustrate its functional and structural coupling to $GABA_A$ receptors. They are similar to properties of the $GABA_A$-regulated Cl^- channel [1-3]. Bicuculline inhibits Cl^-,HCO_3^--ATPase, which indicates that this enzyme is involved in $GABA_A$-induced Cl^-/HCO_3^- exchange across the brain neuronal membrane. Experiments with the enzyme incorporated into proteoliposomes illustrate a strong differentiation of properties of the Cl^- transport system. Moreover, the direction of Cl^- transport depends strongly on the intracellular concentration of Cl^- ions and extracellular concentrations of HCO_3^- ions. Cl^- transport is reversed (ion efflux from the cell) at high concentrations of Cl^- and HCO_3^- in the cell and incubation medium, respectively. These data are consistent with the results of electrophysiological studies of $GABA_A$-induced depolarization. The effect of GABA on the membrane potential in neuronal membranes of adult animals is related to their interaction with $GABA_A$ receptors and increase in Cl^- influx into the neuron, which results in hyperpolarization [10]. Experiments with mature neurons showed that an increase in GABA concentration or incidence of receptor exposure to GABA is accompanied by the transition of neuronal membrane inhibition into membrane excitation [4,6]. All scientists believe that HCO_3^- ions are involved in this process. However, there is no general agreement about the role of Cl^- ions. Some authors hypothesized that $GABA_A$-induced Cl^-/HCO_3^- exchange

is characterized by passive influx of Cl^- ions into the neuron (in exchange for HCO_3^- ions) [8,15]. No strong evidence exists for this phenomenon. Other authors reported that Cl^- efflux from the cell occurs under conditions of $GABA_A$-induced depolarization. The question arises: does Cl^--ATPase have a role in ATP-dependent Cl^- transport into the cell differing from the Cl^- channel and coupled to $GABA_A$ receptors [12,13]? The existence of this ATPase is confirmed by published data on the bicuculline-sensitive $GABA_A$ receptor-regulated Cl^- channel in specific neurons of the brain [6]. This structure binds GABA and induces ATP-dependent Cl^- transport against the electrochemical gradient. We showed that this Cl^-,HCO_3^--ATPase can be detected at high concentrations of Cl^- (~50 mM) and HCO_3^- (~10 mM). This enzyme, probably, hydrolyzes ATP and plays a role in $GABA_A$-induced Cl^-/HCO_3^- exchange. Hydrolytic activity of this ATPase not only provides energy for the process, but also determines a certain direction of Cl^- flux. This process depends not only on the intracellular concentrations of ATP and Cl^- ions, but also on the concentration of HCO_3^- ions. Analysis of the properties and role of $GABA_A$ receptor-coupled Cl^-,HCO_3^--ATPase in anion transport across the neuronal membrane is required to evaluate the pathogenesis of some diseases (*e.g.*, epilepsy).

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