EVIDENCE OF MEMBRANE H^+-ATPase IN RAT BRAIN SYNAPTIC VESICLES

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UDC 612.8.015.1:577.152.361]-088.1

KEY WORDS: synaptic vesicles; brain; proton-ATPase; Na,K-ATPase; dicyclohexylcarbodi-imide; anions.

In the last ten years reports have been published that the membranes of various secretory granules contain proton-ATPase (H^+-ATPase). Hydrolysis of ATP on the outside of granules is accompanied by transmembrane potential generation and the formation of a transmembrane pH difference, i.e., by acidification of the matrix of the granules. The functions of the proton pump in secretory granules are linked with active uptake of transmitters or hormones from the cell cytoplasm, with maintenance of a high level of these substances in the granules, with their release from the granules in the course of exocytosis, with changes in the osmotic properties of the granules, and with transport of anions into the granules [1, 11]. Most attention has been paid to the study of the properties and functions of H^+-ATPase in membranes of the chromaffin granules (CG) of the adrenals, and rather less to membranes of brain synaptic vesicles (SV).

It was accordingly decided to obtain evidence in support of the presence of H^+-ATPase in membranes of brain SV in animals.

EXPERIMENTAL METHOD

The SV fraction was isolated from whole brain of rats weighing 150-200 g [7]. For this purpose, unpurified synaptosomes were obtained (10,000 g, 10 min) from a 10% brain homogenate (0.32M sucrose, 20 mM Tris-HCl, pH 7.4) and subjected to osmotic shock, by suspending the residue in 8 volumes of bidistilled water at 0-4°C for 20-30 min. From the supernatant (15,000 g, 20 min) the SV fraction was sedimented (100,000 g, 60 min) and suspended in isolation medium and kept at −10°C for 1 week. Preparations frozen once were used in the work. Protein was determined by Lowry's method. The fraction of brain mitochondria was obtained by the method in [8]. ATPase activity in the SV fraction was determined (20 min, 37°C) in basic incubation medium of the following compositions (in mM): Tris-HCl (pH 7.4) 25, $MgCl_2$ 2.5, Tris-ATP 2.5, EGTA 0.5, in a volume of 1 ml. In some experiments the medium contained NaCl or KCl, the concentration of which is indicated in the captions to Figs. 1 and 2.

Na,K-ATPase activity was determined as the rate of hydrolysis of ATP in medium containing 100 mM NaCl and 20 mM KCl, after subtracting the rate of hydrolysis in medium containing 120 mM NaCl. Activity measured by this method was inhibited by 98% by 0.5 mM ouabain. After preincubation for 10 min at 37°C (7 μg protein in 1 ml for Na,K-ATPase and 20-25 μg/ml for Mg-ATPase) the reaction was initiated by the addition of Tris-ATP. The reaction was stopped and inorganic phosphate (P_i) was determined as described previously [4].

Tris-ATP was obtained by passing ATP-Na_2 through a column packed with Dowex resin 50w × 2, 50-100 mesh (from "Serva," West Germany). Dicyclohexylcarbodi-imide (DCCD), oligomycin, and carbonylcyanide-m-chlorophenylhydrazone (CCCP) — all from "Serva" — were dissolved in 96% ethanol, the final concentration of which in the samples was 2%. Control samples also contained 2% of ethanol. N-ethylmaleimide and ouabain were obtained from "Serva," and ATP-Na_2 from "Reanal" (Hungary). The remaining reagents were of the chemically pure grade.

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Laboratory of Molecular Pathology and Biochemistry, Institute of General Pathology and Pathological Physiology, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 539-541, November, 1984. Original article submitted April 10, 1984.

0007-4888/84/9811-1487$08.50

EXPERIMENTAL RESULTS

Specific activity of Mg-ATPase in the SV fraction of rat brain was close to that given in the literature [1]. Marked Na,K-ATPase activity also was observed (61 μmoles P_i/mg protein/h), indicating contamination of the SV fraction with synaptosomal cytoplasmic vesicles. Na,K-ATPase activity was blocked by ouabain ($K_{0.5}$ = 1 μM). To study Mg-ATPase activity the measurements were made under conditions ruling out the possibility of manifestation of Na,K-ATPase.

In salt-free (basic) medium Mg-ATPase activity of the SV fraction was 18.8 ± 3.8 (n = 6) moles P_i/mg protein/h. In medium containing 120 mM NaCl, Mg-ATPase activity was 112%, and in medium with 150 mM KCl it was 80% of the initial value. When Ca-ATP (2.5 mM) was used as substrate in salt-free medium, activity fell to 66%.

DCCD (10-200 M), the classical inhibitor of the different H^+-ATPases (and above all, of mitochondrial H^+-ATPase, one which has been studied the most [5]), inhibited Mg-ATPase of the SV fraction considerably (Fig. 1A). It was shown previously that DCCD in a concentration of 40 μM blocks Mg-ATPase of the SV fraction of rat brain by 45%. Dependence of the effect of DCCD on Mg-ATPase of the SV fraction of the brain on concentration, revealed by these experiments, was virtually identical with that for ATPase of CG membranes of the adrenals [9].

It is an interesting fact that DCCD in low concentrations (50 μM) activated Na,K-ATPase activity found in the SV fraction of the brain by 40%, whereas in higher concentrations (200 μm) it inhibited that activity considerably (Fig. 1A). Inhibition of Na,K-ATPase by high concentrations of DCCD has been described in the literature [3], but activation is described now for the first time.

It will be clear from Fig. 1B that oligomycin, a specific blocker of mitochondrial H^+-ATPase [5], had no effect on Mg-ATPase of the SV fraction in concentrations (0.5 μg/ml) which, in the present experiments, induced maximal inhibition of Mg-ATPase activity of the preparation which we isolated from brain mitochondria. Hence it follows, first, that relative to this criterion DCCD-sensitive ATPase of the SV fraction differs from mitochondrial.

Resistance of H^+-ATPase of various secretory granules to oligomycin also has been noted in the literature [1, 11]. However, this resistance to oligomycin is not absolute, for in comparatively high concentrations oligomycin (10-20 μg/ml) moderately inhibited the Mg-ATPase of the SV fraction (Fig. 1B).

Orthovanadate, a powerful endogenous blocker of transport Na-K- and Ca-ATPase [12], in our experiments had virtually no effect in concentrations of 10-100 μM on the Mg-ATPase activity of the SV fraction. At the same time, it considerably inhibited Na,K-ATPase activity in our preparations: by 91% in a concentration of 10 μM and by 99% in a concentration of 100 μM. It has been suggested that orthovanadate inhibits only those ATPases which characteristically form phosphorylated intermediates and does not act on electrogenic H^+-ATPases, an exception being the nonelectrogenic K,H-ATPase of the gastric mucosa [12].

According to data in the literature [13], mitochondrial ATPase is insensitive to N-ethylmaleimide, a blocker of SH groups. The present experiments showed that 0.3 mM N-ethylmaleimide inhibits Mg-ATPase activity of the SV fraction by 25-35% in salt media. This fact is further evidence that Mg-ATPase of SV membranes is different from mitochondrial ATPase.

We found that the protonophore CCCP in a concentration of 20 μM stimulated Mg-ATPase activity of the SV preparation moderately (by 12.5%). The effects varied from one preparation to another. According to data in the literature, the protonophore uncoupler S-13 activates Mg-ATPase of SV of rat brain [15] and of cholinergic SV of the electric organ of the skate [6] weakly.

We know that H^+-ATPase of mitochondria [2, 5], of cholinergic SV of the electric organ of the skate [14], and of secretory granules of the bovine adenohypophysis [10] is activated by anions and, in particular, by bicarbonate. In view of these data, we studied the possible effect of bicarbonate on Mg-ATPase from mitochondria and SV of rat brain. These experiments showed that the presence of KCl or NaCl (up to 60 mM) in the incubation medium had only a weak effect on Mg-ATPase activity of the mitochondria and SV compared with a salt-free medium. It will be clear from Fig. 2 that HCO_3^- stimulated brain mitochondrial Mg-ATPase sharply (by 2.6-2.8 times, in the presence of 60 mM bicarbonate), whereas Mg-ATPase of the SV fraction was stimulated by bicarbonate (20 mM) by only 16-23% ($P < 0.05$). In both cases anionic stimulation was virtually independent on the form (the K^+ or Na^+ salt) in which bicarbonate was

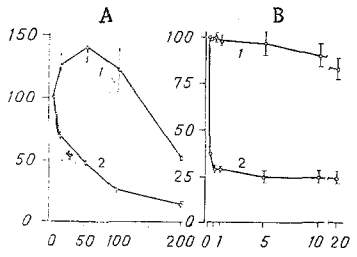

Fig. 1

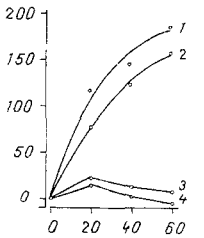

Fig. 2

Fig. 1. Action of DCCD (A) and oligomycin (B) on Mg-ATPase and Na,K-ATPase of SV fraction and mitochondria of rat brain. Abscissa: A) concentration of DCCD (in M), B) concentration of oligomycin (in μg/ml); ordinate, enzyme activity (in % of control). Mg-ATPase activity measured in presence of 120 mM NaCl; protein content in samples 25 μg/ml. For A: 1) Na,K-ATPase, 2) Mg-ATPase of SV fraction; for B: 1) Mg-ATPase of SV, 2) Mg-ATPase of mitochondria. Results of three or four experiments given.

Fig. 2. Stimulation of Mg-ATPases of brain mitochondria (1, 2), and SV (3, 4) by bicarbonate. Abscissa, bicarbonate concentration (in mM); ordinate, activation (in %). Specific activity of Mg-ATPase in salt-free medium taken as 100%. Effect of stimulation calculated by subtracting control activity in presence of corresponding chloride. Mg-ATPase activity of brain mitochondria was 31.8 μmoles P_i/mg protein/h. On addition of $KHCO_3$ or $NaHCO_3$, pH of medium was adjusted to 7.4. Results of two experiments for mitochondria and three to five experiments for SV are given. 1, 3) K^+ salt; 2, 4)Na^+ salt. *$P < 0.05$ compared with corresponding control.

used. Comparison of the effects on Mg-ATPase from mitochondria and SV showed that in the first case the effect of stimulation by HCO_3^- increased with an increase in its concentration from 20 to 60 mM, whereas in the second case the effect was maximal in the presence of 20 mM HCO_3^-, and thereafter gradually disappeared.

When the effect of anionic stimulation is studied it is important to take into consideration the form in whcih the substrate is used. For instance, if ATP-Na_2 was used (the incubation medium contained 5 mM Na^+), "false" anionic stimulation by $KHCO_3$ (20 mM) appeared and was abolished by ouabain. This may have been the reason for inhibition of this stimulation by ouabain that is sometimes observed [14].

Stimulation of Mg-ATPase activity of the SV fraction by CCCP and HCO_3 indicates some degree of similarity between Mg-ATPase and mitochondrial H^+-ATPase. However, the comparatively weak effect of stimulation in these two cases can probably be explained by contamination of the SV preparation by other ATPases.

It can thus be concluded from the results described above that brain SV membranes contain an H^+-ATPase which, in its properties, is similar to the proton pump of mitochondria and of CG membranes of the adrenals. The H^+-ATPase of SV membranes differs from the mitochondrial form in its insensitivity to oligomycin and sensitivity to the thiol reagent N-ethylmaleimide. A detailed study of the H^+-ATPase of brain SV membranes must be all-embracing and must include investigation both of hydrolytic activity and of translocation of H^+ across the vesicle membrane.

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CHOLINERGIC REGULATION OF Na,K-ATPase ACTIVITY FROM PIG KIDNEY

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UDC 612.46.015.1:577.152.311].014.46:577.175.822

KEY WORDS: acetylcholine; acetylcholinesterase; Na^+,K^+-ATPase.

Many investigations have shown that acetylcholine (ACh) can regulate Na,K-ATPase activity. High ACh concentrations inhibit, whereas low stimulate Na,K-ATPase activity of various biological objects [1, 4]. It has been suggested that in Na,K-ATPase there are subunits of acetylcholine receptors [7].

A link also is found between two membrane enzymes, namely Na,K-ATPase and acetylcholinesterase (AChE), but its nature is not yet clear. It has been suggested that this link is effected by means of an intermediary, ACh, for example, which is the substrate for one enzyme and regulator for the other.

According to one view, catalytic centers of both enzymes lie on the same protein subunit [9] and function simultaneously; however, according to another view, the catalytic subunit of AChE is the precursor of the catalytic subunit of Na,K-ATPase. It has been shown that ACh in membrane preparations of Na,K-ATPase and homogenates of various tissues can increase the number of catalytic centers of Na,K-ATPase and reduce their number in AChE [5, 6]. In the investigation described below the effect of ACh was studied on the AChE activity of a purified preparation of Na,K-ATPase, generously provided by the staff of the Institute of Bioorganic Chemistry, Academy of Sciences of the USSR [3].

The preparation is a homogeneous protein with mol. wt. of 540 kilodaltons, containing equal amounts of α- and β-subunits.

EXPERIMENTAL METHOD

The original preparation of Na,K-ATPase, containing 3.6 mg protein in 1 ml of histidine-sucrose medium, was diluted in a solution of 250 mM sucrose and 30 mM histidine, pH 7.2, to a concentration of 40 μg protein/ml. The total sample was divided into 1.5-ml portions and frozen at −12°C for keeping until required. Each portion was thawed before the experiment.

AChE activity was determined as the rate of hydrolysis of ACh by Ellmann's method [8], and Na,K-ATPase activity was determined as accumulation of inorganic phosphorus [10].

EXPERIMENTAL RESULTS

The results of the experiments with ACh are given in Table 1.

The control preparation of Na,K-ATPase did not react significantly to ACh irrespective of the concentration of the regulator used. Preincubation of the preparation at 22°C for 30 min did not change the sensitivity of Na,K-ATPase for ACh. Similar treatment of unpurified preparations in some cases revealed a stimulating action of ACh on Na,K-ATPase [2].

Patent Information Department, Kazan' Medical Institute. Laboratory of Membrane Physiology, Institute of Physiology, Academy of Sciences of the Kazakh SSR, Alma-Ata. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Orekhovich.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 542-543, November, 1984. Original article submitted February 27, 1984.

 0007-4888/84/9811-1490$08.50