ELECTROGENIC CHARACTER OF H^+-ATP-ASE FUNCTION IN RAT BRAIN SYNAPTIC VESICLES

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Secretory granules and, in particular, synaptic vesicles (SV) of neurons contain a protein pump (H^+-ATPase) in their membranes. During ATP hydrolysis on the outer side of the membrane H^+ ions are actively pumped inside SV (or the secretory granules) and the pH within the granules reaches 5.0-5.3. The functioning of the H^+-pump of SV membrane may be connected with vesicular uptake of mediators from the cytoplasm and with maintenance of high concentrations (up to 0.6-0.8 M) of mediators in SV and, possibly, with regulation of exocytosis.

The writers previously [3, 5] obtained proof of the location of the H^+-pump in membranes of brain SV and described some of its properties, in particular, the coupling of its function with the chloride channel. We know that the Mg-ATP-induced pH gradient in lysosomes [6] and in chromaffin granules of the adrenals [7] is coupled with generation of a transmembrane potential (TMP), recorded by means of a voltage-sensitive fluorescent probe diS-C_3-(5).

The aim of this investigation was to determine the electrogenic character of function of the H^+-ATPase of SV membranes, i.e., to study the principles of TMP generation.

EXPERIMENTAL METHOD

SV were isolated from the brains of rats weighing 200-220 g by the method described previously [1]. The SV obtained were suspended in medium containing 250 mM sucrose and 20 mM Tris-HCl, pH 7.4, and kept in the frozen state at −20°C. The preparation used in the experiments had been frozen and thawed once.

TMP of the synaptic vesicles was assessed from the change in fluorescence of the voltage-sensitive probe diS-C_3-(5), in a final concentration in the cuvette of 1 μM. Fluorescence of the probe was excited by light with a wavelength of 620 nm and recorded at a wavelength of 670 nm. The volume of the sample was 2 ml and the final concentration of SV (as protein) was 10 μg/ml. Measurements were made in medium containing 250 mM sucrose and 20 mM Tris-HCl. All changes in the composition of the incubation medium are indicated in the text. The 3,3'-dipropylthiodicarbocyanine — diS-C_3-(5) — probe was generously provided by M. Blaustein (USA).

EXPERIMENTAL RESULTS

It was shown previously [3, 5] that on the addition of Mg-ATP to a suspension of SV, the internal contents of SV become acidified, due to activity of H^+-ATPase. The SV preparations which we obtained also possessed this property. On the addition of Mg-ATP (1.0 mM) to the SV suspension an increase in the intensity of fluorescence (I_{fl}) of the diS-C_3-(5) probe was observed. The increase in fluorescence depended on the SV concentration and reached its peak value when the SV protein concentration was 10 μg/ml, when it was 10% of the fluorescence of the probe before addition of SV. The increase in fluorescence of the probe also depended on the Mg-ATP concentration. It follows from Fig. 1 (curve 1) that this concentration dependence is nonlinear in character. When a solution of Mg-ATP was used, incidentally, the presence of Mg^{2+} and ATP^{2-} ions not forming an Mg-ATP complex had to be taken into account, for they also may influence the test parameter. Accordingly, we studied the effect of Mg^{2+} and ATP^{2-} ions themselves in I_{fl} of the probe bound with SV. ATP in concentrations found in Mg-ATP solutions (0.1-1.5 mM) was shown to have virtually no effect on fluorescence. Meanwhile Mg^{2+} ions led

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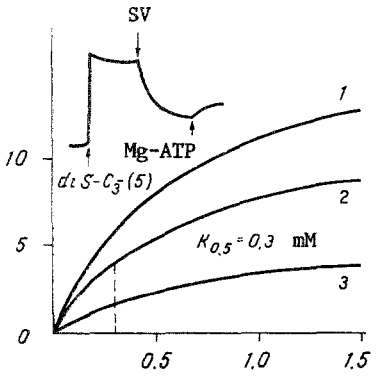

Fig. 1

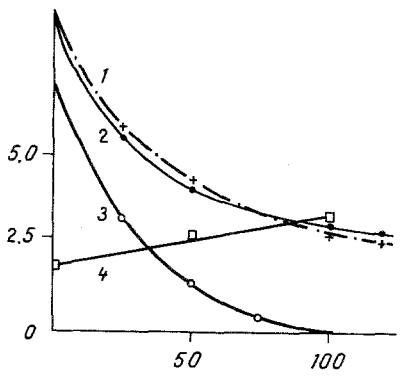

Fig. 2

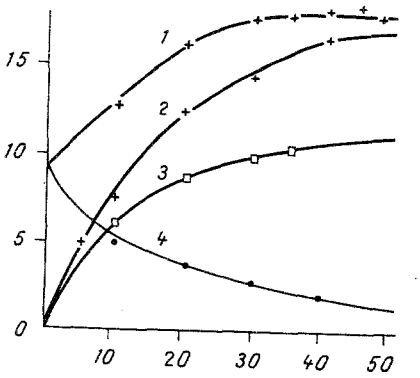

Fig. 3

Fig. 1. Action of Mg-ATP (1) and of $MgCl_2$ (3) on intensity of fluorescence in probe—SV system. Abscissa, Mg-ATP concentration (in mM); ordinate, changes in I_{fl} (in % of I_{fl} of probe before addition of SV). Curve 3 reflects change in fluorescence of SV-bound probe under the influence of free Mg^{2+} ions in concentrations corresponding to Mg-ATP present in solution. Curve 2 shows difference between curves 1 and 3. Here and subsequently results of 5-6 experiments are shown. Typical curve showing change in I_{fl} of probe during successive addition of substances (arrows) is shown in Fig. 1.

Fig. 2. Mg-ATP-induced (1, 2) and Mg^{2+}-induced (4) change in fluoresence of probe in probe—SV system in medium in which sucrose is replaced by NaCl (1) or KCl (2, 4). Abscissa, KCl or NaCl concentration (in mM); ordinate, the same as in Fig. 1. Mg-ATP concentration 1 mM, $MgCl_2$ concentration 0.1 mM. Curve 3 is the difference between curves 2 and 4. In all cases the iso-osmolarity of the medium was maintained.

Fig. 3. Effect of ammonium sulfate (2), ammonium sulfate preceded by 1 mM Mg-ATP (3), and Mg-ATP (1 mM) preceded by various concentrations of ammonium sulfate (4), on fluorescence of probe in presence of SV. Curve 1 is sum of curves 2 and 4. Abscissa, ammonium sulfate concentration (in mM); ordinate, the same as in Fig. 1.

to an increase in I_{fl} of the SV-bound probe. Thus to record the true effect of the Mg-ATP complex, the concentration of free Mg^{2+} ions present in the Mg-ATP solutions had to be calculated (0.1-1.5 mM) and their nonspecific contribution had to be taken into account in the general effect. The calculations were done in accordance with recommendations proposed by Boldyrev [2]. Curve 3 in Fig. 1 shows dependence of the change in fluorescence of the bound probe due to the presence of free Mg^{2+} ions in the Mg-ATP solutions. Considering the high specificity of the test enzyme with respect to the Mg-ATP complex, it can be postulated that both specific and nonspecific components exist in the recorded change of fluorescence of the probe. Thus curve 2 (Fig. 1), which is the difference between curves 1 and 3, evidently describes the specific component, that connected with the action of H^+-ATPase and interaction of Mg-ATP with SV. Using the method of titration of the probe with the object suggested by Ivkova and co-workers [4], we showed that about 90% of the change in fluorescence of the probe is connected with an increase in the quantity of free probe, and 10% with a change in fluorescence of the membrane-bound probe. The results confirm the view that there exist a specific — i.e., due to generation of a TMP (positive within) on SV and leading, correspondingly, to a change in the ratio between free and bound probe [4] — and a nonspecific component, connected with a change in the parameters of fluorescence of the membrane-bound probe, possibly through a change in its microenvironment. It must be recalled that interaction of Mg-ATP with the free probe was not observed.

The aim of the next series of experiments was to prove that the increase recorded in I_{fl} of the probe during interaction between Mg-ATP and SV is the result of appearance of a TMP, due to activity of the H^+-pump.

Disturbance of the structural integrity of SV by the aid of the detergent Triton X-100 or the creation of marked permeability for ions with the aid of the channel former alamethicin (25-150 μg/ml) reduced the effect of Mg-ATP. Incidentally, Triton X-100 was used on concentrations not causing any significant change in the intensity of scattering of light by the SV suspension (maximal concentration 0.015%), i.e., complete destruction of the vesicles did not take place.

The creation of a marked degree of conductance of SV membrane for H^+ by means of carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) also reduced Mg-ATP-induced changes in I_{fl} of the probe. The marked interaction between CCCP and the probe in the absence of SV must be noted, leading to quenching of fluorescence of the probe, which reached 70% with 5 μM CCCP. In the presence of SV, however, this marked quenching of fluorescence did not take place, evidently because of the virtually total binding of CCCP with SV, which made it possible for us to use CCCP in our experiments as a protonophore.

The value of the Mg-ATP-induced increase in I_{fl} of the probe also depended on the composition of the incubation medium and, in particular, on the presence of penetrating anions (in this case Cl^-), capable of shunting the generation of TMP on the SV membrane. We observed that Mg-ATP-induced TMP in SV was increased by 30-40% when a solution of 0.25M sucrose, made up in 20 mM HEPES-Tris, pH 7.4, was used as the incubation medium instead of a solution of 0.25 M sucrose made up in 20 mM Tris-HCl, pH 7.4. It also follows from Fig. 2 that replacing sucrose by KCl or NaCl, leaving the osmotic pressure of the solution unchanged, led to reduction of the Mg-ATP effect. It must be pointed out that the dependence of the effect of Mg-ATP on the KCl concentration was virtually identical with the change in this value on an increase in the NaCl concentration, which indicates that it is Cl^- which plays a role in this process. However, our data do not completely rule out the possibility that monovalent cations may make a contribution to the recorded TMP. Manifestation of specific interaction of Mg-ATP and SV was completely neutralized by Cl^- in concentrations of 80-90 mM (Fig. 2, curve 4). This dependence, incidentally, is logarithmic in character.

It has been shown that ammonium sulfate in concentrations of 5-40 mM, lowering the pH gradient by a mechanism of weak bases [5], leads to a decrease in Mg-ATP-induced change in fluorescence of the probe (Fig. 3, curve 4). Under these circumstances the ammonium sulfate also increased I_{fl} independently in a probe–SV system (Fig. 3, curve 2) and in this case the concentration dependence was nonlinear in character. Against the background of the Mg-ATP-induced increase in I_{fl} of the probe, this action of ammonium sulfate was reduced (Fig. 3, curve 3). The results are thus evidence that ammonium sulfate and Mg-ATP interact with SV with the same result: indirect or direct stimulation of H^+-ATPase. It must be pointed out that the combined effect of ammonium sulfate and Mg-ATP was increased by an increase in the concentration of these compounds, and came out on a plateau when the change in I_{fl} was about 20% (Fig. 3, curve 1). In this case, the highest possible TMP of the SV membranes due to activity of the H^+-pump was thus reached. The creation of conditions under which H^+-ATPase activity is inhibited, i.e., incubation with N-ethylmaleimide [5], led to depression of the Mg-ATP-induced specific change in I_{fl} of the probe in medium in which some of the sucrose had been replaced by KCl. No inhibition was observed, incidentally, in medium consisting purely of sucrose.

The results are thus evidence that during interaction of Mg-ATP with SV there is a redistribution of the potential-sensitive fluorescent probe diS-C_3-(5) and an associated increase in its I_{fl}. This latter effect was found to be dependent on the structural integrity of SV, on permeability of the SV membranes for H^+, and on functional activity of the H^+-pump, and it was shunted in the presence of penetrating Cl^- anions. It can accordingly be concluded that the appearance of an Mg-ATP-induced TMP of SV membranes, due to activity of H^+-ATPase, can be recorded by means of the diS-C_3-(5) probe.

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