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# ATP-DEPENDENT PROTON TRANSLOCATION ACROSS THE RAT BRAIN SYNAPTIC VESICLE MEMBRANE

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UDC 612.8.015

KEY WORDS: synaptic vesicles; rat brain; proton pump; $H^+$-ATPase; acridine orange; valinomycin

Synaptic vesicles (SV) of brain nerve endings possess ATPase activity, which is connected with the storage, uptake, and release of neurotransmitters [1]. It has been suggested that neurotransmitter transport is coupled with the electrochemical $H^+$ potential, the two components of which, namely pH difference and inside-positive electrical potential, are created through the activity of $H^+$-ATPase, located in the SV membrane, which accumulates $H^+$ on account of hydrolysis of ATP. This concept is confirmed by data obtained on various secretory granules: chromaffin granules of the adrenals [9], peptide-containing granules of the neurohypophysis [17], and insulin-containing granules of the pancreas [8].

There is weighty evidence in support of this concept, which has been obtained on SV preparations. For instance, there is a distinct parallel between the action of inhibitors and proton-carrying uncouples on ATPase activity and catecholamine transport in brain SV [11, 18], and acetylcholine transport in SV of the electric organ of the skate [3]. The pH measured in isolated SV of the skate electric organ is low (5.3-5.6) [4, 12]. Meanwhile, some particular features of the ATPase of SV in particular, is weak stimulation by proton-carrying uncouples [2, 18, 19], indicate that $H^+$-ATPase is not present in the SV membrane [19].

An important contribution to the solution of this problem must be made by direct investigation of the ability of brain SV to undertake ATP-dependent proton translocation. To investigate this problem the method of continuous recording of transmembrane $H^+$ gradients by means of the dye acridine orange (AO) was used [5, 7, 10]. In the undissociated form, this weak base passes readily across the membrane and, because of the high pK of its $NH_2$-group, it is distributed in accordance with the $H^+$ concentration gradient on both sides of the membrane [10]. Accumulating inside the vesicles, as its concentration increases the dye undergoes oligomerization and its optical properties are changed [7]. Accumulation of the dye, which is proportional to the $H^+$ gradient, can be recorded either as a decrease in its extinction using the two-wavelength method [5, 7] or as quenching of fluorescence [10], which reflects a decrease in content of the monomer form in the incubation medium. A similar AO probe has been used to investigated ATP-dependent $H^+$ gradients in chromaffin granules of the adrenals [15].

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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' Eksperimental'noi Biologii i Meditsiny, Vol. 99, No. 1, pp. 35-38, January, 1985. Original article submitted June 15, 1984.

 0007-4888/85/9901-0042$09.50 

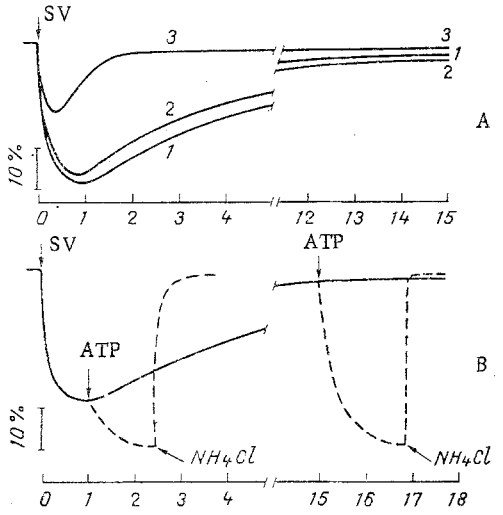


Fig. 1. Effect of ionophores (A) and ATP (B) on fluorescence of AO in SV suspension. Abscissa, time after addition of SV (in min); ordinate, $I_{fl}$ of AO (in %). A: 1) In absence of ionophores, 2) 20 μM CCCP, 3) 20 μM CCCP + 2 μM valinomycin, B: continuous line, in absence of ATP; broken line, after addition of 1.25 mM ATP (with correction for effect due to addition of ATP itself, see text and Fig. 2). Concentration of $NH_4Cl$ was 10 mM.

## EXPERIMENTAL METHOD

The SV fraction was isolated from rat brain by differential centrifugation after treatment of the unpurified synaptosomal fraction by osmotic shock, as described previously [2]. The residue of synaptosome-rich fraction was homogenized in 8 volumes of bidistilled water (from the original weight of tissue), and 20 min later it was centrifuged for 15 min at 20,000g. The SV fraction was precipitated from the supernatant at 1,000g for 60 min. The preparation was suspneded in 0.32 M sucrose, 10 mM Tris-HCl, pH 4.7 (0°C) and kept at −10°C for not more than 1 week. Protein was determined by Lowry's method.

Changes in the transmembrane $H^+$ gradient were recorded [10] as the change in fluorescence of 1 μM AO in 2 ml of medium of basic composition: 0.15 M KCl, 2.5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.4, at room temperature with constant mixing. Tris-ATP (1.25 mM) was usually added 15 min after addition of protein (50-100 μg/ml). Changes in composition of the medium and incubation conditions are mentioned in the text. The various additives were added to the incubation medium in a volume of 10-20 μl, and in the case of alcoholic solutions the corresponding quantity of ethanol was added to the control samples. The intensity of fluorescence ($I_{fl}$) of AO was recorded on a Hitachi MPF-4 (Japan) spectrofluorometer, with wavelengths of exciting and emitted light of 491 and 526 nm, respectively.

Tris-ATP was obtained by passing a solution of ATP-$Na_2$ (from Reanal, Hungary) through a column packed with the ion-exchange resin Dowex 50WX (from Serva, West Germany). Carbonylcyanide-m-chlorophenylhydrazone (CCCP), valinomycin, dicyclohexycarbodiimide (DCCD), oligomycin (these were dissoved in ethanol), N-ethylmaleimide (NEM), oubain, and AO were all obtained from Serva. The remaining reagents were of the chemically pure grade.

## EXPERIMENTAL RESULTS

Addition of SV to the incubation medium at pH 7.4 and 20°C caused rapid quenching of fluorescene of AO (Fig. 1A). During subsequent incubation $I_{fl}$ returned to its initial level more slowly, in the course of 12-15 min; this process, moreover, was slightly accelerated by the protonophore CCCP (20 μM) and was strongly accelerated by the combined action of CCCP and the $K^+$-ionophore valinomycin (2 μM) in the presence of KCl because of $H^+/K^+$ exchange, induced by the two ionophores. These effects show that the acid pH is preserved in SV after isolation and the pH difference causes accumulation of AO and a decrease in its fluorescence [12, 15]. However, a rise of temperature compared with that at which the SV were stored, and the absence of ATP led to fairly rapid equilibration of the pH of the isolated vesicles with the pH of the incubation medium [15, 16].

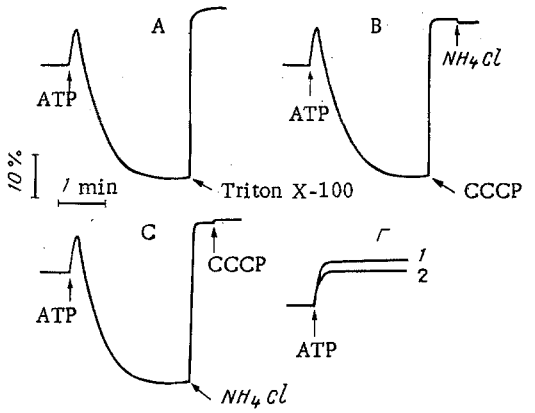


Fig. 2. Effect of detergent (A), CCCP (B), $NH_4Cl$ (C), and $Mg^{++}$ and KCl (D) on ATP-induced changes in fluorescence of AO in SV suspension. 0.025% Triton X-100, 20 M CCCP, 10 mM $NH_4Cl$. D: 1) $Mg^{++}$ not present in incubation medium, 2) KCl replaced by 0.3 Msucrose. Remaineder of legend as to Fig. 1.

Addition of ATP to samples containing SV and $Mg^{++}$ caused a further decrease in $I_{fl}$ of AO (Fig. 1B). The effect of ATP was strongly dependent on the duration of preincubation of SV: The decrease in $I_{fl}$ was small when ATP was added 1 min after the protein, when the original pH gradient was still completely preserved, whereas if ATP was added 15 min later, after spontaneous dissipation of the $H^+$ gradient, a strong and more rapid decrease in AO fluorescence was observed. However, irrespective of the level of AO fluorescence at the time of addition of ATP, the final level was the same (35-40% below the initial value recorded in the absence of SV and ATP). In the next experiments the inhibitory effect of the initial acid pH on ATP-dependent change (which undoubtedly play a regulatory role *in vivo*) was abolished by appropriate preincubation.

We systemically observed a small transient increase in $I_{fl}$ of AO after addition of ATP, which preceded the ATP-induced decrease (Fig. 2). In Fig. 1 this effect was subtracted before presentation of the data. Although the nature of this phenomenon is not absolutely clear [5,10], it did not give rise to any fundamental difficulties in the work.

Observations confirm that ATP-induced changes in $I_{fl}$ of AO in fact reflect accumulation of AO by SP due to the existence of the $H^+$ gradient (Fig. 2). These changes were abolished by detergent, evidence that they were due to accumulation of the probe inside the vesicles, and not to its adsorption on the membrane surface. Furthermore the protonophore CCCP, added either before or after ATP, completely abolished ATP-dependent AO accumulation. A similar action was exhibited by $NH_4Cl$ (10 mM), which is known to destroy $H^+$ gradients [5, 9]. The final level of AO fluorescence after addition of these agents was the same and subsequent addition of the other agent caused no further changes in $I_{fl}$. The degree of quenching of AO fluorescence is thus a measure of the change in transmembrane proton gradient.

For quantitative estimation of the rate of $H^+$ transport, the initial velocity at ATP-induced chagnes in $I_{fl}$ of AO was used and was expressed in percent per minute; the $I_{fl}$ level established after addition of $NH_4Cl$ (or CCCP), and equal (observing the conditions of preincubation, see above) to the level recorded immediately after addition of ATP, was taken as 100%. The initial velocity, measured as the tangent of the angle of the tangent to the initial part of the curve of change of $I_{fl}$, was proportional to protein concentration over a wide range of values (10-150 µg/ml). Total accumulation of $H^+$, determined as the steady-state (minimal) level of AO fluorescence achieved after addition of ATP, according to data in the literature [7], depended on protein concentration only at its low values. Nevertheless, given an equal protein concentration in the experimental and control samples, the two parameters changed parallel to one another under different experimental conditions (see below).

Valinomycin inhibited ATP-dependent $H^+$ translocation strongly (Table 1). What was unexpected was that inhibition by valinomycin, which depended on its concentration, did not depend on whether the incubation medium contained $K^+$ or $Na^+$. This nonspecificity of valinomycin is difficult to explain at present. Similar nonspecificity with respect to $K^+$ and $Na^+$ also was observed during inhibition of acetylcholine uptake by valinomycin and during its uncoupling of ATPase activity of SV in the skate electric organ [3]. Cases when valinomycin exhibited properties evidently unconnected with its generally accepted role of $K^+$-specific ionophore also are known for other membrane system [13].

TABLE 1. Effect of Inhibitors and Ionic Composition of Medium on Initial Velocity of Transport and Total Accumulation of Proteins in SV

| Conditions | $H^+$ transport | |
|---|---|---|
| | initial velocity | total accumulation |
| Inhibitors: | | |
| Valinomycin (0.25 μM) | 80,0 | 62,7 |
| Same, 150 mM NaCl instead of KCl | 55,1 | 49,7 |
| Valinomycin (2.0 μM) | 23,4 | 26,5 |
| Same, 150 mM NaCl instead of KCl | 16,2 | 13,8 |
| DCCD (10 μM) | 84,3 | 78,6 |
| DCCD (50 μM) | 26,4 | 18,6 |
| Ouabain (1 mM) | 111,1 | 114,5 |
| Orthovanadate (200 μM) | 101,1 | 94,5 |
| Oligomycin (2.5 μg/mg protein) | 106,3 | 108,1 |
| Same, 5.0 μg/mg protein | 94,8 | 108,3 |
| N-ethylmaleimide (50 μM) | 14,7 | 32,0 |
| $Ag^+$ (25 μM) | 0 | 0 |
| $Cu^{2+}$ (50 μM) | 4,6 | 5,4 |
| $Zn^{2+}$ (100 μM) | 6,9 | 16,7 |
| $Cd^{2+}$ (100 μM) | 35,6 | 57,1 |
| Change in ionic composition: | | |
| $Mn^{2+}$ (2.5 mM) instead of $Mg^{2+}$ | 115,0 | 116,8 |
| $Ca^{2+}$ (2.5 mM) instead of $Mg^{2+}$ | 0 | 0 |
| $Ca^{2+}$ and $Mg^{2+}$ (1.25 mM) | 49,3 | 71,3 |
| $Ba^{2+}$ and $Mg^{2+}$ (1.25 mM) | 105,0 | 110,9 |
| EGTA (0.5 mM) | 104,0 | 91,8 |
| NaCl (150 mM) instead of KCl | 94,2 | 82,7 |
| LiCl (150 mM) instead of KCl | 73,7 | 60,3 |
| $K_2SO_4$ (1000 mM) instead of KCl | 0 | 0 |
| $KHCO_3$ 150 mM, pH adjusted with HCl, instead of KCl | 30,2 | 60,7 |

Legend. Results expressed in % of control in medium of standard ionic composition (see Experimental Method section). In experiment with ouabain, 130 mM NaCl and 20 mM KCl were used instead of 150 mM KCl. Mean results of 3-5 experiments shown.

To determine the connection between $H^+$ translocation and ATPase activity of SV the action of some known inhibitors of membrane ATPases was examined (Table 1). DCCD, a blocker of all $H^+$-ATPases, inhibited $H^+$ transport in SV, and inhibition was total in the presence of 100 μM DCCD. Similar but incomplete inhibition of ATPase activity of SV preparations by DCCD was observed previously [2, 7]. Ouabain, an inhibitor of Na,K-ATPase, did not affect ATP-dependent translocation of $H^+$ even with a ratio of $Na^+$ to $K^+$ ions favorable for activity of Na,K-ATPase. Orthovanadate, a powerful inhibitor of several ion-transport ATPase, including gastric $K^+/H^+$-ATPase [14], did not affect $H^+$ transport even in high concentration (200 μM). Insensitivity to vanadate is a characteristic feature of $H^+$-ATPases of the $F_o \cdot F_1$ type and is interpreted as an indication of the absence of intermediate phosphoenzyme in the cephalytic cycle [14]. The results are evidence that $H^+$ transport in SV is effected by an $H^+$-ATPase which functions as an $H^+$-pump.

Similar $H^+$-ATPases are found in membranes of several secretory granules [8, 9, 17]. Unlike mitochondrial $H^+$-ATPase, they are insenitive to its specific inhibitor, oligomycin. In our experiments $H^+$-translocase activity also was not inhibited by oligomycin in concentrations which completely block $H^+$ transport in submitochondrial particles [5]. The writers showed previously that ATP-hydrolase activity of SV is insensitive to oligomycin [2].

NEM, a blocker of protein thiol groups, inhibited $H^+$ translocation from SV (Table 1) and inhibition was total in the presence of 100 μM NEM. This reagent is known to block the $H^+$-pump of chromaffin granules [6] and to inhibit ATPase activity of SV [2] in low concentrations. Some ions of intermediate metals, known as blockers of protein thiol groups, also inhibited the $H^+$-pump of SV in low concentrations. By the effectiveness of their inhibition they were arranged in the following order: $Ag^+ > Cu^{2+} > Zn^{2+} > Cd^{2+}$

In the absence of bivalent cations ATP did not induce $H^+$ translocation (Fig. 2). Mg-ATP and Mn-ATP complexes stimulated $H^+$ transport almost equally, but Ca-ATP was ineffective (Table 1). Similar specificity with regard to bivalent cations has been observed for $H^+$-pumps of secretory granules [8]. In the presence of Mg-ATP, $Ca^{++}$ ions (but not $Ba^{++}$) had an inhibitory action.

Replacement of the salt medium by an isoosmotic solution of sucrose prevented ATP-dependent $H^+$ transport (Fig. 2). The same result was obtained by replacing the penetrating anion $Cl^-$ by the nonpenetrating anion $SO_4^{2-}$ (potassium salts, Table 1). In the presence of $Cl^-$, $H^+$ transport was unchanged by replacement of $K^+$ by $Na^+$, and was slightly reduced in medium with $Li^+$. This specificity with regard to anions and comparative insensitivity to replacement of monovalent cations are also characteristic of the $H^+$-pump of chromaffin granules [9], which can be explained by the electrogenic character of $H^+$ translocation. In the absence of penetrating anions, electrogenic transfer of an uncompensated positive charge leads to a rapid increase of membrane potential, which prevents further proton translocation.

These results, together with those of a study of ATPase activity of SV [2], demonstrate convincingly that an $H^+$-ATPase, which as proton pump is responsible for transmembrane transport of $H^+$ ions and maintains a low pH within SV, may be localized in the membrane of SV. The properties of the $H^+$-pump of SV are similar to those of the analogous $H^+$-pumps in secretory granules [8, 17]. Some special features which distinguish the ATPase activity of SV, namely weak stimulation by uncoupling agents [2, 18, 10] and incomplete inhibition by DCCD and NEM [2], can probably be attributed to the presence of other ATPases, distinct from the proton ATPase, in SV preparations, whose precise localization is not yet known.

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