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Na^+ transport by reconstituted Na^+,K^+-ATPase in the presence of various nucleotides

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The ability of ATP, CTP, ITP, GTP, UTP and two synthetic ATP analogs to provide for ouabain-sensitive Na^+ accumulation into proteoliposomes with a reconstituted Na^+,K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.37) was investigated. A correlation between the proton-accepting properties of the nucleotides and their ability to provide for active transport was found. The proton-accepting properties of the substrate seem to be a necessary condition for the shift from the K-form to Na form of Na^+,K^+- ATPase – an immutable step in the active translocation of Na^+ and K^+ through the Na^+ pump.

$(Na^+ + K^+)$-ATPase; Reconstitution; Na^+ transport; Substrate specificity

1. INTRODUCTION

Recently we have investigated the pattern of hydrolysis of five nucleotides by Na^+,K^+-ATPase purified from duck salt glands and found the maximal rate of hydrolysis to decrease in the following order: ATP > CTP > ITP > GTP ⩾ UTP [1]. The activating effect of these substrates on the K^+-phosphatase reaction in the presence of Na^+ decreases in a similar way, the activation being mediated through nucleotide hydrolysis [2,3]. The hydrolysis of ATP and CTP occurs via complicated kinetics with two apparent K_m values, whereas that of GTP and UTP follows the usual Michaelis-Menten kinetics; ITP occupies an intermediate position [1].

Thus, the properties of ATP and CTP as substrates of Na^+,K^+-ATPase are very similar. We explained this in terms of the ability of ATP (CTP) to accept a proton by means of the nitrogen atom in the first position of the purine base (in the third position of the pyrimidine base, respectively) [3]. This assumption corresponds well to the data of Skou and Esmann, providing evidence that ATP binding to the K form of Na^+,K^+-ATPase facilitates release of protons from the enzyme system, and thereby facilitates transformation from the K to Na form of the enzyme [4]. This transformation is believed to be a rate-limiting step in the hydrolytic cycle of Na^+,K^+-ATPase pumping Na^+ and K^+ [5]. If our hypothesis [3] is valid, the ability of the nucleotides to provide for active transport has to depend on their proton-accepting properties.

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Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

2. MATERIALS AND METHODS

Na^+,K^+-ATPase was isolated and purified from duck salt glands as described [6]. The specific Na^+,K^+-ATPase activity of the final preparations varied from 18 to 25 μmol P_i/mg protein per min at 37°C.

The cholate-dialysis procedure developed by Jorgensen and Anner [7] was used for reconstitution of Na^+,K^+-ATPase into phosphatidylcholine

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liposomes. The NaCl/KCl ratio in the reconstitution medium was constant, i.e. 20 mM/50 mM. Na^+ transport was measured at 25°C. The reaction was initiated by the simultaneous addition of NTP (5 mM) and $^{22}Na^+$ (1–2 μCi) to the liposome suspension (250–300 μl) preincubated for 5–10 min at 25°C. In some experiments the ionophores valinomycin and FCCP were used at a final concentration of 1 μM. Aliquots of samples (50 μl) were taken at definite time intervals and applied to ion-exchange columns (1.3 × 1 cm) packed with CM-Sephadex G-25 in the Tris form. The liposomes were eluted with 1 ml of a chilled 0.25 M sucrose solution for 30–60 s. The $^{22}Na^+$ content inside liposomes was counted using an Ultragamma counter (LKB). The enzyme activity was determined from the P_i production as in [6].

Valinomycin, ouabain, ATP and CTP were obtained from Serva. ITP, choline chloride and sodium cholate were purchased from Sigma; GTP and UTP from Boehringer and FCCP from Fluka. N_1-oxy-ATP and N_1-methoxy-ATP were kindly supplied by Professor E.S. Severin (Institute of Molecular Biology, USSR Academy of Sciences, Moscow). Egg lecithin was obtained from the Bacterial Reagents Laboratory (Kharkov, USSR) and $^{22}Na^+$ from Isotope (USSR).

3. RESULTS AND DISCUSSION

In the presence of ATP, liposomes with reconstituted Na^+,K^+-ATPase provide for Na^+ accumulation, which markedly exceeds the passive Na^+ transport into liposomes (fig.1A). ATP-driven Na^+ accumulation is completely inhibited by ouabain present in the liposomes (fig.1B). The time course of Na^+ transport is non-linear.

We supposed that depletion of the ATP pool outside and the K^+ pool inside liposomes could be a limiting factor for Na^+ accumulation. The activity of Na^+,K^+-ATPase incorporated into proteoliposomes with outside-oriented active sites is 5–11 μmol P_i/ml suspension per h at 37°C. Hence, the loss of ATP due to the function of reconstituted Na^+,K^+-ATPase in a typical experiment (ATP concentration, 5 mM; sample volume, 300 μl; time of recording, 10 min; incubation temperature, 25°C) should not exceed 20%. ATP utilization by Na^+,K^+-ATPase molecules not incorporated into sealed vesicles also cannot be a limiting factor, since inhibition of these enzyme molecules by ouabain present outside vesicles does not influence the kinetics of Na^+ transport (fig.1A).

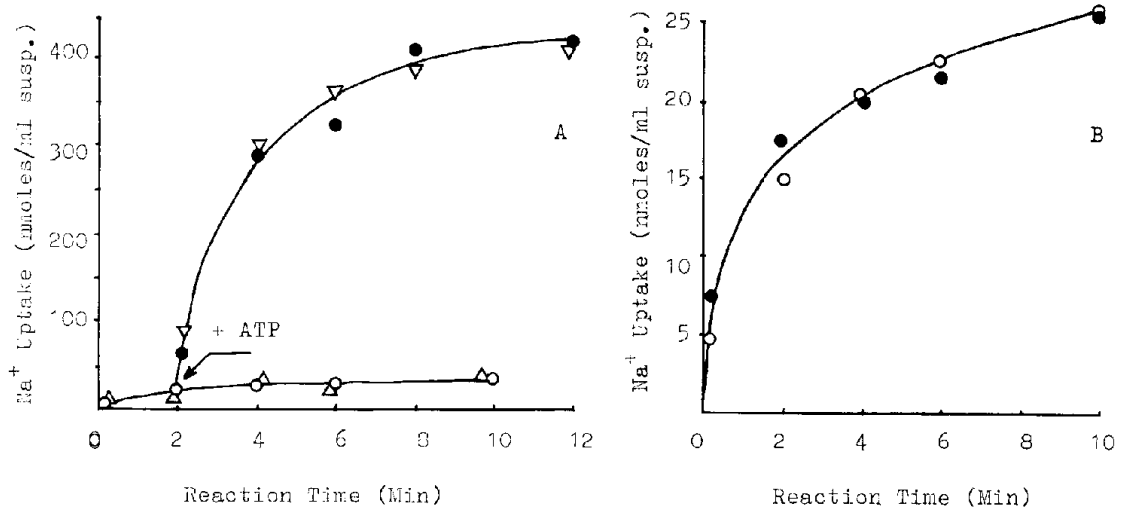

Fig.1. Na^+ accumulation by proteoliposomes with a reconstituted Na^+,K^+ pump. (A) Proteoliposomes were prepared in the absence of ouabain; passive (○) and active (●) Na^+ transport was measured in the absence of ouabain; passive (△) and active (▽) Na^+ transport was measured in the presence of 1 mM ouabain added to the suspension immediately prior to assay. In these experiments ATP (5 mM) was added to samples 2 min after addition of $^{22}Na^+$. (B) Proteoliposomes were prepared in the presence of 1 mM ouabain; Na^+ transport was measured in the absence (○) and presence (●) of 5 mM ATP.

We also could see no difference between the time courses of Na^+ accumulation in the absence and presence of the ionophores valinomycin and FCCP, at least during the first 5 min of the reaction (not shown). Such a combination of ionophores is known to restore the K^+ pool inside liposomes [8,9]. It may be concluded that depletion of the ATP pool outside and the K^+ pool inside liposomes does not limit Na^+ influx.

When analysing the dependence of Na^+ transport on ATP concentration we found the K_m value for ATP to be equal to 345 μM (fig.2). This is in good agreement with the second K_m value for ATP hydrolysis by the same enzyme, i.e. 330 μM [1]. Thus, it may be supposed that the affinity of Na^+,K^+-ATPase for the substrate before and after reconstitution is the same.

We compared Na^+ accumulation by proteoliposomes in the presence of different nucleotides. CTP provides for Na^+ transport almost to the same extent as ATP; ITP does so, but 4-times poorer than ATP (table 1). GTP and UTP do not provide for Na^+ accumulation at all. It is noteworthy that the most efficient substrates are those which can accept protons at neutral pH values. The pK_a value for the N_1 (N_3) atom of the purine (pyrimidine) base of nucleosides decreases in the following order: adenosine, 3.5; cytidine, 4.2; inosine, 8.8; guanosine and uridine, 9.2 [10,11]. (The corresponding values for the nucleotides are in general about 0.5 units higher.) This means that at neutral pH values the extent of protonation of the nucleotides increases from ATP to UTP. Therefore, ATP and CTP seem to be superior to ITP, GTP and UTP as proton acceptors.

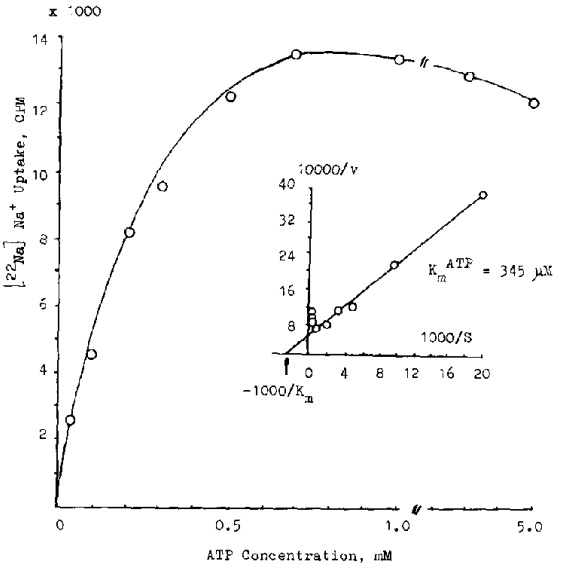

Fig.2. Dependence of active $^{22}Na^+$ transport on ATP concentration (50–5000 μM). For calculations we used the values of $^{22}Na^+$ accumulation obtained 2 min after initiation of the reaction.

Table 1

Active Na^+ accumulation by proteoliposomes with a reconstituted Na^+,K^+ pump

[Substrate] (mM)	$v_{acc.}$ (μmol Na^+/ml suspension per h)[a]
ATP (5)	4.84 ± 0.78 (4)[b]
CTP (5)	5.18 ± 1.34 (4)
ITP (5)	1.26 ± 0.22 (4)
ATP (3)	4.17 ± 0.54 (2)
N_1-Oxy-ATP (3)	6.37 ± 1.73 (2)
N_1-Methoxy-ATP (3)	1.56 ± 0.12 (2)

[a] To estimate the rate of Na^+ accumulation ($v_{acc.}$) we used for calculations the values of accumulation obtained 3 min after initiation of the reaction

[b] The figures in parentheses designate the number of experiments with different preparations of the reconstituted Na^+,K^+-ATPase

From our experiments with ATP analogs (structural formulas shown in fig.3) we obtained conclusive evidence that the proton-accepting properties of the substrate are important for the transport function. N_1-oxy-ATP provides for Na^+ accumulation even better than ATP, whereas N_1-methoxy-ATP is much less effective (table 1). The pK_a value for the substituent group at position 1 of the purine base of N_1-oxy-ATP is 2.6 [12].

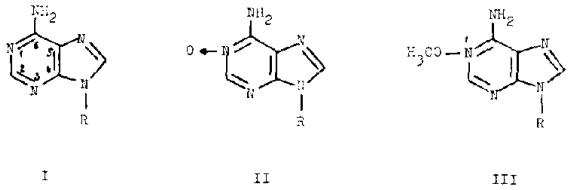

Fig.3. Structural formulas of ATP (I), N_1-oxy-ATP (II) and N_1-methoxy-ATP (III). R, ribose 5′-triphosphate tail of the nucleotide molecule.

This means that the N_1 locus of this analog is deprotonated at neutral pH values. The presence of a partial negative charge on the oxygen atom may explain the proton-accepting properties of N_1-oxy-ATP. The authentic pK_a value for N_1-methoxy-ATP is unknown but its structure suggests exceedingly low proton-accepting capacity as compared with that of N_1-oxy-ATP.

Thus, we have found a correlation between the structural peculiarities of the nucleotides and their ability to provide for active Na^+ transport. We believe that these peculiarities determine the ability of nucleotides to convert the K form of Na^+,K^+-ATPase to the Na form through the deprotonation of an amino acid residue in the active center of the enzyme. Previously, we suggested alteration of the enzyme affinity for Na^+ and K^+ during the hydrolytic cycle to be a critical factor in the coupling of hydrolytic and transport functions of Na^+,K^+-ATPase [13]. The present data support this hypothesis.

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