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ACETYL PHOSPHATE CAN ACT AS A SUBSTRATE FOR Na^+ TRANSPORT BY ($Na^+ + K^+$)-ATPase

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Experiments using liposomes with ($Na^+ + K^+$)-ATPase incorporated showed that in the presence of extravesicular Mg^{2+}, acetyl phosphate was able to stimulate Na^+ uptake when the liposomes contained Na^+ or choline and were K^+-free; this acetyl phosphate-dependent Na^+ transport was similar to the ATP-dependent transport observed with 0.003 mM or 3 mM ATP. When the intravesicular solution contained K^+, there was an ATP-dependent Na^+ uptake which was large with 3 mM ATP and small (about the size seen in K^+-free liposomes) with 0.003 mM ATP; in this case, although acetyl phosphate produced a slight activation of Na^+ transport, the effect was not statistically significant. All ATP and acetyl phosphate-stimulated Na^+ transport disappeared in the absence of extravesicular Mg^{2+} or in the presence of ouabain in the intravesicular solution. These results are consistent with the hypothesis that, at the concentration used, acetyl phosphate can replace ATP in the catalytic but not in the regulatory site of the ($Na^+ + K^+$)-ATPase and active Na^+ transport system. This suggests that as far as the early stages of the pump cycle are concerned the role of ATP is simply to phosphorylate.

Acetyl phosphate, apart from being substrate for the K^+-dependent phosphatase activity of ($Na^+ + K^+$)-ATPase, has been shown to phosphorylate the enzyme in the presence of Mg^{2+} and Na^+ [1–4]. The ATP- and acetyl phosphate-dependent phosphorylation reactions exhibit cross-competition with each other. In addition, the acid stable phosphoprotein formed from acetyl phosphate shares with that obtained from ATP the following properties:
(1) electrophoretic mobility and chemical reactivity;
(2) maximal yield of phosphoprotein;
(3) Na^+ concentration needed for half-maximal phosphorylation; and
(4) half-life at 0°C. Furthermore, there is indirect evidence [4] suggesting that K^+ can accelerate dephosphorylation of acetyl phosphate-phosphorylated enzyme. All these observations have been taken as indicating that acetyl phosphate can also act as a substrate for the ($Na^+ + K^+$)-ATPase reaction [1–4]. On the other hand, there are other experimental results which do not agree with the view just expressed; these are:
(1) no acetyl phosphate-ADP or acetyl phosphate-acetate exchange could be detected [4–6];
(2) in squid axons acetyl phosphate does not sustain any appreciable active Na^+ transport [7]; and
(3) it has been reported [6] that if the phosphorylation reaction is stopped with detergent instead of acid a phosphoprotein can be obtained with ATP but not with acetyl phosphate in the incubation solution. The aim of the present work was to investigate the possibility that acetyl phosphate

can replace ATP in the $(Na^+ + K^+)$-ATPase reaction following the effects of acetyl phosphate and ATP on Na^+ uptake by liposomes with $(Na^+ + K^+)$-ATPase incorporated.

The experiments were performed with $(Na^+ + K^+)$-ATPase partially purified from pig kidneys according to Jørgensen [8]. The initial specific activity was 12–15 units/mg. Protein was determined by the procedure of Lowry et al. [9] with modifications [10] using bovine serum albumin as standard. Liposomes were made with L-α-phosphatidylcholine; their preparation as well as the incorporation of $(Na^+ + K^+)$-ATPase were as described in Ref. 11. To change the composition of extravesicular media the liposomes were centrifuged at 1000 rpm for 3 min through a syringe filled with Sephadex G-50 equilibrated with a solution of the desired composition. In our hands, the vesicles had potassium as contaminant (about 0.2 mM); to obtain vesicles free of K^+ the phospholipids were converted to Tris salts as indicated in Ref. 12. The degree of lipid oxidation, which never exceeded 1%, was determined as in Ref. 13. In Na^+ transport experiments liposomes with $(Na^+ + K^+)$-ATPase incorporated were preheated for 2 min at 20°C and immediately diluted in an equal volume of incubation solution containing 5 μCi/ml of $^{22}Na^+$. Sodium uptake was followed for 2 min at 20°C and pH 7.0; uptake was linear during that time under all experimental conditions investigated. Aliquots of 30–40 μl were then centrifuged at 1000 rpm for 3 min at 4°C through 1 ml Sephadex G-50–40 columns pre-equilibrated with 160 mM Tris-HCl at pH 7.0. Sodium uptake was estimated from the radioactivity present in the effluent; control experiments showed that all extravesicular Na^+ remained trapped in the Sephadex column. Solutions were prepared with deionized bidistilled water. The composition of solutions varied in different experiments and is detailed in the legend of Table I. NaCl and KCl were Baker Ultrex and all other reagents were analytical grade. Carrier free $^{22}Na^+$ was purchased from New England Nuclear (U.S.A.). ATP (vanadium free), acetyl phosphate, ouabain, cholic acid and L-α-phosphatidyl-choline were from Sigma, U.S.A. ATP and acetyl phosphate were converted into Tris salts by passing them through Amberlite R-120 columns. Radioactivity was determined by liquid-scintillation counting.

The results of these experiments are summarized in Table I. In liposomes free of K^+ and containing either choline chloride (A) or NaCl (B) incubated in the presence of extravesicular Mg^{2+} there was a small but distinct, and statistically significant, ATP-dependent uptake of Na^+; this uptake was similar with 3 mM and 0.003 mM ATP. Acetyl phosphate (5–10 mM) was also able to sustain a statistically significant Na^+ influx under these conditions; the magnitudes with acetyl phosphate were slightly smaller than with ATP but the differences between the two phosphate compounds were not significant. On the other hand, when there was K^+ in the intravesicular media (C) (always in the presence of extravesicular Mg^{2+}) the picture changed:

(i) The ATP-dependent Na^+ influx was much larger with 3 mM ATP than with 0.003 mM ATP and

(ii) with 5–10 mM acetyl phosphate there was a slight stimulation of Na^+ transport, but in this case the magnitude of these increases were not statistically significant. A fundamental observation in Table I is that when there was no Mg^{2+} in the extravesicular solution (and 1 mM EDTA was included) or 1 mM ouabain inside the liposomes, no ATP- or acetyl phosphate-dependent Na^+ transport could be detected under any of the experimental conditions investigated. It is also important to stress that when checked, total ATP hydrolysis at 0.003 mM concentration at the end of the 2-min incubation period amounted to about 30% of that initially present. This puts the final ATP concentration around 0.002 mM (averaging about 0.0025 mM), which is enough to saturate the catalytic site of the enzyme [14–17].

Considering the different intravesicular cation composition (Table I) the ATP-dependent uptake of Na^+ can be attributed to three different modes of operation of the Na^+, K^+ pump: uncoupled (choline liposomes), Na_o^+-stimulated (Na^+-liposomes) and Na_i^+-K_o^+ coupled transport (K^+-liposomes). This interpretation is supported by the characteristics of the ATP requirements in the different conditions. In choline and Na^+ liposomes ATP stimulation of Na^+ transport is already saturated at 0.003 mM; this is similar to what has been described for the uncoupled and Na_o^+-stimulated ATPase activity and Na^+ trans-

TABLE I

EFFECTS OF ATP AND ACETYL PHOSPHATE ON Na^+ UPTAKE IN LIPOSOMES WITH ($Na^+ + K^+$)-ATPase INCORPORATED

Solution compositions were as indicated in the table plus the following ligands: (mM) Intravesicular: imidazole (pH (20°C) 7.0), 25; EDTA-Tris, 1. Extravesicular: (A, A'): choline chloride, 120; imidazole (pH (20°C) 7.0), 25; $^{22}NaCl$, 9; (B, B' and C, C'): Tris-HCl (pH (20°C) 7.0), 190; $^{22}NaCl$, 9. ATP- or acetyl phosphate-dependent Na^+ uptake corresponds to the difference in uptake observed in the presence and absence of the phosphate compounds. Temperature was 20°C. Each entry is the mean +S.E. of quadruplicate determinations. Note: (i) intravesicular concentrations refer to those present in the solutions where ($Na^+ + K^+$)-ATPase was incorporated into liposomes; (ii) extravesicular concentrations are those obtained after mixing the liposomes suspensions with the uptake solutions; (iii) Mg^{2+}-free extravesicular solutions contained 1 mM EDTA; (iv) Na^+ uptake in the absence of ATP or acetyl phosphate had the following values (nmol Na^+/10 µl original vesicles per 2 min): (A', A): 0.017±0.008; (B, B'): 0.089±0.010; (C, C'): 0.040±0.008. AcP, acetyl phosphate; ChCl, choline chloride; Ouab., ouabain.

Expt. No.	Solution composition (mM)							Na^+ uptake ATP- or AcP-dependent (nmol/10 µl/liposomes per 2 min)
	Extravesicular			Intravesicular				
	ATP	AcP	$MgCl_2$	ChCl	NaCl	KCl	Ouab.	
A	3	–	3	130	–	–	–	0.076±0.018
	0.003	–	0.5	130	–	–	–	0.089±0.020
	–	5	0.5	130	–	–	–	0.050±0.008
	–	10	1.0	130	–	–	–	0.049±0.015
	–	5	–	130	–	–	–	0.006±0.010
A'	0.003	–	0.5	130	–	–	1	0.006±0.009
	–	5	0.5	130	–	–	1	−0.004±0.012
B	3	–	3	–	200	–	–	0.092±0.015
	0.003	–	0.5	–	200	–	–	0.083±0.016
	–	5	0.5	–	200	–	–	0.064±0.017
	–	5	–	–	200	–	–	−0.008±0.009
B'	0.003	–	0.5	–	200	–	1	−0.010±0.012
	–	5	0.5	–	200	–	1	−0.005±0.011
C	3	–	3	–	–	200	–	1.720±0.050
	0.003	–	0.5	–	–	200	–	0.046±0.015
	–	5	0.5	–	–	200	–	0.025±0.015
	–	10	1	–	–	200	–	0.027±0.016
	3	–	–	–	–	200	–	0.010±0.014
	–	10	–	–	–	200	–	−0.009±0.015
C'	3	–	3	–	–	200	1	−0.010±0.013
	0.003	–	0.5	–	–	200	1	−0.013±0.016
	–	10	1	–	–	200	1	−0.007±0.017

port in resealed red cell ghosts [18] and the ATPase activity by ($Na^+ + K^+$)-ATPase in K^+-free Na^+ solutions (Na^+-ATPase) [14–17], where only a high-affinity ATP site is apparent. On the other hand, in K^+-liposomes the ATP requirements are those of the ($Na^+ + K^+$)-coupled transport and ATPase reaction, which need high ATP concentrations to reach maximal rates [14–17]. All the above, plus the fact that ATP and acetyl phosphate-dependent Na^+ fluxes disappear in the absence of extravesicular Mg^{2+} or in ouabain-containing liposomes, strongly indicates that what were measured as phosphate compounds-dependent Na^+ fluxes were indeed fluxes of Na^+ through the active Na^+, K^+-transport mechanism; this is so even when the actual fluxes did not take place against an electrochemical gradient. On these grounds one can asses that acetyl phosphate, at the concentrations used, can replace ATP in the catalytic site of the enzyme, but not on the regulatory one. This is consistent with the apparent affinity reported for ($Na^+ + Mg^{2+}$)-dependent enzyme phosphorylation from acetyl phosphate, which as a $K_{0.5}$ value for acetyl phosphate around 2–3 mM

[4,6]. The fact that in K^+-containing liposomes the acetyl phosphate-dependent Na^+ uptake did not reach statistical significance may reflect the inability of acetyl phosphate to fuel Na^+-K^+ exchange, or might be a result of the dispersion inherent to this methodology and the low levels of fluxes. A possible explanation could be the simultaneous stimulation of phosphatase and ATPase activities by acetyl phosphate, diverting enzyme away from the ATPase reaction. With Na^+ and Mg^{2+} in the extravesicular side and 200 mM K^+ in the intravesicular solution some K^+ accumulation in the extravesicular media is not unlikely; this would create the conditions for phosphatase activity to take place [14–17]. The capability of acetyl phosphate to sustain Na^+ transport through the complete cycle of the Na^+ pump reaction is possible on thermodynamic grounds, for the free energy of hydrolysis at pH 7 and 25°C is about -8 kcal · mol for ATP [19] and -11 kcal · mol for acetyl phosphate [20]. Therefore, these results indicate that acetyl phosphate can replace ATP as substrate for the Na^+ pump running the complete cycle in the forward direction, at least when the pump is operating in the uncoupled or Na^+-stimulated mode. In turn, this suggests that as far as the early stages of the pump cycle are concerned the role of ATP is simply to phosphorylate. Finally, some comments on Na^+ fluxes independently of ATP or acetyl phosphate are called for. In the absence of any phosphate compounds Na^+ uptake was higher in Na^+- than in K^+-liposomes, and this in turn higher than in liposomes containing choline (see legend to Table I); all these differences were statistically significant. This could represent the ability of $(Na^+ + K^+)$-ATPase to passively exchange monovalent cations in the absence of ATP [12,21]. However, there are two arguments against this idea;

(i) ouabain did not have any effect within each group of liposomes [12], and

(ii) these results cannot distinguish passive cation exchange from variable leakage between liposomes with $(Na^+ + K^+)$-ATPase incorporated under different ionic conditions.

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