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Evidence for the presence of an ATP transport system in brush-border membrane vesicles isolated from the kidney cortex

Ada Elgavish [a,*] and Gabriel A. Elgavish [b]

Laboratory of Membrane Biology [a], and Department of Biochemistry and the NMR Core Facility of the Comprehensive Cancer Center [b], University of Alabama in Birmingham, Birmingham, AL 35294 (U S A)

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Results of ^{31}P-NMR studies and transport experiments using the radioactive tracer technique are presented. They point to the conclusion that ATP is taken up into isolated renal brush-border membrane vesicles, possibly by a carrier-mediated mechanism.

Early studies on the metabolism of ^{32}P-labelled nucleotides in the whole mammalian animal [1] or in tissue slices and in cell suspension [2] suggested that dephosphorylation had to precede uptake. Such evidence led to the generalization that plasma membranes have very low permeability to nucleotides. However, some studies on fibroblasts [3], perfused kidneys [4] and kidney slices [5] indicated that, added exogenously, nucleotides accumulated in the cells. Most of these studies were conducted in vivo or in isolated, perfused organs. In such systems the observed results were necessarily a combination of processes occurring in the plasma membrane and in the cytoplasmic compartment. To avoid the complications inherent in such systems, the present study was initiated to investigate whether the isolated renal brush-border membrane was permeable to ATP.

Brush-border membrane vesicles from the proximal tubule of rabbit kidneys were prepared as described previously [6,7]. The quality of the membrane preparations, evaluated by specific activities of enzyme markers, was the same as reported earlier [6]. In particular, the effect of inhibitors on Mg^{2+}-ATPase activity was tested. Ouabain (1.5 mM) had no significant inhibitory effect on the ATPase activity at pH 7.5 or pH 9.2, indicating that no significant contamination by basolateral membrane ($Na^+ + K^+$)-ATPase was found in our membrane preparations. Oligomycin (20 μM) had no effect at pH 9.2 and a maximal inhibitory effect of 20% at pH 7.5. At similar concentrations, the renal-cortex mitochondrial ATPase is inhibited over 95% [8]. Moreover, in our preparation, the specific ATPase activity was 100–200 nmol/min per mg protein. Therefore, the maximum 20% of this activity which was oligomycin inhibitable and may have been due to mitochondrial contamination was 20–40 nmol/min per mg protein. However, crude preparations of mitochondria, such as may be expected to contaminate the brush-border membrane preparation have been shown to have a specific ATPase activity of about 1000 nmol/min per mg protein [16]. Therefore, the 20–40 nmol/min per mg protein of oligomycin-inhibitable activity in our preparation may represent not more than 20–40 μg mitochondrial protein out of each

* To whom correspondence should be addressed at (present address) Department of Pharmacology, University of Alabama in Birmingham, University Station, Box 191, Birmingham, AL 35294, U S A.

mg of total brush-border membrane protein. Accordingly, not more than 2–4% of the intravesicular volume in this preparation may have been of mitochondrial nature.

ATP uptake by brush-border membrane vesicles was measured by a Millipore filtration technique, using 0.45 μm filters, as described previously [7,9]. The temperature of the uptake medium was 37°C. The composition of extravesicular medium varied depending on the objective of the experiment and is given in detail with the results. [^{32}P]ATP and [^{3}H]ATP were purchased from New England Nuclear.

Protein was determined by the procedure of Lowry et al. [10] using bovine serum albumin as standard.

Samples of fresh brush-border membrane vesicles were prepared for ^{31}P-NMR experiments. Isoosmotic conditions were kept in all experiments. Spectra were recorded in the FT mode, at 80.9 MHz, on a Bruker CXP 200/300 spectrometer of the UAB Cancer Center NMR Core Facility (supported by NIH grant CA-13148). A repetition time of 3 s was used to avoid signal suppression. Differentiation between the intravesicular and extravesicular resonances of each phosphate was achieved by using a paramagnetic relaxation reagent. The reagent used in these experiments was Gd(EDTA) [11]. It has already been shown [12] that membranes are not permeable to this metal chelate. Therefore, due to the formation of short-lived, weak complexes of Gd(EDTA) with the extravesicular ATP and inorganic phosphate, but not with their intravesicular counterparts, this paramagentic chelate was expected to have a broadening effect on the extravesicular phosphate species only. Sharp peaks of the intravesicular phosphate species should remain unaffected.

A typical time-course of [^{3}H]ATP uptake into the brush-border membrane vesicles is given in Fig. 1. It was important to resolve the question whether ATP taken up by the brush-border membrane preparations represented binding to the membrane or ATP transport across the membrane into an intravesicular space. Two lines of evidence suggested that the latter was the case. The intravesicular space was varied by altering the osmolarity of the medium with sucrose, a relatively impermeable solute that is not degraded in the kidney

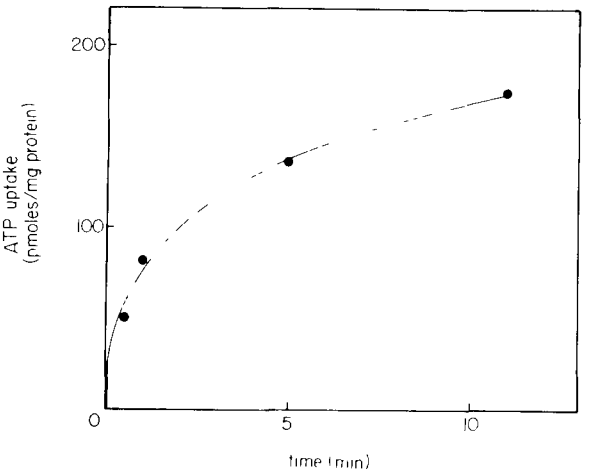

Fig 1. ATP transport in renal brush-border membrane vesicles The membrane vesicles were loaded with 300 mM sucrose, 50 mM Tris-HCl (pH 7 5) ATP uptake was measured under the following conditions 300 mM sucrose, 50 mM Tris-HCl (pH 7 5), 0 5 mM CDTA, 0 5 mM EGTA, 0.1 mM ATP, [^{3}H]ATP

[13]. With a given concentration of substrate, the amount taken up at equilibrium should be dependent on the relative intravesicular space, and this volume should be inversely proportional to the osmolarity of the medium. As shown in Fig. 2, the

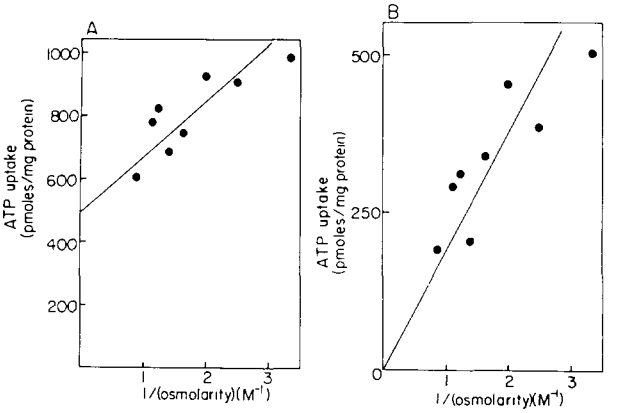

Fig. 2. The effect of medium osmolarity on ATP uptake into renal brush-border membrane vesicles Membrane vesicles were loaded with 300 mM sucrose, 50 mM Tris-HCl (pH 7 5) Uptake of ATP (60 min, 37°C) was measured under the following conditions 0.5 mM CDTA, 0 5 mM EGTA, 100 mM NaCl, 50 mM Tris-HCl (pH 7 5) and various concentrations of sucrose (100, 200, 300, 400, 500, 600, 700, 800 mM) (A) ATP uptake without subtraction of 'zero uptake' values (for details see results) (B) ATP transport with 'zero uptake' values subtracted

uptake was indeed proportional to the inverse osmolarity and, thus, to the intravesicular space. The extent of the binding to the membrane was obtained by extrapolating to infinite medium osmolarity (zero intravesicular space) and represented about 50% of the total 'uptake' at 300 mosM. An estimate for the membrane-bound component was also found by the 'zero-time uptake'. The latter was the amount of membrane-associated ATP measured at time zero of the uptake kinetics before any transport across the membrane could have taken place. This method assumes that the binding is a process much faster than cross-membrane transport. In Fig. 2b such a 'zero-time uptake', performed for each value of the intravesicular space was subtracted from the corresponding ATP uptake measured at 60 min. As shown (Fig. 2b), in this case extrapolation to infinite medium osmolarity (zero space) indeed resulted in zero uptake. For the rest of the study such 'zero-time uptake' measurements, which represent binding to the membrane vesicle and the filter, accompanied each experiment. The bound component was obtained in this manner, then subtracted from the total uptake measurement.

Although the above results seemed to indicate the presence of transport across the brush-border membrane, the radioactive-label technique was affected by several practical drawbacks and the interpretation of the results involved certain assumptions. Moreover, using solely this technique it would have been impossible to conclude whether ATP, ADP, AMP or adenosine were accumulating in the vesicles. Even ^{32}P-label on the phosphate moiety could not answer this question since the hydrolysis-generated inorganic phosphate was quickly transported through the brush-border membrane. To solve this problem we utilized ^{31}P-NMR. The advantage of this method was the simultaneous monitoring of intravesicular and extravesicular phosphate compounds. Therefore, no physical separation of the vesicles from the medium was needed. The NMR measurements were done noninvasively. They did not perturb the system in equilibrium and there was no destruction of the membranes. In summary, the ^{31}P-NMR results were not plagued by any of the drawbacks of the radioactive technique. However, since ATP concentrations higher than the physiological levels had to be used in order to have detectable intravesicular resonances, the NMR study was carried out only to establish the chemical identity of

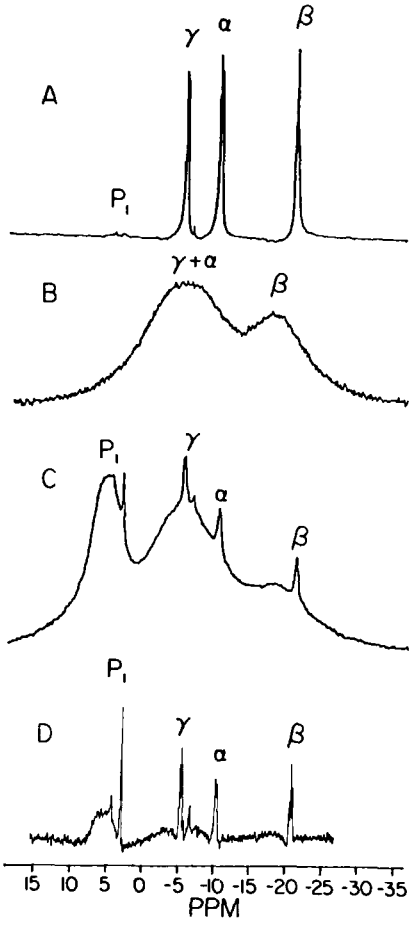

Fig. 3. Observation of intravesicular ATP in renal brush-border membrane vesicles using ^{31}P-NMR Membranes were loaded with 575 mM sucrose, 50 mM Tris-HCl (pH 7 5) The ATP-uptake was carried out under the following conditions. 150 mM disodium ATP, 0.5 mM CDTA, 0.5 mM EGTA, Trizma base to bring pH to 7.5 (A) The ^{31}P-NMR spectrum of the brush-border membrane vesicles after reaching equilibrium with the uptake medium (60 min). (B) The ^{31}P-NMR spectrum of the ATP-uptake buffer to which 27 mM Gd(EDTA) had been added (C) The ^{31}P-NMR spectrum of the brush-border membrane sample in (A) to which 27 mM Gd(EDTA) had been added. (Note· the approximately 3 ppm shift of the broad, Gd affected signals is due to the bulk susceptibility effect in the extravesicular medium) (D) The ^{31}P-NMR convolution difference spectrum derived from (C) Linebroadening parameters of 5 Hz and 100 Hz were used. Note the improved resolution of the expected splitting pattern of the α and γ doublets and the β triplet of the ATP.

the uptaken substrates. Once this was established, quantitative kinetics at physiological concentrations of ATP were done, using radioactive techniques.

For ^{31}P-NMR measurements a 2 ml sample of renal brush border membrane vesicles (15 mg protein) was equilibrated for about 1 h (30°C) with a buffer containing 150 mM ATP, 0.5 mM CDTA, 0.5 mM EGTA, Tris (pH 7.5) and then it was placed in the probe of the NMR spectrometer. The spectrum is shown in Fig. 3A. Large peaks of the α-, β- and γ-phosphates of ATP and a negligibly small peak of orthophosphate were observed. In order to separate the otherwise isochroneous resonances of intravesicular and extravesicular ATP, the aqueous relaxation reagent Gd(EDTA) (27 mM) was added [11] and the sample was scanned for several hours. The latter had a broadening effect on the peaks of all three phosphates of the extravesicular ATP (Fig. 3C). A separate set of sharp phosphate resonances, evidently unaffected by the extravesicular relaxation reagent, proved the existence of an intravesicular pool of ATP and P_i. The larger P_i peak in Fig. 3C as compared to Fig 3A is the result of ATP hydrolysis which occurs in these membranes at a slow rate as a result of ATPase activity in the absence of Mg^{2+} (Elgavish and Elgavish, unpublished data). The effect of Gd(EDTA) on the α-, β- and γ-phosphate peaks of ATP in the buffer used for membrane preparations, but without the membranes, is given as a control in Fig. 3B. As expected, only broad peaks were observed. Fig. 3D depicts a 'convolution difference spectrum' obtained from the data of Fig. 3C by once filtering the free-induction decay with a small linebroadening parameter (approx. 5 Hz) and then filtering the original free-induction decay with a large linebroadening parameter (approx. 100 Hz) and Fourier Transform the difference between the two. In this manner, the broad signals are almost entirely removed. The resulting spectrum is superior to Fig. 3C in emphasizing the narrow intravesicular resonances. Based on the ^{31}P-NMR spectra [14], the extravesicular and intravesicular pH values were found to be 7.6, identical to the pH of the solution in which the membranes had been dispersed. The determination of the intravesicular pH using ^{31}P-NMR is based on the pH dependence of the chemical shift position of intravesicular orthophosphate relative to an orthophosphoric acid reference external to the sample. Independently, the chemical shift difference between the β-, and γ-phosphates of ATP is also pH sensitive [14]. This difference gave the same result for the intravesicular and extravesicular pH, i.e. 7.6. Since no Mg^{2+} was present this result was not affected by Mg binding to ATP.

The ^{31}P-NMR study led to the conclusion that after 60 min incubation with extravesicular ATP, ATP was found in the intravesicular space. The uptake cannot be explained by a fast opening-sealing process of the vesicles in the presence of ATP. Such an event would have caused rapid equilibration of the paramagnetic reagent as well, and would have prevented the observed differentiation between the intravesicular and extravesicular ATP signals. Both the ^{31}P-NMR studies and the studies carried out with radioactively labeled ATP support the idea that an ATP transport system is present

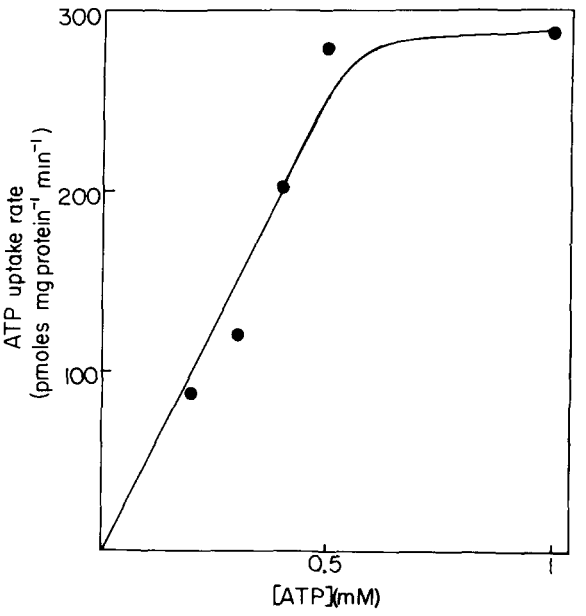

Fig 4 The initial ATP-uptake rate as a function of ATP concentration in the uptake medium. The membrane vesicles were loaded with 300 mM sucrose, 50 mM Tris-HCl (pH 7 5) Initial uptake rates were measured under the following conditions· 100 mM sucrose, 100 mM NaCl, 0.5 mM CDTA, 0 5 mM EGTA, 50 mM Tris-HCl (pH 7 5), [^{3}H]ATP and various concentrations of ATP (0 2, 0 3, 0 4; 0 5, 1 mM)

in the renal membrane vesicles. The possibility that the ATP transport occurs into a contaminating fraction of mitochondria was excluded by the results of the ^{31}P-NMR study. The ratio of the total intravesicular to extravesicular volume as evident in Fig. 1C is much higher than expected had the intravesicular signal been due to intramitochondrial ATP. Therefore, we conclude that an ATP transport system is present in the renal membrane vesicles. It is also possible that, like in many other systems, in the isolated vesicles ATP was dephoshorylated, internalized as adenosine and subsequently phosphorylated back to ATP [15]. However, this is an unlikely possibility since the transport experiments in our study were conducted in the absence of Mg^{2+} (0.5 mM CDTA, 0.5 mM EGTA). The saturability of the uptake at high extravesicular ATP concentration has indicated that the uptake may have proceeded by a carrier mediated process with an apparent K_m of 0.3 mM (Fig. 4).

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References

1 Roll, P.M., Weinfeld, H., Carroll, E. and Brown, G.B (1956) J. Biol Chem 220, 439–454
2 Leibman, K C. and Heidelberger, C. (1975) J Biol Chem. 216, 823–830
3 Plunkett, W., Lapi, L., Ortiz, P.J. and Cohen, S.S. (1974) Proc Natl. Acad. Sci USA 71, 73–77
4 Widemann, M J., Hems, D.A. and Krebs, H A. (1969) Biochem J. 115, 1–10
5 Chaudry, I.H., Sayeed, M.M and Baue, A E (1975) Can J. Physiol Pharmacol. 54, 742–749
6 Beck, J C. and Sacktor, B (1978) J. Biol Chem 253, 5531–5535
7 Elgavish, A., Rifkind, J. and Sacktor, B (1983) J. Membrane Biol 72, 85–91
8 Liang, C.T. and Sacktor, B (1976) Arch. Biochem. Biophys 176, 285–297
9 Aronson, P S. and Sacktor, B. (1975) J. Biol Chem 250, 6032–6039
10 Lowry, O H., Rosebrough, N J., Farr, A.L and Randall, R J (1951) J. Biol Chem 193, 265–275
11 Elgavish, G.A. and Reuben, J (1976) J. Am. Chem Soc. 98, 4755–4759
12 Degani, H. and Elgavish, G A (1978) FEBS Lett 90, 357–360
13 Sacktor, B. (1977) Curr. Top Bioenerg. 6, 39–81
14 Moon, R D. and Richards, J H (1975) J Biol Chem 216, 823–830
15 Paterson, A R P., Harley, E R., Kolassa, N. and Cass, C E (1981) in Nucleosides and Cancer Treatment (Tattersall, M H N. and Fox, R.M.J., eds) pp. 3–17, Academic Press, New York
16 Vogel, G (1979) Methods Enzymol. 55, 317–319