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ATP dependence of anion uptake by isolated vacuoles: requirement for excess Mg^{2+}

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Vacuoles were isolated from barley mesophyll protoplasts. [^{14}C]Malate or $^{36}Cl^-$ were taken up from the surrounding medium. Uptake was only slightly increased in the presence of equimolar levels of ATP and Mg^{2+} (as magnesium gluconate). In the presence of excess Mg^{2+} in the medium, ATP-stimulated uptake of malate and chloride increased several-fold. Stimulation by excess Mg^{2+} was not observed for ATP-stimulated amino acid uptake by isolated vacuoles. Stimulation of uptake by excess Mg^{2+} was observed at all malate concentrations upto 10 mmol · l^{-1}. The content of Mg^{2+} needed for half-maximum stimulation was about 3.5 mmol · l^{-1} in the presence of 1 mmol · l^{-1} ATP. The increase in Mg^{2+} concentration had no effect on the tonoplast ATPase activity.

Introduction

Vacuoles function as transient or permanent storage compartment of plant cells. Metabolites, ions and products of the secondary metabolism are deposited inside the vacuoles of various plant tissues. This function requires transport across the tonoplast membrane. Transporter proteins catalyze the transport into and out of the vacuoles. Transport has been characterized for sucrose [1–3] amino acids [4–6], malate [7], alkaloids [8] inorganic anions [9,10] and sodium [11]. Ion channels have been identified at the tonoplast membrane by patch clamp analysis [12]. The observations have revealed the existence of specific carriers which are located in the tonoplast. They facilitate either mediated diffusion or energized transport by gradient coupling. The source of energy is the proton motive force which is built up by the action of primary pumps, the tonoplast H^+-ATPase and the H^+-pumping pyrophosphatase [13].

Little is known about the regulation of these various transport systems. Ca^{2+} is involved in the activation of ion channels (see also Ref. 12). Binding of ATP to the amino acid carrier is a regulatory device in amino acid transport [5,6].

Here we report that Mg^{2+} in excess of the Mg^{2+} complexed by ATP is required for efficient anion transport across the tonoplast.

Material and Methods

Plant material. Barley (*Hordeum vulgare,* var. Gerbel) was grown in soil in a growth chamber. During the day/night cycle (14 h/10 h) the temperature was 20 ° C, and 18 ° C, respectively.

Isolation of vacuoles. Protoplasts and vacuoles were isolated and purified as described by Martinoia et al. [7] and Kaiser and Heber [3]. Vacuoles were liberated from the protoplasts by mechanical lysis.

Transport experiments. Uptake of malate, chloride and alanine by isolated vacuoles was measured at 20 ° C. To separate the vacuoles from the incubation medium, the silicon oil layer centrifugation technique was employed as previously described [3,7]. For each condition and time point, five polypropylene microcentrifugation tubes (with a capacity of 400 μl) were prepared as follows: 40 μl of vacuole suspension were added to 60 μl of medium containing 67% Percoll, 0.35 mol · l^{-1} sorbitol, 45 mmol · l^{-1} potassium gluconate, 30 mmol · l^{-1} Hepes-KOH (pH 7.0), 3.3 mmol · l^{-1} dithiothreitol, 0.3% (w/v) purified bovine serum albumine and other solutes as indicated. The samples were overlayered with 150 μl phenylmethyl silicone oil (AR 200, Wacker Chemie, München, F.R.G.) and 40 μl of H_2O on top of it. Substrate uptake was terminated by centrifugation at 10000 × g for 30 s. Intact vacuoles floated through the silicone layer into the aqueous phase which was recovered and used for measurements. For the transport of malate, chloride and alanine, the assay contained the substrates at concentrations as indicated with a radioactivity of 5 to 6 kBq. ^3H_2O was also included (5.5 kBq).

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It equilibrates rapidly between medium and vacuoles. The radioactivity of ^3H_2O in the upper phase after centrifugation was used to quantify the recovery of vacuoles.

For the experiment shown in Fig. 3, the Percoll medium was added only immediately before the silicon oil centrifugation. The incubation was performed in an assay volume of 360 μl. The incubation mixture consisted of 350 mmol · l^{-1} sorbitol, 30 mmol · l^{-1} Hepes (imidazole, pH 7.0), 1 mmol · l^{-1} malate, 2 mmol · l^{-1} dithiothreitol, 30 mmol · l^{-1} potassium gluconate and 1 mmol · l^{-1} ATP. After 20 min of incubation, 240 μl of Percoll-containing medium (100% (v/v)) were added. Immediately after the addition, aliquots of 100 μl of the mixture were placed on the bottom of polypropylene microcentrifugation tubes, rapidly overlayered with silicon oil and water and centrifuged as described above.

Measurement of ATPase activity. ATP hydrolysis by intact vacuoles was measured under conditions similar to the uptake experiments. The incubation medium consisted of 350 mmol · l^{-1} glycine betaine, 30 mmol · l^{-1} Hepes (imidazole, pH 7.6), 30 mmol · l^{-1} potassium gluconate, 2 mmol · l^{-1} dithiothreitol, 0.1 mmol · l^{-1} orthovanadate, 0.1 mmol · l^{-1} sodium molybdate and 1 mmol · l^{-1} ATP. In the absence of Mg^{2+}, ethylenediamine tetraacetic acid was added at a final concentration of 1 mmol · l^{-1}. Alternatively, Mg^{2+} was added as gluconate salt at a concentration of either 1 or 5 mmol · l^{-1}. ADP released by hydrolysis of ATP was determined spectrophotometrically. For this, the extracts were treated with 6% $HClO_4$ on ice, neutralized with 5 mol · l^{-1} K_2CO_3/0.25 mol · l^{-1} Tris and the supernatant was used for the enzymatic determination of the formed ADP [14].

Uptake of sucrose by liposomes. Phosphatidylcholine-liposomes were prepared by a freeze/thaw cycle and consecutive sonification as described by Flügge and Heldt [15]. The medium consisted of 360 mmol · l^{-1} sorbitol, 20 mmol · l^{-1} potassium gluconate, Tricine-KOH (pH 7.2) and [^{14}C]sucrose (3.3 kBq; specific activity: 10 MBq/μmol) was added to the 10% liposome solution (final volume 200 μl). Uptake was terminated by applying the samples to a 10 ml Sephadex G-75 column to separate the liposomes from the radioactivity in the supsension medium. Radioactivity in the liposomes was determined.

Results

Binding of Mg^{2+} by ATP inhibits anion uptake by isolated vacuoles

Fig. 1 shows the effect of increasing ATP concentrations on alanine, malate and chloride uptake by isolated vacuoles and on sucrose uptake by phosphatidylcholine liposomes. The magnesium content was kept constant. Sucrose uptake by liposomes was measured as a control to exclude possible effects of Mg^{2+} and ATP on protein-free lipid membranes. It was unchanged over the whole range of ATP concentrations. Amino acid transport increased with ATP up to a concentration of 1 mmol · l^{-1} ATP. It was unaffected by higher ATP concentrations. This dependence of alanine uptake on ATP was similar to that previously reported, although the transport was saturated at lower ATP levels [6]. However, a completely different and somewhat peculiar dependence on ATP concentration was observed for malate and chloride uptake.

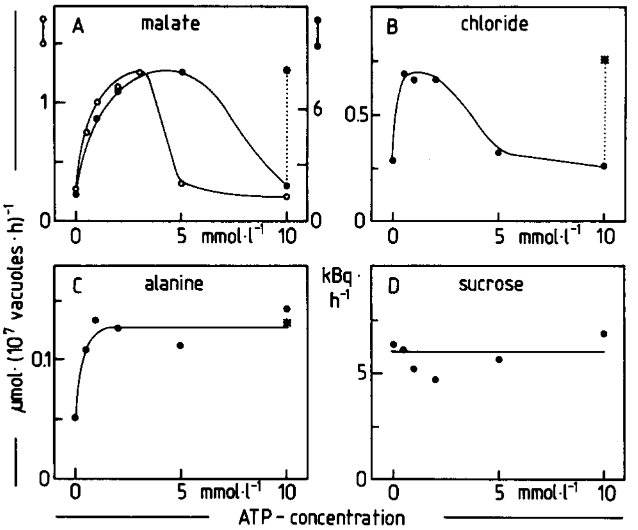

Fig. 1. ATP-dependent uptake of [^{14}C]malate. $^{36}Cl^-$ and [^{14}C]alanine by isolated barley vacuoles. (A) Rates of malate uptake in the presence of 1 mmol·l^{-1} (○) and 10 mmol·l^{-1} (●) malate. The magnesium gluconate concentration was either 10 (○) or 20 mmol·l^{-1} (●). At the ATP concentration of 10 mmol·l^{-1}, malate uptake was also measured in the presence of 40 mmol·l^{-1} magnesium gluconate (*). (B) Relationship between chloride uptake (1 mmol·l^{-1}) and ATP concentration. The magnesium gluconate concentration was 10 mmol·l^{-1}. Addition of 40 mmol·l^{-1} magnesium gluconate reversed ATP inhibition of chloride uptake (*). (C) and (D) are control experiments. (C) Uptake of [^{14}C]alanine by isolated vacuoles. The alanine concentration was 1 mmol·l^{-1}, the magnesium gluconate concentration was 20 mmol·l^{-1}. The rate of alanine uptake was also measured in the presence of 10 mmol·l^{-1} ATP but without magnesium added (*). (D) ATP concentration dependence of [^{14}C]sucrose uptake by phosphatidylcholine-liposomes. The magnesium gluconate concentration was 10 mmol·l^{-1}.

Uptake of chloride and malate anions was low in the absence of ATP. It increased with ATP concentration. However, a further increase in ATP levels caused an inhibition of malate and chloride uptake. In the presence of 10 mmol · l^{-1} Mg^{2+}, suppression of the ATP stimulated anion uptake to the level observed in the absence of ATP was obtained at ATP concentrations higher than 5 mmol · l^{-1}. Inhibition of malate uptake was shifted to higher ATP concentrations when the Mg^{2+} concentration was 20 mmol · l^{-1}. Under these conditions, an increase of the Mg^{2+} concentration to 40 mmol · l^{-1} reestablished the maximal rate of malate transport. Excess of Mg^{2+} also reversed ATP inhibition of chloride uptake.

ATP is known to bind divalent cations such as Mg^{2+}. The stability coefficient of the MgATP complex is high. At equal molar concentrations of ATP and Mg^{2+}, most of the Mg^{2+} is complexed by ATP (see also Ref. 16). The result shows a requirement for Mg^{2+} of anion uptake by isolated vacuoles in addition to the Mg^{2+} which is complexed to ATP to form the substrate for the tonoplast ATPase. Although gluconic acid also binds Mg^{2+}, the stability constant of the Mg^{2+}-gluconate complex ($\log K^{\text{gluconic acid}}{}_{\text{Mg}} = 0.7$) is by more than three orders of magnitude smaller than the stability constant of MgATP complex ($\log K^{\text{ATP}}{}_{\text{Mg}} = 4.0$–$4.9$). At equimolar concentrations of ATP and Mg^{2+}, more than 99% of the Mg^{2+} is bound by ATP. In the presence of 3.5 $\text{mmol} \cdot \text{l}^{-1}$ Mg and under the experimental conditions of Fig. 3 (1 $\text{mmol} \cdot \text{l}^{-1}$ ATP, 30 $\text{mmol} \cdot \text{l}^{-1}$ potassium gluconate), the free Mg^{2+} concentration is about 20 $\mu\text{mol} \cdot \text{l}^{-1}$. Gluconate salts were used in the experiments for two reasons: (i) Anions such as chloride decrease the stability of the isolated vacuoles. (ii) Isolated vacuoles take up inorganic anions. This will interfere with the measured uptake of malate or other ions. Particularly in the experiments where high Mg^{2+} concentrations were added it seemed advisable to use magnesium gluconate instead of $MgCl_2$.

Magnesium-stimulation of malate uptake at varying malate concentrations

Malate uptake was only slightly stimulated by ATP at low Mg^{2+} concentrations irrespective of the malate concentration. After increasing the Mg^{2+} content of the suspension medium to 20 $\text{mmol} \cdot \text{l}^{-1}$, malate uptake increased irrespective of the malate concentration (Fig. 2). A saturable component was only observed at equimolar concentrations of ATP and Mg^{2+}.

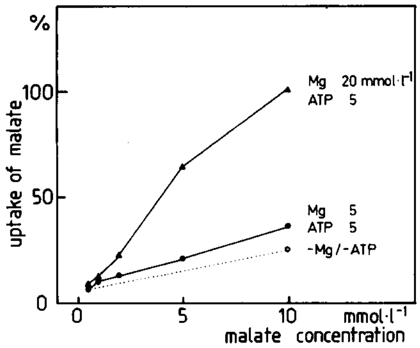

Fig. 2. Concentration dependence of malate uptake by vacuoles. Uptake was measured in the absence of ATP and Mg (○), in the presence of equal molar concentrations of ATP and magnesium gluconate (5 $\text{mmol} \cdot \text{l}^{-1}$) (●) and at an increased Mg concentration of 20 $\text{mmol} \cdot \text{l}^{-1}$ (△). The result is the mean of three experiments (five replicates each). Uptake in the presence of 10 $\text{mmol} \cdot \text{l}^{-1}$ malate, 5 $\text{mmol} \cdot \text{l}^{-1}$ ATP and 20 $\text{mmol} \cdot \text{l}^{-1}$ magnesium gluconate was set to 100% and corresponds to 4.5 ± 0.4 $\mu\text{mol} \cdot (10^7 \text{ vacuoles})^{-1} \cdot \text{h}^{-1}$. The mean standard deviation (S.D._{n-1}) was below 10%. Sodium molybdate was added at a final concentration of 0.1 $\text{mmol} \cdot \text{l}^{-1}$.

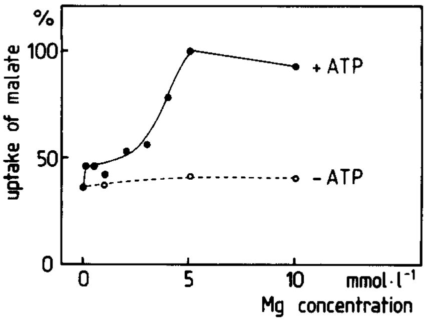

Fig. 3. Mg^{2+}-dependent malate uptake of isolated vacuoles. The uptake of malate was determined after 20 min of incubation. The malate and the ATP concentration was 1 $\text{mmol} \cdot \text{l}^{-1}$ each. The uptake in the presence of 5 $\text{mmol} \cdot \text{l}^{-1}$ magnesium gluconate was set to 100% and corresponds to 295 ± 62 $\text{nmol} \cdot (10^7 \text{ vacuoles})^{-1}$. The data are means of three experiments (five replicas each).

Mg^{2+} concentration dependence of malate uptake

In the experiments described above, the incubation medium contained 40% Percoll which may also bind cations. To investigate the Mg^{2+} dependence of malate uptake, Percoll was added only at the end of the incubation period. It was needed to stabilize the silicone oil step gradient during centrifugation. Mg^{2+} at concentrations between 0 and 2 $\text{mmol} \cdot \text{l}^{-1}$ had only a small effect on ATP-dependent malate uptake (Fig. 3). Uptake was stimulated in a sigmoidal manner by increasing the Mg^{2+} content up to 5 $\text{mmol} \cdot \text{l}^{-1}$. No further stimulation was observed at higher concentrations. The Mg^{2+} content needed for half maximal stimulation was in the range of 3 to 4 $\text{mmol} \cdot \text{l}^{-1}$ Mg^{2+}.

Table I compares the effect of various magnesium salts on ATP-stimulated malate uptake. The pH of the incubation mixtures was checked after addition of the magnesium salts. ATP was present at a concentration of 5 $\text{mmol} \cdot \text{l}^{-1}$. Magnesium gluconate stimulated the rate of malate uptake when its concentration was increased from 5 to 20 $\text{mmol} \cdot \text{l}^{-1}$. The stimulatory effect of increasing $MgCl_2$ concentrations on malate uptake was in principle similar to that of magnesium gluconate.

TABLE I

Mg^{2+} concentration dependence of ATP-stimulated malate uptake

Mg^{2+} was added as gluconate, chloride or sulfate salts. The ATP concentration was 5 $\text{mmol} \cdot \text{l}^{-1}$, the malate concentration 1 $\text{mmol} \cdot \text{l}^{-1}$. The standard deviation ($n = 4$–8) was below ± 18%.

	Uptake rates ($\mu\text{mol} \cdot (10^7 \text{ vacuoles})^{-1} \cdot \text{h}^{-1}$)			
Mg^{2+} concn. ($\text{mmol} \cdot \text{l}^{-1}$):	1	5	10	20
Magnesium gluconate	0.22	0.27	0.30	0.90
$MgSO_4$	0.19	0.49	0.41	0.29
$MgCl_2$	0.21	0.15	0.30	0.46

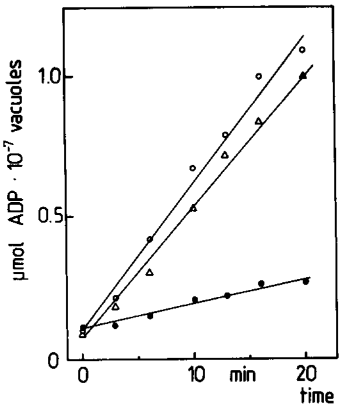

Fig. 4. ATP hydrolysis by isolated vacuoles in dependence of the Mg^{2+} concentration. The assay contained either 1 mmol·l^{-1} EDTA (●), 1 mmol·l^{-1} magnesium gluconate (○) or 5 mmol·l^{-1} magnesium gluconate (△). The ATP concentration was 1 mmol·l^{-1}.

However, the rate observed in the presence of 20 mmol ·l^{-1} $MgCl_2$ was only half of that in the presence of 20 mmol·l^{-1} magnesium gluconate. Cl^- and SO_4^{2-} are known to inhibit malate uptake [7]. This may partly explain why $MgSO_4$ stimulated malate uptake at low concentrations and inhibited uptake at higher concentrations. The result shows specific, probably in part, competitive effects of anions on malate uptake. Anion effects have to be distinguished from the effect of Mg^{2+}. Furthermore, magnesium gluconate was most effective in stimulating malate uptake.

Mg^{2+} concentration dependence of ATPase activity

Fig. 4 shows Mg^{2+} concentration dependence of ATPase activity as measured by the appearance of ADP in the medium. The assay was composed as in the uptake experiments shown in Fig. 3. Low rates of ATP hydrolysis were observed in the presence of EDTA. Although vacuoles contain an endogenous pool of Mg^{2+} which may partly be released either by breakage of vacuoles or by efflux from the vacuoles, the Mg^{2+} concentration in the medium was maintained low by excess EDTA. Therefore the basal rate of ATP hydrolysis may be due to some molybdate-insensitivity of acid phosphatases. The rate of ATP hydrolysis increased several-fold in the presence of 1 mmol·l^{-1} Mg^{2+} and was not further stimulated by additional Mg^{2+}.

Conclusions

Transport of malate and chloride across the tonoplast membrane is stimulated by MgATP [7,9]. An ATP-dependent proton pump transports H^+ into the intravacuolar space [13]. Anion transport is secondarily coupled to the protonmotive force (pmf) generated by electrogenic proton transport. Dissipation of the pmf inhibits ATP-stimulated transport [7]. However, stimulation of anion uptake by the addition of ATP and Mg^{2+} is variable. Our incubation medium was optimized for vacuolar stability and lacked all interfering inorganic anions. We generally observed a stimulation of anion transport in the presence of ATP by increasing the Mg^{2+} concentration. The stability constant of the MgATP complex is high; at equal molar concentrations of ATP and Mg^{2+}, the free Mg^{2+} concentration in the medium is very low. In addition, Mg^{2+} may be bound to negative surface charges of the vacuoles and to malate.

Our results indicate that Mg^{2+} in addition to that complexed by ATP is required for energized anion uptake by vacuoles. The ATPase activity is not increased by additional Mg^{2+}. Therefore, we conclude that either the anion carrier itself or the maintenance of the pmf across the tonoplast membrane require Mg^{2+}. Chloride and malate are transported through a tonoplast-localized Ca^{2+}-regulated channel [17]. Recently, Martinoia et al. [18] investigated the inhibitor-sensitivity of chloride and malate transport. Their results suggest that malate may also be transported by another transport system which does not catalyze chloride translocation. The Mg^{2+} effect on the two types of carriers has yet to be established. From our data it is not possible to compute the absolute requirement of anion transport for Mg^{2+}. The apparent affinity of about 3 mmol·l^{-1} suggested by Fig. 3 is likely to overestimate the real affinity because of binding of Mg^{2+} in addition to complex formation with ATP. This suggests that the cytosolic free Mg^{2+} concentration is close or in excess to the requirement of the transport system for Mg^{2+}.

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References

1 Guy, M., Reinhold, L. and Michaeli, D. (1979) Plant Physiol. 64, 61–64.
2 Doll, S., Rodier, F. and Willenbrink, J. (1979) Planta 144, 407–411.
3 Kaiser, G. and Heber, U. (1984) Planta 161, 562–568.
4 Homeyer, U., Litek, K., Huchzermeyer, B. and Schultz, G. (1989) Plant Physiol. 89, 1388–1393.
5 Dietz, K.-J., Martinoia, E. and Heber, U. (1989) Biochim. Biophys. Acta 984, 57–62.
6 Dietz, K.-J., Jäger, R., Kaiser, G. and Martinoia, E. (1990) Plant Physiol. 92, 123–129.
7 Martinoia, E., Flügge, U.-I., Kaiser, G., Heber, U. and Heldt, H.W. (1985) Biochim. Biophys. Acta 806, 311–319.
8 Deus-Neumann, B. and Zenk, M.H. (1984) Planta 162, 250–260.
9 Martinoia, E., Schramm, M.J., Kaiser, G., Kaiser, W.M. and Heber, U. (1986) Plant Physiol. 80, 895–901.
10 Kaiser, G., Martinoia, E., Schröppel-Meier, G. and Heber, U. (1989) J. Plant Physiol. 133, 756–763.

11 Garbarino, J. and DuPont, F.M. (1988) Plant Physiol. 86, 231–236.

12 Hedrich, R. and Schroeder, J.I. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 539–569.

13 Rea, P.A. and Sanders, D. (1987) Physiol. Plant. 71, 131–141.

14 Jaworek, D. and Welsch, J. (1985) in Methods of Enzymatic Analysis, Vol. 7 (Bergmeyer, H.U., ed.), pp. 365–370, Verlag Chemie, Weinheim.

15 Flügge, U.I. and Heldt, H.W. (1981) Biochim. Biophys. Acta 638, 296–304.

16 Piazza, G.J. and Gibbs, M. (1983) Plant Physiol. 71, 680–687.

17 Hedrich, R. and Neher, E. (1987 Nature 329, 833–835.

18 Martinoia, E., Vogt, E. and Amrhein, N. (1990) FEBS Lett. 261, 109–111.