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Proton and anion transport at the tonoplast in crassulacean-acid-metabolism plants: specificity of the malate-influx system in *Kalanchoë daigremontiana*

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Abstract. Tonoplast vesicles were prepared from leaf mesophyll homogenates of the crassulacean-acid-metabolism plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie to study the effects of anions on ATP- and inorganic-pyrophosphate (PPi)-dependent H^+ transport. In the presence of gramicidin, substrate hydrolysis by the tonoplast ATPase was characteristically stimulated by chloride and inhibited by nitrate, but was unaffected by malate and a wide range of other organic-acid anions; the PPiase was anion-insensitive. Malate was more effective than chloride both in stimulating ATP- and PPi-dependent vesicle acidification (measured as quinacrine-fluorescence quenching) and in dissipating a pre-existing inside-positive membrane potential (measured as oxonol-V-fluorescence quenching), indicating that malate was more readily transported across the tonoplast. Certain other four-carbon dicarboxylates also supported high rates of vesicle acidification, their order of effectiveness being fumarate $\gg$ malate $\simeq$ succinate $>$ oxalacetate $\simeq$ tartrate; the five-carbon dicarboxylates 2-oxoglutarate and glutarate were also transported, although at lower rates. Experiments with non-naturally occurring anions indicated that the malate transporter was not stereospecific, but that it required the *trans*-carboxyl configuration for transport. Shorter-chain or longer-chain dicarboxylates were not transported, and neither were monocarboxylates, the amino-acid anions aspartate and glutamate, nor the tricarboxylate isocitrate. The non-permeant anions maleate and tartronate appeared to be competitive inhibitors of malate transport but did not affect chloride transport, indicating that malate and chloride influx at the tonoplast might be mediated by separate transporters.

Key words: Anion transport – ATPase (tonoplast) – Crassulacean acid metabolism – *Kalanchoë* – Malate – Proton transport – Pyrophosphatase

Introduction

Solute transport across the tonoplast is involved in many physiologically important processes, such as homeostasis of cytoplasmic composition, maintenance of cell turgor and sequestration of secondary metabolites. The driving force for solute transport is provided by the proton-motive force, which can be generated by two H^+-translocating enzymes at the tonoplast, an ATPase and an inorganic pyrophosphatase (PPiase; for a review see Sze 1985; Marin 1987; Rea and Sanders 1987). By enhancing the transmembrane pH difference (Δ pH, inside acid) and electrical potential difference ($\Delta\psi$, inside positive), these primary H^+ pumps energize the secondary transport and accumulation of a wide range of solutes in plant vacuoles (Guern et al. 1987; Poole 1988).

The present study investigated the relationship between ATP- and PPi-dependent H^+ transport and secondary anion movements across the tonoplast of plants exhibiting crassulacean acid metabolism (CAM). These plants accumulate large amounts of malic acid in the vacuoles of chlorenchymatous cells following nocturnal fixation of CO_2 in the cytosol (Lüttge 1987; Smith 1987; Lüttge and Smith 1988). The process has an overall stoichiometry of 2 mol H^+ accumulated per mol

Abbreviations: BTP = 1,3-bis-[tris(hydroxymethyl)methylamino]-propane; CAM = crassulacean acid metabolism: oxonol V = bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; Δ pH = transmembrane pH difference; PPi = inorganic pyrophosphate; PPiase = inorganic pyrophosphatase; quinacrine = 6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine dihydrochloride; $\Delta\psi$ = transmembrane electrical potential difference

malate, and appears to be directly energized by the proton pumps at the tonoplast. These create an inside-positive $\Delta\psi$ of about 25 mV (Rona et al. 1980), which is thought to drive the electrophoretic influx of malate^{2-} anions from the cytosol. Currently, this hypothesis is supported by several observations: (i) the tonoplast of CAM plants possesses both an ATPase activity (Aoki and Nishida 1984; Smith et al. 1984a, b; Jochem and Lüttge 1987; Balsamo and Uribe 1988) and a PPiase activity (Marquardt and Lüttge 1987); (ii) both these enzymes can drive electrogenic H^+ transport across the tonoplast (Jochem and Lüttge 1987; Marquardt and Lüttge 1987); (iii) malate acts as a permeant anion, dissipating an inside-positive $\Delta\psi$ across the tonoplast (Jochem and Lüttge 1987); and (iv) uptake of malate by intact vacuoles from CAM plants can be stimulated by ATP (Nishida and Tominaga 1987). As yet, however, little is known about the molecular properties of the malate transporter.

In this paper, the malate-influx system at the tonoplast of CAM plants has been further characterized by studying the effects of a wide range of anions on ATP- and PPi-dependent H^+ transport. Since permeant anions dissipate $\Delta\psi$ and thereby increase Δ pH, rates of vesicle acidification can be used to determine the relative permeability of the tonoplast to various anions (Bennett and Spanswick 1983; Kaestner and Sze 1987; Pope and Leigh 1987). Some anions also have a direct action on the tonoplast ATPase activity, notably Cl^- which is stimulatory and NO_3^- which is inhibitory (Walker and Leigh 1981a; Sze 1985). However, the PPiase activity is insensitive to anions (Walker and Leigh 1981b; Rea and Poole 1985) and is particularly useful in assaying the effects of different anions on vesicle acidification (Kaestner and Sze 1987; Pope and Leigh 1987). For CAM plants, malate is known to support high rates of H^+ transport in tonoplast vesicles (Jochem and Lüttge 1987; Struve and Lüttge 1987), but it appears to be a relatively impermeant anion in vesicles from non-CAM species (Kaestner and Sze 1987; Pope and Leigh 1987). Thus, a wide range of related organic-acid anions were used in the present experiments to investigate the molecular specificity of the malate-influx system at the tonoplast in CAM plants.

Material and methods

Plant material. Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie were propagated vegetatively and grown in John Innes No. 3 potting compost in a heated glasshouse. Natural lighting was supplemented by mercury-vapour lamps (400 W MBF; Thorn EMI, London, UK) for 12 h daily. When five to eight months old, plants were transferred to a reverse-phase controlled-environment room, where they were illuminated by a combination of metal-halide fluorescent lamps (400 W MBIF/BU; Thorn) and tungsten lamps (PAR 38 150 W Flood; General Electric Co., Wembley, Middx., UK) for 12 h daily at a photosynthetic photon flux density (400–700 nm) of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at mid-plant height (measured with an LI-190SB quantum sensor; Li-Cor Inc., Lincoln, Neb., USA). Air temperature was maintained at 25° C (light)/14° C (darkness), with a relative humidity of approx. 35% (light)/70% (darkness).

Tonoplast isolation. Plants were maintained in the controlled-environment room for at least 7 d prior to experimentation. Fully expanded leaves were harvested 2 h into the dark cycle, when leaf malic-acid content was close to its minimum value. Tonoplast fractions from the mesophyll tissue were isolated according to the method of Bremberger et al. (1988) and Haschke et al. (1988) with minor modifications. The leaf epidermises, midrib and margins were removed and the mesophyll tissue was homogenized in a commercial food blender in a medium (typically $100 \cdot 10^{-6}$ m^3 medium to 40 g tissue) containing the following: 450 $mol \cdot m^{-3}$ mannitol, 3.0 $mol \cdot m^{-3}$ $MgSO_4$, 10 $mol \cdot m^{-3}$ ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1.0 $mol \cdot m^{-3}$ dithiothreitol (DTT), 0.5% (w/v) polyvinylpyrrolidone (PVP-40), 2.0% (w/v) bovine serum albumin (Fraction V), 100 $mol \cdot m^{-3}$ *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine) buffered to pH 8.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris). The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at 5000·*g* for 10 min. The resulting supernatant was layered over a 25% (w/v) sucrose cushion containing 1.0 $mol \cdot m^{-3}$ DTT and 5.0 $mol \cdot m^{-3}$ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffered to pH 8.0 with Tris. The gradients were centrifuged at 100000·*g* for 60 min in a TST 41.14 rotor using a Superspeed 65 ultracentrifuge (both from M.S.E., Crawley, Sussex, UK). Tonoplast vesicles were removed from the interface using a Pasteur pipette and diluted 1:1 (v/v) with a medium containing 150 $mol \cdot m^{-3}$ mannitol, 1.0 $mol \cdot m^{-3}$ DTT and 25 $mol \cdot m^{-3}$ 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) buffered to pH 8.0 with 2-(*N*-morpholino)ethanesulfonic acid (Mes). Vesicles were then pelleted at 100000·*g* for 30 min in an MSA (M.S.E.) 50.10 rotor. The pellet was finally resuspended in a medium containing 150 $mol \cdot m^{-3}$ mannitol, 1.0 $mol \cdot m^{-3}$ DTT and 25 $mol \cdot m^{-3}$ BTP buffered to pH 8.0 with Mes. All steps were performed at 4° C. Preparations were stored at −40° C and used within one week.

Hydrolytic assays. Rates of ATP hydrolysis were measured at 38° C on samples containing approx. 10 g protein $\cdot m^{-3}$ in an assay medium consisting of 6.0 $mol \cdot m^{-3}$ $MgSO_4$, 3.0 $mol \cdot m^{-3}$ Tris-ATP, 200 $mmol \cdot m^{-3}$ sodium azide, 100 $mmol \cdot m^{-3}$ sodium molybdate, 100 $mmol \cdot m^{-3}$ sodium orthovanadate, 1.6 $mmol \cdot m^{-3}$ gramicidin and 50 $mol \cdot m^{-3}$ Tricine buffered to pH 8.0 with Tris. Further anions were added from stock solutions of the free acids buffered to pH 8.0 with Tricine-Tris. Rates of ATP hydrolysis were determined from inorganic orthophosphate (Pi) release according to the method of Smith et al. (1984a).

Inorganic pyrophosphate (PPi) hydrolysis was measured at 38° C on samples containing approx. 1.0 g protein $\cdot m^{-3}$ in an assay medium of 7.5 $mol \cdot m^{-3}$ $MgSO_4$, 500 $mmol \cdot m^{-3}$ Na_4PPi, 50 $mol \cdot m^{-3}$ KOH, 100 $mmol \cdot m^{-3}$ sodium molybdate, 1.6 $mmol \cdot m^{-3}$ gramicidin and 50 $mol \cdot m^{-3}$ Tricine buffered to pH 8.0 with Tris. Further anions were added from stock solutions of the free acids buffered to pH 8.0 with Tricine-Tris. Hydrolysis of PPi was determined from Pi release as above.

Measurement of Δ pH. Acidification of tonoplast vesicles in the presence of ATP or PPi was monitored by following the fluorescence quenching of 6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine dihydrochloride (quinacrine) according to the method of Bennett and Spanswick (1983). Initial rates of H^+ transport at 25° C (at which temperature Δ pH formation was close to optimal) were determined from the initial rates of fluorescence quenching upon the addition of either Tris-ATP (final concentration 3.0 mol·m^{-3}) or Na_4PPi (final concentration 500 mmol·m^{-3}) to the reaction media. For assays of ATP-dependent H^+ transport, the reaction medium (volume 500·10^{-9} m^3) contained approx. 20–40 µg protein, 3.0 mmol·m^{-3} quinacrine, 6.0 mol·m^{-3} $MgSO_4$, 0.3 mol·m^{-3} disodium ethylenediaminetetraacetate (EDTA), 150 mol·m^{-3} mannitol and 25 mol·m^{-3} BTP buffered to pH 8.0 with Mes. For measurements of PPi-dependent H^+ transport, the reaction medium was identical except that the $MgSO_4$ concentration was increased to 7.5 mol·m^{-3} and the medium also contained 100 mol·m^{-3} K-Mes. Further anions were added from stock solutions of the free acids buffered to pH 8.0 with BTP. Fluorescence quenching was measured using a model LS-5B luminescence spectrometer (Perkin-Elmer, Beaconsfield, Bucks., UK) with excitation at 427 nm and emission at 495 nm, both with a slit width of 5 nm.

Membrane-potential measurements. The membrane-potential difference (Δψ: inside positive) across the tonoplast was determined from the fluorescence quenching of bis-(3-phenyl-5-oxo-isoxazol-4-yl)pentamethine oxonol (oxonol V) according to the method of Scherman and Henry (1980), with excitation at 585 nm and emission at 645 nm, both with a slit width of 5 nm. Aliquots of tonoplast preparation (approx. 60 g protein·m^{-3}) were equilibrated in a reaction medium identical to that used for measurement of Δ pH (as above), except that quinacrine was replaced by 4 mmol·m^{-3} oxonol V. Membrane-potential changes were initiated by the addition of either Tris-ATP (final concentration 3.0 mol·m^{-3}) or Na_4PPi (final concentration 500 mmol·m^{-3}). When a steady-state Δψ was achieved, BTP-anions were added from stock solutions prepared in an identical reaction medium 'spiked' with equivalent amounts of membrane protein and either Tris-ATP or Na_4PPi to prevent changes in fluorescence caused by dilution.

Protein determination. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Sources of chemicals. All chemicals were of standard analytical grade. Oxonol V was obtained from Molecular Probes Inc. (Eugene, Ore., USA). DL-isocitric and L-malic acids were obtained from BDH (Poole, Dorset, UK). Butylmalonic, citraconic, citramalic, itaconic, mesaconic, oxalacetic and phenylsuccinic acids were obtained from Aldrich Chemical Co. (Gillingham, Dorset, UK). Quinacrine, Na_4PPi, Tris-ATP, gramicidin D and the other organic acids were obtained from Sigma Chemical Co. (Poole, Dorset, UK).

Results

Characteristics of tonoplast vesicles. Bremberger et al. (1988) have shown that a tonoplast fraction can be obtained from leaf mesophyll homogenates of *Kalanchoë daigremontiana* by a combination of differential and sucrose density gradient centrifugation. The ATPase activity of this fraction is sensitive to nitrate but insensitive to azide and vanadate (Bremberger et al. 1988), characteristics specific to the tonoplast ATPase. In the present experiments, we found that this tonoplast fraction contained transport-competent vesicles, with the addition of either Mg-ATP or Mg-PPi to the medium resulting in electrogenic H^+ transport into the vesicles. Quinacrine-fluorescence quenching, observed in the presence of permeant anions, indicated acidification of the vesicular lumen (Fig. 1 A, C), while oxonol-V-fluorescence quenching, in the absence of permeant anions, indicated the generation of an inside-positive Δψ across the vesicle membrane (Fig. 1 B, D).

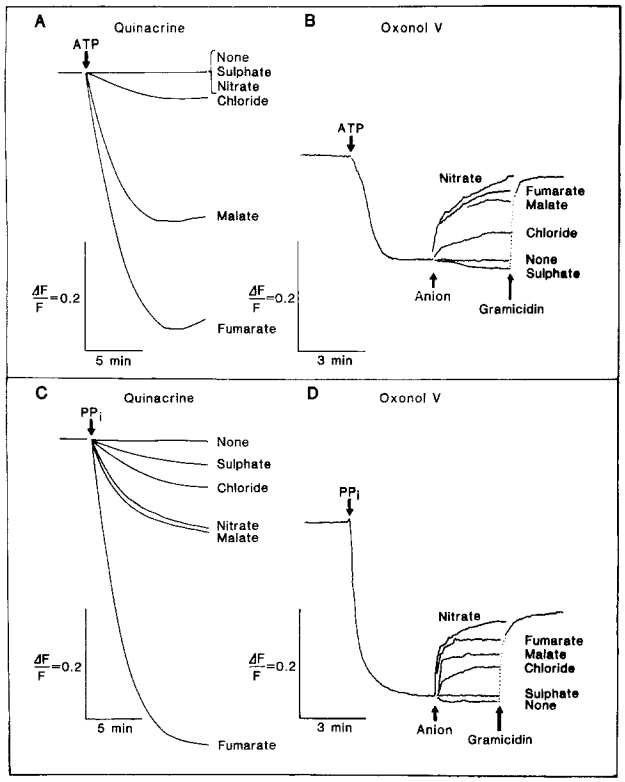

Fig. 1 A–D. Effect of anions on electrogenic ATP- and PPi-dependent proton transport in tonoplast vesicles of *Kalanchoë daigremontiana.* Vesicle acidification on addition of substrate (**A**, **B**: 3.0 mol·m^{-3} ATP; **C**, **D**: 0.5 mol·m^{-3} PPi) was monitored by the quenching of quinacrine fluorescence (**A**, **C**), and generation of an inside-positive Δψ by the quenching of oxonol V (**B**, **D**). For ATPase assays, anions were supplied as 50 mol·m^{-3} BTP-salts; for PPiase assays, anions were supplied as 50 mol m^{-3} K-salts. Gramicidin D was added to a final concentration of 1.6 mmol·m^{-3} as indicated. F=fluorescence intensity (arbitrary units)

Initial rates of quinacrine-fluorescence quenching, which are a measure of the initial rates of vesicle acidification (Bennett and Spanswick 1983), were greatly dependent upon the nature of the anion present. Malate supported higher rates of vesi-

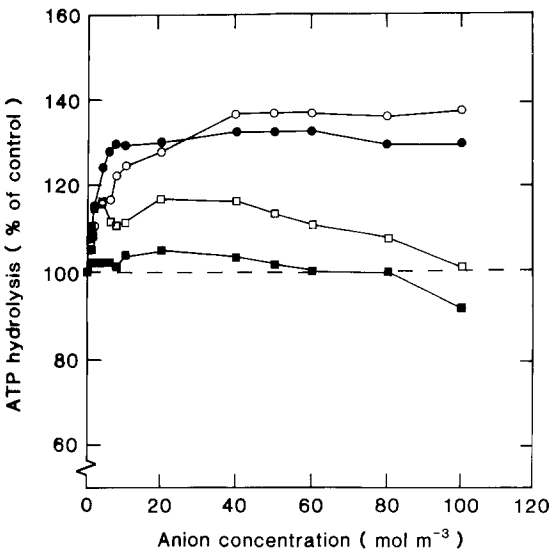

Fig. 2. Dependence of ATP hydrolysis in tonoplast vesicles of *Kalanchoë* on chloride (●, ○) and malate (■, □) concentration in the presence (●, ■) and absence (○, □) of 1.6 mmol·m^{-3} gramicidin D. Rates are expressed as a percentage of those observed in the absence of added anions, which were 23.0 μmol·h^{-1}·(mg protein)$^{-1}$ in the presence and 17.8 μmol·h^{-1}·(mg protein)$^{-1}$ in the absence of gramicidin

cle acidification than chloride, as observed previously in tonoplast vesicles from *K. daigremontiana* (Jochem and Lüttge 1987), but even higher rates were observed in the presence of another four-carbon dicarboxylate, fumarate (Fig. 1 A, C). Sulphate supported only low rates of vesicle acidification, whereas nitrate, although having a direct inhibitory effect on the ATPase, supported a high rate of PPi-dependent vesicle acidification. Addition of anions to vesicles already energized by Mg-ATP or Mg-PPi dissipated the inside-positive $\Delta\psi$ with a similar order of effectiveness, namely fumarate > malate > chloride > sulphate (Fig. 1 B, D). Nitrate, however, dissipated $\Delta\psi$ to a greater extent than expected from its effect on pH formation by the PPiase (Fig. 1 C, D).

Direct effects of anions on ATP and PPi hydrolysis. To investigate further the transport characteristics of tonoplast vesicles, it is important to separate direct effects of ions on the H^+-translocating enzymes from indirect effects resulting from their transport. These can be distinguished using the H^+-conducting ionophore gramicidin, which abolishes the proton-motive force across the tonoplast and allows direct effects of anions on the ATPase and PPiase to be observed (Bennett and Spanswick 1983; Sze 1985; Griffith et al. 1986).

To compare the effects of inorganic and organic-acid anions on the tonoplast ATPase, ATP hydrolysis was assayed with or without gramicidin in the presence of chloride or malate (Fig. 2). In the absence of gramicidin, both chloride and malate stimulated ATP hydrolysis (by a maximum of 38% and 17%, respectively), as observed by Jochem et al. (1984) and Jochem and Lüttge (1987). Gramicidin reduced the chloride concentration required for half-maximal stimulation of ATP hydrolysis from approx. 6 to 2 mol·m^{-3}, but had little effect on the maximal percentage stimulation of ATP hydrolysis. However, gramicidin abolished the stimulation of ATP hydrolysis by malate (Fig. 2), indicating that there was no direct action of malate on the ATPase.

Nitrate had a direct inhibitory effect on both ATP hydrolysis and ATP-dependent H^+ transport in the tonoplast fraction. In the presence of gramicidin, ATP hydrolysis was reduced to 30% of its control value by 50 mol·m^{-3} nitrate. The ATP-dependent transport of H^+, with either chloride or malate as the permeant anion, was more sensitive to nitrate, being half-maximally inhibited by 0.5 mol·m^{-3} nitrate and completely abolished by 50 mol·m^{-3} nitrate (data not shown). This result indicates that all ATP-dependent H^+ transport in this fraction was energized by the tonoplast ATPase.

As observed in other studies (Rea and Poole 1985; Kaestner and Sze 1987; Pope and Leigh 1987), PPi hydrolysis assayed in the presence of gramicidin was insensitive to chloride, malate and nitrate (data not shown).

Effects of organic-acid anions on ATP- and PPi-dependent H^+ transport. The fact that malate supports high rates of ATP- and PPi-dependent H^+ transport (Fig. 1) appears to be a property specific to tonoplast vesicles from CAM plants (Jochem and Lüttge 1987; Marquardt and Lüttge 1987; Struve and Lüttge 1987). Since even higher rates of H^+ transport were observed in the presence of fumarate (Fig. 1), we investigated the specificity of this effect further with respect to organic-acid anion structure.

Some organic-acid anions complex significant amounts of Mg^{2+} (Martell and Smith 1977), so optimal conditions for the assay of ATPase and PPiase activities were first determined by varying total Mg concentration at fixed concentrations of ATP (3.0 mol·m^{-3}) or PPi (0.5 mol·m^{-3}). Both ATPase and PPiase activities exhibited saturation-type curves with increasing total Mg concentrations (data not shown) and neither activity was significantly reduced at excess total Mg concentrations, contrary to reports for the PPiase in other

Table 1. Effect of organic-acid anions (present as their BTP-salts at 50 mol·m^{-3}) and their stereoisomers on ATP and PPi hydrolysis, assayed in the presence of 1.6 mmol·m^{-3} gramicidin D, and on ATP- and PPi-dependent proton transport, measured as initial rates of quinacrine-fluorescence quenching, in tonoplast vesicles of *Kalanchoë*. Results are expressed relative to hydrolytic activities measured in the absence of added anions and to rates of proton transport measured in the presence of L-malate (100%), and are given as means ±SE (number of experiments). Specific activities (100%) for ATP and PPi hydrolysis averaged 13.5 and 36.1 μmol·h^{-1}·(mg protein)$^{-1}$, respectively, and for ATP- and PPi-dependent quinacrine-fluorescence quenching averaged 483 and 314%·min^{-1}·(mg protein)$^{-1}$, respectively

Anion	ATPase		PPiase	
	Hydrolytic activity	Proton transport	Hydrolytic activity	Proton transport
None	100	12±1 (15)	100	7±2 (8)
L-Malate	94±4 (13)	100	96±4 (10)	100
A. *Monocarboxylates*				
L-Lactate	85±4 (6)	6±2 (3)	97± 4 (3)	8±1 (3)
Pyruvate	NA	3±0 (3)	NA	5±2 (3)
n-Butyrate	116±6 (4)	5±2 (3)	98± 4 (4)	3±2 (3)
Valerate	102±6 (4)	8±3 (3)	98± 2 (4)	4±2 (3)
B. *Dicarboxylates*				
Oxalate	NA	14±6 (4)	NA	10±5 (4)
Malonate	88±3 (6)	17±4 (4)	92± 9 (6)	21±5 (4)
Tartronate	NA	9±1 (3)	NA	12±5 (3)
Fumarate	114±7 (7)	257±22 (35)	100± 5 (6)	209±21 (16)
Succinate	105±1 (5)	116±13 (4)	112± 7 (3)	75±13 (5)
D-Malate	100±12 (5)	77±10 (10)	83± 6 (6)	88±7 (8)
Oxalacetate	NA	60±22 (4)	NA	105±10 (4)
L-Aspartate	103±3 (4)	11±3 (3)	125± 1 (3)	10±4 (3)
L-Tartrate	NA	60±2 (3)	NA	123±15 (8)
D-Tartrate	NA	59±17 (4)	NA	81±6 (3)
L-Glutarate	105±11 (4)	42±8 (3)	94± 2 (4)	21±5 (6)
2-Oxoglutarate	93±4 (4)	44±3 (4)	60± 6 (6)	83±5 (6)
L-Glutamate	118±8 (4)	17±6 (5)	123± 5 (3)	13±4 (4)
Adipate	93±7 (4)	3±1 (3)	92± 3 (4)	4±2 (3)
C. *Tricarboxylates*				
DL-Isocitrate	95±11 (4)	10±5 (3)	78± 6 (6)	6±1 (3)

NA = Not assayed

species (Chanson et al. 1985; Wang et al. 1986; Pope and Leigh 1987). Thus, in the present experiments, assays could be performed with a sufficient concentration of total Mg (6.0 mol·m^{-3} for the ATPase and 7.5 mol·m^{-3} for the PPiase) to prevent significant substrate depletion via complexation of Mg^{2+} by organic-acid anions.

The majority of organic-acid anions tested had no significant effect on either ATP or PPi hydrolysis in the presence of gramicidin, but rates of H^+ transport were strongly dependent on the nature of the anion present (Table 1). For several anions, rates of H^+ transport differed somewhat between the ATP- and PPi-dependent assays, perhaps on account of the K-Mes present in the PPiase assays. Nevertheless it was clear that only a limited number of organic-acid anions supported rates of vesicle acidification significantly above background, their approximate order of effectiveness being fumarate ≫ malate ≃ succinate > oxalacetate ≃ tartrate > 2-oxoglutarate > glutarate. Thus 1,4-dicarboxylates appeared to be most effective in supporting H^+ transport, with 1,5-dicarboxylates less so. Comparison of L-malate with its stereoisomer D-malate, and of L-tartrate with D-tartrate, indicated that the anion-stimulation of H^+ transport was not markedly stereospecific (Table 1). None of the monocarboxylates tested supported H^+ transport, and neither did shorter-chain (oxalate, malonate) nor longer-chain (adipate) dicarboxylates. Also, no H^+ transport was observed in the presence of the dicarboxylic amino-acid anions aspartate and glutamate or the free tricarboxylate isocitrate.

The dependence of H^+ transport on anion concentration showed saturation kinetics for both chloride and the 1,4-dicarboxylates fumarate, malate and succinate (Fig. 3). Half-maximal stimula-

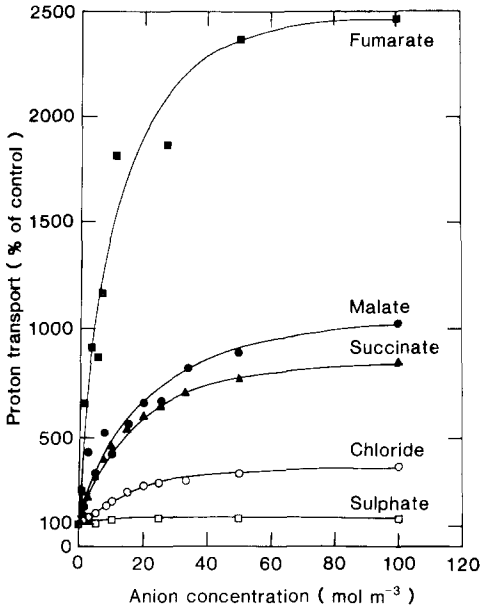

Fig. 3. Effect of fumarate, malate, succinate, chloride and sulphate concentration on the initial rate of ATP-dependent proton transport in tonoplast vesicles of *Kalanchoë*. Proton transport is expressed relative to the rate observed in the absence of added anions (100%). Data are from one experiment, representative of a total of five, in which the average concentrations for half-maximal stimulation of proton transport were as follows: fumarate, 12 mol·m^{-3}; malate, 14 mol·m^{-3}; succinate, 14 mol·m^{-3}; chloride, 17 mol·m^{-3}

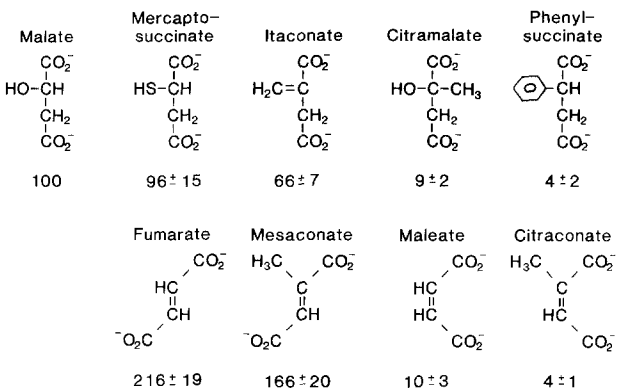

Fig. 4. Effects of several non-naturally occurring organic-acid anions (50 mol·m^{-3}) on initial rates of PPi-dependent proton transport in tonoplast vesicles of *Kalanchoë*. Rates are represented by *numbers* under the structures of the individual anions and expressed as percentages of those observed in the presence of 50 mol·m^{-3} L-malate. Values are means ±SE from five experiments. In the absence of added anions, initial rates of proton transport averaged 7±2% of those in the presence of L-malate

tion of H^+ transport occurred at a chloride concentration of 17 mol·m^{-3}, significantly higher than that for chloride stimulation of ATP hydrolysis (compare Fig. 2); for the dicarboxylates, half-maximal stimulation occurred at concentrations between 12 and 14 mol·m^{-3} (Fig. 3). The malate concentration giving half-maximal stimulation of ATP-dependent H^+ transport (14.0±1.2 mol·m^{-3}) did not differ significantly from that for malate stimulation of PPi-dependent H^+ transport (11.7±2.7 mol·m^{-3}, both means ±SE from five experiments), consistent with this stimulation being the result of malate transport into the vesicles.

The specificity of dicarboxylate stimulation of H^+ transport was further studied by comparing a range of non-naturally occurring 1,4-dicarboxylates with malate and fumarate (Fig. 4). Mercaptosuccinate supported similar rates of PPi-dependent H^+ transport to malate. Itaconate, however, supported lower rates of H^+ transport than malate, and citramalate and phenylsuccinate did not stimulate vesicle acidification, indicating that steric factors are important in determining rates of dicarboxylate transport. Whereas fumarate supported rates of H^+ transport approximately double those of malate, its *cis* geometric isomer, maleate, did not support H^+ transport, implying that a *trans* conformation is essential for 1,4-dicarboxylate transport. Similarly, mesaconate (2-methylfumarate) supported high rates of H^+ transport (although significantly less than fumarate), whereas citraconate (2-methylmaleate) was ineffective.

Evidence for discrete chloride and malate transport mechanisms. From the current evidence, it is not clear whether chloride and dicarboxylate anions utilize the same transport pathway across the tonoplast. If separate pathways exist, it may be possible to identify anions that selectively inhibit either chloride or malate transport. To test for such effects, anions were selected that were non-permeant, and consequently would not compete with chloride or malate by dissipating the driving force for anion transport. Non-permeant anions were added at 50 mol·m^{-3} to a medium containing either 10 mol·m^{-3} chloride or 10 mol·m^{-3} malate, concentrations close to those giving half-maximal stimulation of H^+ transport (compare Fig. 3). Several competing anions reduced rates of H^+ transport observed both in the presence of chloride and of malate (Table 2). However, two anions, maleate and tartronate, inhibited H^+ transport in the presence of malate but not of chloride. The inhibition by maleate showed competitive kinetics (Fig. 5): the malate concentration for half-maximal stimulation of H^+ transport increased progressively from 11 mol·m^{-3} in the absence of maleate to 19 mol·m^{-3} in the presence of 40 mol·m^{-3} maleate, whereas the V_{max} for H^+ transport was unaffected.

Table 2. Effect of non-permeant, competing anions at 50 mol·m^{-3} on the initial rates of PPi-dependent proton transport in tonoplast vesicles of *Kalanchoë* in the presence of either 10 mol·m^{-3} chloride or 10 mol·m^{-3} malate. All anions were added as their BTP-salts. Values are means ±SE (three experiments) and are expressed as percentages of the rates observed in the presence of malate and chloride alone

Competing anion (50 mol·m^{-3})	Initial rate of PPi-dependent H^+ transport (%)	
	Chloride (10 mol·m^{-3})	Malate (10 mol·m^{-3})
None	100	100
Sulphate	148±16	135±14
Maleate	107±15	63±4
Tartronate	147±1	27±3
Butylmalonate	60±2	35±3
Phenylsuccinate	42±4	24±4

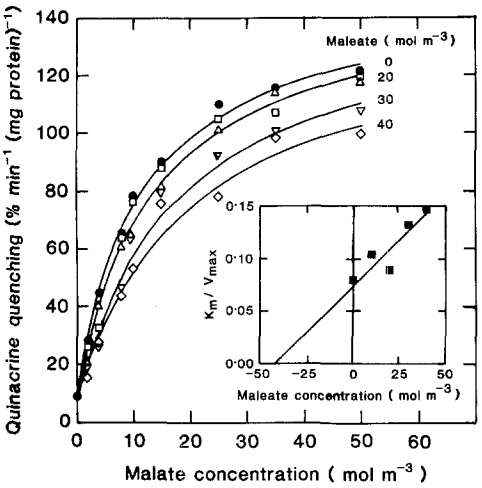

Fig. 5. Effect of maleate concentration on the initial rate of PPi-dependent quinacrine-fluorescence quenching by tonoplast vesicles of *Kalanchoë* in the presence of L-malate. Maleate concentrations were 0 (●), 10 (□), 20 (△), 30 (▽) and 40 (◇) mol·m^{-3}. Curves were fitted by nonlinear regression analysis using the computer program Multifit (Walmsley and Lowe 1986); for clarity, no curve is shown for 10 mol·m^{-3} maleate. Maximal quinacrine-fluorescence quenching (V_{max}) averaged 139±2%·min^{-1}·(mg protein)$^{-1}$ (mean ±SE, five estimates). *Inset* is a plot of K_m/V_{max} for the malate stimulation of PPi-dependent quinacrine-fluorescence quenching against maleate concentration, from which the K_i (maleate) was calculated by extrapolation to the *abscissa*

The K_i for maleate inhibition of H^+ transport in the presence of malate was approx. 42 mol·m^{-3} (Fig. 5, insert).

Discussion

Primary ATP- and PPi-dependent H^+ transport into tonoplast vesicles has been used in this study to investigate the characteristics of secondary anion transport at the tonoplast. Permeant, transportable anions should be very effective in charge compensation, and will consequently minimize the transmembrane $\Delta\psi$ but maximize the Δ pH generated by the primary H^+-translocating enzymes (Fig. 1). However, to use this approach in studying anion transport, indirect effects on H^+ transport such as these must be clearly distinguished from any direct effects of anions on the ATPase or PPiase.

Consistent with the findings for other species (Sze 1985; Rea and Sanders 1987), the tonoplast ATPase of *Kalanchoë daigremontiana* mesophyll cells was directly stimulated by chloride (Fig. 2) and inhibited by nitrate, whereas the PPiase was anion-insensitive (see also Jochem and Lüttge 1987; Marquardt and Lüttge 1987). Jochem and Lüttge (1987) have suggested that malate may interact directly with the tonoplast ATPase of *K. daigremontiana*, based on an apparent increase in the sensitivity of the tonoplast ATPase to nitrate and changes in the substrate dependence of the ATPase in the presence of malate. In the present experiments, malate stimulated ATP hydrolysis in the absence of the H^+-conducting ionophore gramicidin, but apparently only by acting as a permeant anion, since this effect was abolished by gramicidin (Fig. 2). Indeed, the hydrolytic activities of the ATPase and PPiase were little affected by a wide range of organic-acid anions (Table 1). We therefore conclude that the tonoplast ATPase of *K. daigremontiana* is sensitive to chloride and nitrate but not to organic-acid anions.

In assays of ATP- and PPi-dependent H^+ transport, certain organic-acid anions such as malate and fumarate supported considerably higher rates of vesicle acidification than chloride (Fig. 1A, C). With the exception of nitrate, these anions also dissipated a pre-existing inside-positive $\Delta\psi$ with an equivalent order of effectiveness (Fig. 1B, D), indicating that the effects on Δ pH formation were the result of electrophoretic anion transport into the vesicles. The cause of the anomalous nitrate effect is not clear at present, but might be some form of H^+-coupled nitrate transport at the tonoplast such as NO_3^-/H^+ antiport (compare Schumaker and Sze 1987). For the other anions tested, the rates of vesicle acidification could be taken to reflect the relative permeability of the tonoplast to those anions. In a similar way, acidification of endomembrane vesicles by the vacuolar-type H^+-ATPase of animal cells is constrained by the nature of the anions available for charge compensation (for a review see Mellman et al. 1986; Van Dyke 1988).

From the wide range of organic-acid anions tested, the malate-influx system at the tonoplast of *K. daigremontiana* appears to be specific for four-carbon and five-carbon dicarboxylates (Table 1). Monocarboxylates did not support vesicle acidification, and neither did shorter-chain nor longer-chain dicarboxylates. The transport system did not distinguish between stereoisomers (Table 1), as noted for D- and L-malate by Jochem and Lüttge (1987), but appeared to require the *trans*-carboxyl geometric configuration (Fig. 4). Fumarate, which is fixed by the carbon-carbon double bond in the *trans* configuration, was transported approximately twice as rapidly as malate and succinate, which are free to adopt either *cis* or *trans* configurations, and maleate, the *cis* isomer of fumarate, was not transported.

Investigating the effects of substitutions in the four-carbon dicarboxylates showed that steric factors were important in determining rates of anion transport (Table 1, Fig. 4). Several substitutions appeared not to impair transport, such as mercapto substitution (2-mercaptosuccinate), keto substitution (oxalacetate), and 2,3-hydroxyl substitution (tartrate). However, the presence of a methylene group, as in itaconate, significantly reduced anion transport, and the methyl group in citramalate (2-methylmalate) completely prevented transport. The presence of a methyl group also reduced transport in the unsaturated, *trans* dicarboxylate mesaconate (methylfumarate) compared with fumarate. Interestingly, five-carbon dicarboxylates were also transported (Table 1), although at a reduced rate compared with malate. In the case of 2-oxoglutarate, the presence of the carbonyl group at the C-2 position may render the molecule sufficiently similar to the four-carbon dicarboxylates to permit transport.

The stimulation of Δ pH formation saturated with increasing anion concentration for both inorganic and organic-acid anions (Figs. 3, 5), indicating that anion transport was mediated by proteinaceous porters. Half-maximal stimulation of ATP-dependent H^+ transport occurred at a relatively high chloride concentration (17 $mol \cdot m^{-3}$), similar to values found in other studies using this technique (Bennett and Spanswick 1983; Churchill and Sze 1983; Lew and Spanswick 1985; Giannini and Briskin 1987; Jochem and Lüttge 1987; Pope and Leigh 1987). These values also agree with those observed in direct studies of $^{36}Cl^-$ uptake (Hager and Biber 1984; Hager et al. 1986; Martinoia et al. 1986), as well as those obtained using the chloride-sensitive fluorescent probe 6-methoxy-1-(3-sulfonatopropyl)quinolinium (Pope and Leigh 1988). Values for the half-maximal stimulation of H^+ transport by four-carbon dicarboxylates were of the same order (12–14 $mol \cdot m^{-3}$), as observed for malate in *K. daigremontiana* by Jochem and Lüttge (1987). Corresponding values for [^{14}C]malate uptake into intact vacuoles of *K. daigremontiana* (Buser-Suter et al. 1982; Nishida and Tominaga 1987), as well as *Hordeum vulgare* (Martinoia et al. 1985) and *Catharanthus roseus* (Marigo et al. 1988), were almost an order of magnitude lower, but these apparent kinetic differences might be caused partly by the different transport characteristics of intact vacuoles and tonoplast vesicles (Guern et al. 1989).

In view of the similar anion-concentration-dependence of H^+ transport on chloride and malate, the question arises whether these anions utilize the same or different transport pathways across the tonoplast. Evidence from patch-clamp recordings indicates that some tonoplast ion channels can conduct both chloride and malate (for a review see Hedrich and Schroeder 1989), but other studies with tonoplast vesicles isolated from C_3 (*Avena sativa*) and C_4 (*Zea mays*) species have indicated that malate is a relatively impermeant anion compared with chloride (Mettler et al. 1982; Kaestner and Sze 1987; Pope and Leigh 1987). High rates of H^+ transport in the presence of malate seem to be a property of tonoplast vesicles from CAM plants (Jochem and Lüttge 1987; Marquardt and Lüttge 1987). Indeed, induction of CAM in *Mesembryanthemum crystallinum* by salt stress appears to be associated with an increase in the permeability of the tonoplast to malate (Struve and Lüttge 1987). Possibly, therefore, malate transport may be associated with a tonoplast protein distinct from the chloride transporter. The present experiments showed that malate transport can be competitively inhibited by non-permeant organic-acid anions, such as maleate and tartronate, that are without effect on chloride transport (Table 2, Fig. 5). Hager et al. (1986) also noted that the enhancement of ATP-dependent H^+ transport in barley tonoplast vesicles by chloride and succinate is additive. Thus, there is some evidence for distinct transport pathways for chloride and dicarboxylates, but more direct studies to attempt to identify these anion transporters are clearly required.

Based on the present transport experiments, the tonoplast malate-influx system in CAM plants appears to have quite different characteristics from other known carboxylate transporters. For example, malate transport in mitochondria and chloroplasts is generally thought to be mediated by high-affinity, electroneutral exchange systems (for a re-

view see LaNoue and Schoolwerth 1979; Heldt and Flügge 1987), whereas malate transport at the tonoplast appears to occur by a low-affinity, electrophoretic mechanism. However, recent experiments indicate that malate uniport may also be an important mechanism of anion transport in plant mitochondria (Zoglowek et al. 1988) and in the peribacterioid membrane of soybean nodules infected with *Bradyrhizobium japonicum* (Udvardi et al. 1988). Assuming that the dicarboxylates which are transported across the tonoplast all utilize the same transport system, the putative tonoplast malate transporter would also appear to share similarities in its substrate specificity with several other carboxylate-transport systems. For example, the 2-oxoglutarate/malate exchanger of chloroplasts is specific for 2-oxoglutarate, malate, succinate, fumarate and glutarate (Woo et al. 1987), and the electrogenic dicarboxylate transport system of the bacterial plasma membrane can transport succinate, fumarate, malate and tartrate (Kay et al. 1987). Thus, further studies would be useful both to characterize the membrane components associated with malate influx into the vacuole and to clarify the relationship of the tonoplast malate transporter in CAM plants to other carboxylate-transport systems.

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