Malate and malate-channel antibodies inhibit electrogenic and ATP-dependent citrate transport across the tonoplast of citrus juice cells

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Summary

Citrus juice cells accumulate high levels of citric acid in their vacuoles when compared to other organic ions including malate. Uptake of citrate into tonoplast vesicles from Citrus juice cells was investigated in the presence of malate, and after incubation with antibodies raised against the vacuolar malate-specific channel of Kalanchoë diagremontiana leaves. Antibodies against the vacuolar malate channel immunoreacted with a protein of similar size in tonoplast extracts from three Citrus varieties differing in citric acid content. Malate channel antibodies inhibited both ΔµH⁺-dependent and ΔµH⁺-independent ATP-dependent citrate transport, indicating common domains in both transport systems and to the malate-specific channel of Kalanchoë diagremontiana leaves. Malate strongly inhibited electrogenic citrate transport, whereas ATP-dependent citrate uptake was less affected. Kinetic analysis of citrate transport in the presence of malate confirmed the existence of two citrate transport mechanisms and indicated that both citrate and malate share a common transport channel across the tonoplast of Citrus juice cells.

Key words: Citrus – tonoplast transport – vacuolar pH

Abbreviations: ΔµH⁺ = electrochemical potential. – MOPS = 3-(N-morpholino)propanesulfonic acid. – MES = 2-(N-morpholino)ethanesulfonic acid. – BTP = bis-tris propane. – DTT = dithiothreitol

Introduction

Several mechanisms have been proposed to describe the transport of citrate into the vacuole of plant cells (Brune et al. 1998, Canel et al. 1995, Marin and Chrestin 1985, Marin et al. 1981, Oleski et al. 1987, Rentsch and Martinoia 1991). Whereas some of these transport systems directly involve a component of the ΔµH⁺, a ΔµH⁺-independent ATP-dependent citrate pump has been described for citrus juice cells (Brune et al. 1998, Canel et al. 1995). In experiments using tonoplast vesicles from a low-acid citrus variety (Canel et al. 1995) as well as with the highly acidic 'Persian lime' (Citrus aurantifolia; Brune et al. 1998), citrate uptake was stimulated by ATP in the absence of a ΔµH⁺. For low acid varieties such as 'pummelo 2240' and 'Sweet lime' (Citrus limettioides), with trans-tonoplast pH gradients of approximately 1.5 units
(vacuolar pH of 5.5), uptake of citrate energized by the ATP-citrate pump or driven by the $\Delta\psi$ of $\approx$25 mV would be sufficient to account for the low concentrations found in vivo (Brune et al. 1998). However, the efficacy of the ATP-dependent citrate pump in acidic citrus varieties has never been investigated given the difficulty in establishing artificial trans-ent citrate pump in acidic citrus varieties has never been investigated. Nonetheless, given the difficulty in establishing artificial trans-ent citrate pump in acidic citrus varieties has never been investigated, the efficacy of the ATP-dependent citrate pump or driven by the $\Delta\psi$ of $\approx$25 mV would be sufficient to account for the low concentrations found in vivo in the vacuole of Citrus juice cells.

Materials and Methods

Plant material

-Acid- limes {Citrus aurantifolia}, -Sweet- limes {Citrus imiticioides} and -Valencia- orange {Citrus sinensis L.} were collected from the orchards located at the Citrus Research and Education Center in Lake Alfred, FL. Fruit were taken to the lab and immediately used for tonoplast isolation.

Tonoplast isolation

For the isolation of tonoplast vesicles from acid lime, sweet lime and orange juice cells, we followed the procedure of Müller et al. (1996) with few modifications. Approximately 150 mL of juice cell extract was squeezed directly into 100 mL homogenization buffer (Müller et al. 1996). For acid limes, the buffer concentration was increased to 2mol/L MOPS/KOH (pH 8.0) to neutralize the increase in acidity. The juice was directly added to the stirring solution to keep exposure of the membranes to the low pH as short as possible. The homogenate was filtered through 200 µm pore nylon mesh and centrifuged at 7,000g for 10 min. The supernatant was further centrifuged for 2 h at 100,000g. The resulting pellet was resuspended in resuspension buffer (Müller et al. 1996), layered on an 8, 17, 26 % sucrose gradient and centrifuged for 90 min at 100,000g. Membrane vesicles at the 8/17 and 17/26 sucrose interfaces were collected, pelleted at 100,000g for 30 min and resuspended in 1 mL of storage buffer containing 50 mmol/L MES/BTP (pH 7.5), 1 mmol/L DTT, 1 mmol/L EDTA, 20 mmol/L KCl. Vesicles were stored in aliquots at $-80^\circ$C until needed. All isolation procedures were conducted at 4°C.

Sodium-dodecyl sulfate gel electrophoresis and western blotting

Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels containing 12.5 % acrylamide and by using the Laemmli buffer system (Laemmli 1970). All lanes contained 10 µg of membrane protein. Proteins were transferred from SDS-PAGs to a nitrocellulose membrane (0.2 µm) following the western-blot method of Towbin et al. (1979) in a semi-dry transblot chamber (BioRad). Immunostaining was performed using alkaline phosphatase-coupled secondary goat-anti-rabbit IgG-antibodies (Sigma, St. Louis, MO). Primary antiserum used was a specific polyclonal anti-serum against a fraction of Triton X-100 solubilized and hydroxyapatite-purified from tonoplast proteins of K. diagremontiana, which was active in malate transport when reconstituted in liposomes (anti-HA; Ratajczak et al. 1994, Steiger et al. 1997), and an affinity-purified anti-serum against a 32-kDa peptide from this hydroxyapatite fraction (anti-32kDa, Lüttege et al. 2000).

Citrate uptake into tonoplast vesicles

Uptake with tonoplast vesicles were performed using a vacuum separation method at 625 mm Hg constant vacuum. Stock solutions of $[^{14}C]$citric acid (Sigma, St. Louis, MO) were prepared at a radiolabel concentration of 25 µCi/µmol. Tonoplast vesicles (24 µg membrane protein) were incubated at 30°C in a solution containing 50 mmol/L HEPES/BTP/KOH (pH 7.5), 25 mmol/L KCl, 1 mmol/L DTT, 0.01 mg/mL BSA, ATP 3 mmol/L, 0.5 mmol/L MgSO$_4$ and 10 µmol/L gramicidin were added as required. For uptake experiments in the presence of antibodies against malate transporter we used a total of 488 µg of antibody protein (anti-HA) and 24 µg of total tonoplast protein. Control experiments were run with Ig at the same concentration of protein (488 µg) against 24 µg of tonoplast protein.

Uptake was started by addition of $[^{14}C]$citrate to a final concentration of 100 µmol/L and stopped at indicated times by pipetting 100 µL aliquots onto pre-rinsed cellulose nitrate filters (pore size 0.22 µm, 25 mm in diameter; Whatman Int. Ltd., Maidstone, UK). After vacuum was applied, the vesicles were washed with 5 mL storage buffer at pH 7.5.

Radioactivity retained in the vesicles was determined by scintillation spectroscopy after immersing the filter discs in 5 mL of Scintiverse BD SX 18-4 (Fisher Scientific, Pittsburgh, PA).

Protein determination

Protein was determined by the Coomassie blue protein assay (Bradford 1976) using bovine plasma gamma globulin as a standard.

Results and Discussion

Citrus fruits accumulate high levels of citric acid often reaching concentrations above 350 mmol/L (Sinclair 1984). On the contrary, malate constitutes only a small fraction of the total organic acids, rarely exceeding 10 % of the total organic acid pool. Previous studies have indicated that transport of citrate into the vacuole of Citrus juice cells occurs by way of two distinct mechanisms. One system is driven by the proton motive force ($\Delta\mu^H+$), whereas the other is a $\Delta\mu^H+$-independent, ATP-dependent citrate carrier (Brune et al. 1998, Canel et al. 1995). Neither system has been characterized in terms of specificity towards other organic ions such as malate, nor their similarity to the malate channel of leaf vacuoles has
been investigated. According to literature reports, malate \(^{-2}\) and citrate \(^{-3}\) share a common transport system (Oleski et al. 1987, Rentsch and Martinoia 1991), a situation that can be further elucidated in the high citrate accumulating cells of Citrus fruits.

Tonoplast protein samples from juice cells of three distinctive Citrus cultivars cross-reacted with antibodies (anti-32 kDa; Lüttge et al. 2000) raised against the highly selective vacuolar malate transporter of Kalanchoë diagremontiana leaves (Hafke et al. 2003; Fig. 1). The selected cultivars encompass the range of vacuolar citric acid content and corresponding pH’s found in Citrus fruits. ‘Acid lime’, with the lowest vacuolar pH of approximately 2.0 (Echeverría and Burns 1989, Echeverría et al. 1992), accumulates citric acid to concentrations of over 350 mmol/L, whereas ‘Sweet lime’ accumulates no more than 0.2 mmol/L citric acid while maintaining a vacuolar pH around 5.5 (Echeverría and Burns 1989). Juice cells from ‘Valencia orange’ accumulate citric acid to concentrations of over 350 mmol/L, whereas ‘Sweet lime’ accumulates no more than 0.2 mmol/L citric acid while maintaining a vacuolar pH around 5.5 (Echeverría and Burns 1989). Juice cells from ‘Valencia orange’ are somewhat intermediate, storing roughly 50 mmol/L citric acid with a vacuolar pH of nearly 3.0 (Echeverría and Burns 1989). In all three samples, antibodies cross-reacted with a protein band of \(\approx 33\) kDa (Fig. 1). Interestingly, based on equal amount of tonoplast protein, band densities were comparable in all three cultivars despite the enormous differences in vacuolar citric acid content and virtual absence of malic acid. The labeled protein was similar in weight to the 32 kDa malate channel of Kalanchoë, and slightly larger than the 27 kDa protein identified as the malate transporter in barley tonoplast (Rentisch and Martinoia 1991). A band of \(\approx 62\) kDa was also observed (data not shown), most likely representing aggregates of the same protein. Therefore, although malate constitutes only a small fraction of the organic acids accumulated by Citrus fruits, an analogous protein to the malate channel of Kalanchoë diagremontiana appears to be present in the tonoplast of Citrus juice cells. Since acid lime contains the highest levels of citric acid, further characterization of the transport mechanisms was performed using tonoplast from acid lime juice cells. Figure 2 shows the increasing immunoreactivity to anti-32 kDa antibodies with increased tonoplast purity in the sucrose gradient (Brune et al. 2002). This denotes an enrichment of the 32 kDa protein in tonoplast samples as compared to other endomembrane systems.

To examine the possibility of a mutual channel for citrate and malate at the tonoplast of Citrus juice cells, we carried out competition experiments with malate. Figure 3 shows a decline in \(^{14}\)Ccitric acid accumulation into tonoplast vesicles with increasing malate concentrations. At 0.1 mmol/L citrate and 0.45 mmol/L malate, and in the presence of 2.5 mmol/L ATP, citrate uptake was reduced by 42 % (Table 1, \([8 + ATP]/(7 + ATP)\) x 100). ATP was included in the reaction medium in order to maintain the established \(\Delta G^+\) gradient by the action of V-ATPase. In the absence of ATP, uptake of malate \(^{-2}\) into energized vesicles (regardless of which transport mechanism) would dissipate the established \(\Delta G^+\) after protonation) and consequently reduce the driving force for citrate \(^{-3}\) uptake, potentially producing conflicting results. Even under these favorable conditions, uptake of citrate was substantially reduced. Nevertheless, given that the Citrus juice cell tonoplast contains an ATP-dependent citrate transport system, the data do not allow us to differentiate whether malate inhibition of cit-
rate uptake was mediated through $\Delta \mu H^+$-dependent ion channels, by inhibiting the ATP-dependent citrate pump, or both. To distinguish between these two possibilities, further experiments were conducted. Table 1 summarizes a series of experiments in which tonoplast vesicles were subjected to various treatments including competition with malate, use of protonionophores, malate channel antibodies and respective combinations, all in the presence and absence of ATP. In many cases, the experiments were aimed at isolating individual citrate transport systems to enable particular studies. For most experiments, treatment 1 with gramicidin and no added ATP served as control, since it represented the amount of citrate that would penetrate by diffusion. Regardless of the treatment, citrate uptake was always higher in the presence of ATP (Table 1), further supporting the existence of a separate and unique ATP-dependent citrate transport system.

Malate at 0.1 mmol/L reduced citrate transport via the ATP-dependent citrate pump by 20%. This inhibition is evident when comparing citrate uptake into de-energized vesicles plus ATP $[(1 + ATP) – (1 – ATP)]$ where $\Delta \mu H^+$ is not allowed to form in the presence of gramicidin, with similar vesicles plus malate $[(6 + ATP) – (1 – ATP)]$. Dissipation of $\Delta \mu H^+$ by gramicidin would abolish electrogenic citrate uptake through channels and would limit uptake to the ATP-dependent citrate pump. Therefore, it appears that the ATP-dependent citrate pump is not specific for citrate but shows some partial affinity for malate.

In contrast to the ATP-dependent citrate pump, inhibition by malate of $\Delta \mu H^+$-energized citrate transport was considerably higher. When malate and citrate were present in the medium at equal concentrations, citrate uptake into energized vesicles was inhibited 67% $[(2 – ATP) – (7 – ATP)/(2 – ATP)] \times 100$. For this comparison, $(6 – ATP)$ would serve as control since malate $-2$ would compete with citrate through any common channel even in the absence of a $\Delta \mu H^+$. Therefore, at equal ion concentrations in the medium, malate appears to be preferentially transported across the organic acid channel.

The effect of anti-32 kDa antibody on citrate transport (with and without ATP) was substantially different, providing further evidence of the existence of two citrate transport mechanisms. In the absence of antibody, citrate uptake by the ATP-dependent citrate transporter was 2.64 nmol citrate · mg protein$^{-1}$ after 40 min $[(1 + ATP) – (1 – ATP)]$ whereas in the presence of the antibody, citrate uptake was notably reduced to 1.85 nmol citrate · mg protein$^{-1}$ after 40 min $[(3 + ATP) – (3 – ATP)]$. Therefore, antibodies against malate channel decreased citrate uptake by the ATP-dependent citrate transporter about 30%. The same results were obtained when we compare $[(2 + ATP) – (2 – ATP)]$ to $[(4 + ATP) – (4 – ATP)]$. A much higher inhibitory effect was noted for the $\Delta \mu H^+$-
energized citrate transport where a 65% inhibition was noted [(4-ATP)/(2-ATP)] × 100.

The data presented in this communication demonstrate that neither of the two previously described mechanisms for citrate transport into the vacuole of Citrus juice cells is citrate-specific, despite the great differences in concentration between citrate and other organic anions found in their vacuoles. Instead, both ∆µH⁺-dependent channel and the ∆µH⁺-independent ATP-dependent citrate transporter show various degrees of affinity for malate. Furthermore, the data also demonstrate that ∆µH⁺-dependent channel and the ∆µH⁺-independent ATP-dependent citrate pump share common protein domains with each other and with the malate-specific channel of Kalanchoë diagremontiana, given that both were recognized and their transport was inhibited by anti-32 kDa channel antibodies. Finally, it is highly likely that in Citrus juice cells, the ∆µH⁺-dependent channel mediates transport of both citrate and malate as has been previously suggested for other cells (Rentsch and Martinoia 1991). Since malate seems to be preferentially recognized by the ∆µH⁺-dependent channel (see above), higher levels of citric acid in the juice cells may just simply reflect a higher rate of synthesis and not a preferential transport mechanism.

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References
