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Protoplasmic pH Modifies Water and Solute Transfer in *Beta vulgaris* Root Vacuoles

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Abstract. Volume changes were studied in *Beta vulgaris* storage root vacuoles, using video microscopy, when exposed to hypotonic conditions. The osmotic gradient was either step-applied or progressively imposed in perfusion experiments. Preincubation at low pH (6.6) or with $HgCl_2$ strongly reduced the vacuoles' water permeability, measured in step experiments. Furthermore, the volumetric response depended on the rate with which the aniso-osmotic condition was established. In perfusion experiments a ''plateau value'' (osmotic equilibrium or steady-state volume value) was observed, which was significantly lower than the theoretically expected one. Furthermore, if vacuoles were preincubated in presence of $HgCl_2$ or at low pH and then the hypo-osmotic challenge was applied in perfusion experiments, a still lower ''plateau value'' was observed. This reduction was concentration-dependent and completely reversible. In these conditions, when $HgCl_2$ concentration was 300 μM or medium pH was 6.6, the volume change was abolished. In other experiments, when urea iso-osmotically replaced mannitol, a reversible, pH-dependent volumetric response was observed. These results can be interpreted accepting that 1) mercury-sensitive water channels, present in the studied structure, were blocked by low pH during the hypo-osmotic challenge; 2) modification of water permeability prevents excessive swelling during the osmotic shock; 3) the effectiveness of this last mechanism depended on the osmotic challenge rate; and 4) additionally, urea reflection coefficients were also modified by reduced medium pH.

Key words: Aquaporin — *Beta vulgaris* — Urea — Permeability — Volume regulation

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Introduction

Plants have developed water balance control mechanisms that make possible their survival in environments that do not necessarily provide the best conditions for growth and development. Nevertheless, water deficit as well as strong soil salinity and/or acidity represent important negative challenges to the normal physiology of crops and plants (Katsuhara et al., 1989; Cruz, Jordan & Drew, 1992; Neumann, 1997).

Vacuoles represent 90% of the total volume in plant cells and are responsible for turgence maintenance, cell volume regulation and pH homeostasis between the cytoplasm and the vacuole content (normally acid) (Taiz, 1992; Marty, 1999). In 1993, Maurel et al. observed that the tonoplast protein γ-TIP from *Arabidopsis thaliana* performs as a water-specific channel in *Xenopus* oocytes, and in 1996 the characterization of a new tonoplast aquaporin (α-TIP), sensitive to mercury at a unique site, was published (Daniels et al., 1996). Since these works, many other TIPs have been identified (Maurel, 1997) and water relations in plant tissues have been reconsidered in terms of these new findings (Schäffner, 1998, Tyerman et al., 1999).

Medium acidification regulates water permeability in animal tissues in which AQP2, AQP3 and AQP4 are normally expressed (Parisi, Wietzerbin & Bourguet, 1983; Parisi & Bourguet, 1984; Parisi & Wietzerbin, 1984). More recently it has also been reported that proton concentration can regulate the osmotic permeability of *Xenopus laevis* oocytes expressing AQP3 (which transports water and glycerol; Zeuthen & Klaerke, 1999) and, in certain circumstances, of AQP0 (Nemeth-Cahalan & Hall, 2000). At present, there is no evidence of pH-dependent water channels in plants.

In a previous work we focused our interest in understanding water movements on *Beta vulgaris* storage roots, and we were able to describe a trans-

cellular water movement sensitive to mercurial compounds (Amodeo et al., 1999). In this species a tonoplast intrinsic protein (TIP) has been described in the root parenchyma (Marty-Mazars et al., 1995) and the osmotic permeability of isolated vacuoles has been reported confirming the functionality of mercury-sensitive water channels (Morillon & Lassalles, 1999).

We focused our work on studying water and solute transfers in isolated vacuoles. Our results show that medium acidification reduces water permeability in vacuoles isolated from *Beta vulgaris* storage roots, while the steady-state volume observed after a hypo-osmotic shock seems to be dependent on the velocity with which the osmotic gradient is applied. Furthermore, the medium pH regulated, in a complex manner, urea movements. Reversible effects on mercury-sensitive water channels and changes in the reflection coefficient (σ) for the employed solutes would play an important role in the described phenomena.

Material and Methods

Vacuole Isolation from Parenchyma of *Beta vulgaris* Storage Roots

Beta vulgaris L. plants were grown in the field and harvested after 90 days, transferred to the laboratory and maintained in moist soil until the experiments. Vacuoles were mechanically isolated according to Leigh and Branton (1976). Briefly, the storage roots were cut in 1-cm^3 sections and incubated during 20 min in a solution containing 2 M sucrose. These sections were collected with an absorbent material, and then transferred to a solution containing 400 mM mannitol, 1 mM EDTA, 20 mM Tris-Mes, pH 7.6, final osmolarity: 490 mOsm kg^{-1} (control solution). The slices were finely chopped using a sharp razor blade. In these conditions vacuoles were released to the medium.

Volume-Change Studies Using Videomicroscopy

Step Tonicity-Change Experiments

To test the vacuole water permeability, a small drop (20 µl) of a solution (490 mOsm kg^{-1}) containing vacuoles was placed in the middle of a slide, close to a second drop (same volume) containing only solution. One vacuole was tracked in the videomicroscope system. Then a small pipette was used to collect the tracked vacuole in about 0.1 µl of solution (*see* below) and to transfer it to the second drop (isotonic or hypotonic; Fig. 1*A*). The close position of the drops allowed fast refocusing. When the vacuole was changed to a solution containing the same osmolarity, no volume change was observed. The volume of the solution inside the pipette containing the selected vacuole was calculated considering the diameter and used length of the pipettes (Fig. 1*B*). The mean volume captured to transport the vacuoles with the pipette was 0.11 ± 0.02 µl, representing less than 0.5% of the drop volume. Evaporation caused less than a 1% increase in osmolarity in 120 seconds. Since all the measurements were finished within the first 20 seconds, it is assumed that final osmolarity remained constant.

Perfusion Experiments

The aim of this protocol was to study the "final" relative volume (V/Vo) (which we will refer to as "plateau value") achieved after a gradual osmotic challenge. In these experiments, 10 µl of the solution containing vacuoles were transferred to a small observation chamber (chamber final volume 500 µl) with an interchangeable 6-mm coverslip at the bottom. The volume-change time course could be followed in isolated vacuoles suspended in the medium. A perfusion system was developed to exchange solutions. This system consists of three 60-ml syringes with valves that warrant a fixed flux. Dead space of the system was 250 µl, and the perfusion flow was 430 µl min^{-1}. The observation chamber medium reached 95% concentration of a new perfusion solution in 60 seconds. A single vacuole could be recorded, first when the perfusion solution was iso-osmotic; then the perfusion solution was switched in order to apply different protocols. Perfusion was maintained all the time during the experiments.

Videomicroscopy Setup. Volume Measurements

Vacuoles could be observed by transmitted light using 300 × magnification in an inverted Olympus IMT-2 microscope connected through a digital video camera (Electrim EDC-1000) (Fig. 1*C*). Images were digitized through a PC computer acquisition board (total amplification 1,300 ×). Single vacuole images could be recorded if necessary, every 2 sec up to 10 min. In both types of protocol, vacuole diameters were measured from the stored images employing commercial image software (Optimetric 1.0; Bioscan, USA) and a special calibrated microscope slide. The diameter of each vacuole was measured three times from each acquired image. Vacuole volumes were calculated considering that vacuoles are spherical. Mean diameter in control solution was 27.47 ± 0.37 µm (n = 140). Three to five independent isolations were performed to test each experimental condition. Results are expressed as relative volume change over time.

Hypotonic Challenges and Inhibition Tests

A hypotonic medium is one in which cells swell, while a hypo-osmotic medium simply has a lower osmotic concentration than the physiological osmotic value. We therefore applied the terms tonicity and osmolarity upon this above-mentioned definition.

Unless indicated, control solution was 400 mM mannitol, 1 mM EDTA, 20 mM Tris-Mes, pH 7.6, final osmolarity: 490 mOsm kg^{-1}. Osmotic challenges were made possible by reducing final mannitol concentration, from iso-osmolarity (490 mOsm kg^{-1}) to a hypo-osmotic condition (290 mOsm kg^{-1}). Furthermore, in some experiments iso-osmolarity was maintained constant (490 mOsm kg^{-1}) but mannitol was partially replaced by 100 mM urea, in order to verify if isotonicity was affected.

To test inhibition through mercurial compounds, vacuoles were incubated in the presence of $HgCl_2$ at different final concentrations (0.3 to 300 µM). Reversibility was tested in the presence of 5 mM β-mercaptoethanol. In pH experiments vacuoles were perfused in hypo-osmotic conditions at different proton concentrations, which were obtained by manipulating the employed buffer pH (Tris-Mes 20 mM), increasing or lowering the pH in one unit. All the osmolarities of the employed solutions were measured and adjusted using an osmometer (Wescor 5100C).

Measurement of Water Permeability

The transmembrane water flux (J_v) was calculated from the slope of the linear portion of the relative volume change (V/V_o) time course

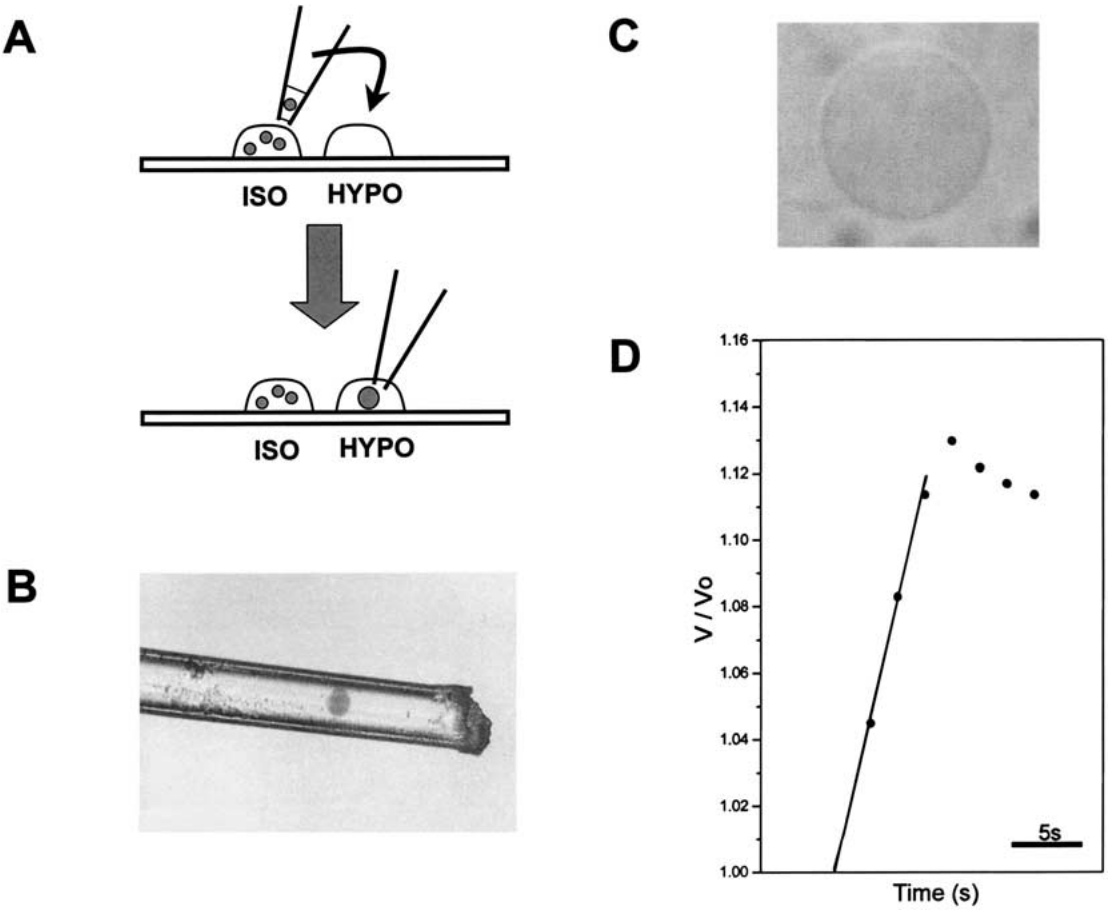

Fig. 1. Step-tonicity change experiments. (*A*) Experimental setup. A small pipette collects a vacuole and transfers it to a hypotonic drop. (*B*) A photograph of a vacuole (diameter: 27.47 ± 0.37 μm, mean ± SEM, $n = 140$) inside the pipette, (*C*) A single vacuole under the inverted microscope. (*D*) Representative experiment showing the variation in volume for a vacuole transferred to a hypotonic medium.

plot in step experiments (as an example, *see* Fig. 1*D*). The apparent osmotic water permeability coefficient ($P_{os\text{-}ap}$, μm sec^{-1}) was calculated from the following equation:

$$P_{os-ap} = J_v[V_w.S(Osm_o - Osm_i)]$$

where: V_w is the water partial molar volume, S the initial vacuole surface and (Osm_o - Osm_i) the applied osmotic gradient.

Results

Vacuole Response to Hypotonic Conditions Obtained by Modifying Mannitol Concentration

Figure 1 describes the setup for "step-tonicity-change" experiments (*see* Materials and Methods). Figure 1*D* presents the observed results when the vacuoles were exposed to hypo-osmotic conditions (290 mOsm kg^{-1}) by modifying mannitol concentration: a fast swelling response was recorded. The rate of volume change allowed to calculate an "apparent osmotic water permeability" ($P_{os\text{-}ap}$). The mean relative volume change 20 sec after imposing the 200-mOsm gradient went up to 1.14 ± 0.01 (mean ± SEM, $n = 6$). Nevertheless, vacuoles became extremely difficult to track for longer periods under these experimental conditions. No volume difference was observed when a selected vacuole was changed to an iso-osmotic solution (490 mOsm kg^{-1}).

We therefore performed experiments using the "perfusion" setup. First, an initial equilibrium period with an iso-osmotic solution was allowed. The vacuoles arrived at an osmotic equilibrium or steady state with the control solution as indicated by no volume change observed up to 10 min (control experiments, Fig. 2, iso-iso conditions). Results obtained by reducing mannitol concentration in the external medium are also presented in this figure. In this case and in all similar experiments, the first point after the osmotic challenge was recorded 60 sec after the beginning of the perfusion process, the time in which 95% removal of the bath was obtained (*see* Methods). It can be remarked that: 1) A rather slow swelling was observed and 2) The observed final relative volume (1.08 ± 0.01; mean ± SEM, $n = 14$) was lower than the one observed in "step-tonicity-challenge"

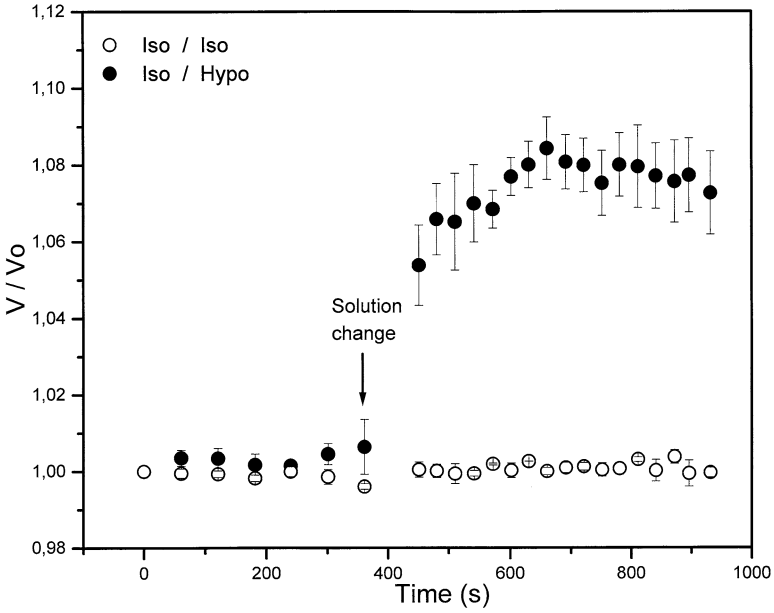

Fig. 2. Volume changes induced by a hypotonic gradient in isolated vacuoles (perfusion experiments). In control conditions (*white circles*) the perfusion solution was iso-osmotic (490 mOsm kg^{-1}) throughout the experiment. In experimental condition (*black circles*), after an initial equilibration period, an osmotic challenge was introduced by altering mannitol final concentration (final osmolarity 290 mOsm kg^{-1}). Mean relative volume changes ± SEM for 12 to 14 vacuoles obtained from 3 to 4 independent extractions.

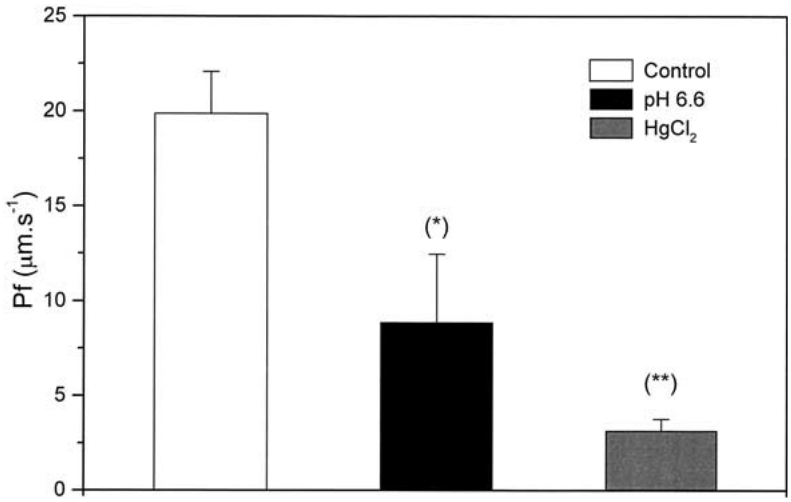

Fig. 3. Permeability values *Pf* measured in step-tonicity-change experiments. Vacuoles were preincubated in control iso-osmotic solution ($n = 6$), or in the presence of 300 µM $HgCl_2$ ($n = 4$) or at pH 6.6 ($n = 4$). Then a hypo-osmotic challenge was applied. Data are expressed as mean ± SEM.

experiments and quite different from the expected one (1.70), according to the applied gradient.

Mercurial Compounds Reversibly Inhibited Vacuole Volume Changes under an Osmotic Gradient

It is known that $HgCl_2$ blocks many aquaporins, thus reducing water permeability (Preston et al., 1993; Zhang et al., 1993; Daniels et al., 1996). When vacuoles were pre-incubated in presence of $HgCl_2$ (iso-osmotic conditions) and then a step hypo-osmotic challenge was applied, a dramatic reduction (84%) in the calculated $P_{os\text{-}ap}$ was observed (control, 19.87 ± 2.21 µm sec^{-1}; experimental, 3.18 ± 0.59 µm sec^{-1}, $p < 0.01$, Fig. 3). These results confirm previous results (Maggio & Joly, 1995; Morillon & Lassalles, 1999), indicating that mercury-sensitive water channels contribute to the elevated P_{os} values observed in *Beta vulgaris* root vacuoles and other plant structures.

In other experiments, $HgCl_2$ -pretreated vacuoles were exposed to the hypotonic gradient-perfusion experiments. As compared with control conditions, a lower "plateau value" was observed. This reduction was concentration-dependent and when $HgCl_2$ was increased up to 300 µM, the volume change was completely abolished (Fig. 4*A* and *B*). It must be stressed here that a change in water permeability will only modify the rate of volume change as a function of time and not the final volume. Another mechanism must then be underlying the results observed in perfusion experiments. Furthermore, $HgCl_2$ did not induce relative volume changes in iso-osmotic conditions (Fig. 5*A*) and $HgCl_2$ inhibition of the osmotic response was completely reverted when the

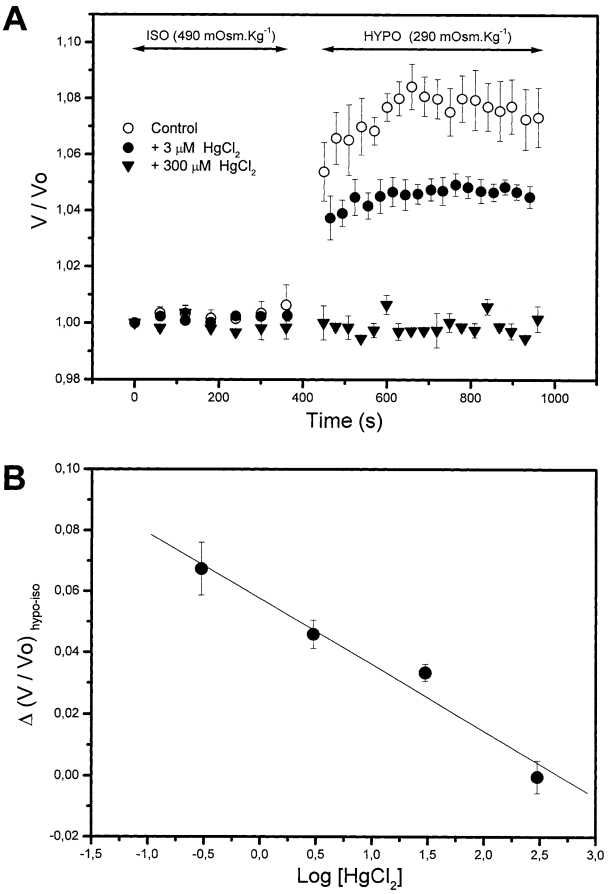

Fig. 4. Effects of mercurial compounds under a hypo-osmotic challenge (perfusion experiments). (*A*) Preincubation for 5 min with different $HgCl_2$ concentrations. Data are expressed as relative volume changes (mean ± SEM for 4 to 14 vacuoles obtained from 2 to 5 independent extractions. (*B*) Dose-response plot for $HgCl_2$ of final "plateau values." Used concentrations were 0.3, 3, 30 and 300 μM $HgCl_2$. Each point represents the mean difference between V/V_o in hypo-osmotic conditions (t = 720 sec) and V/V_o in iso-osmotic conditions (t = 180 sec) (n = 4 to 14 vacuoles obtained from 2 to 5 independent extractions).

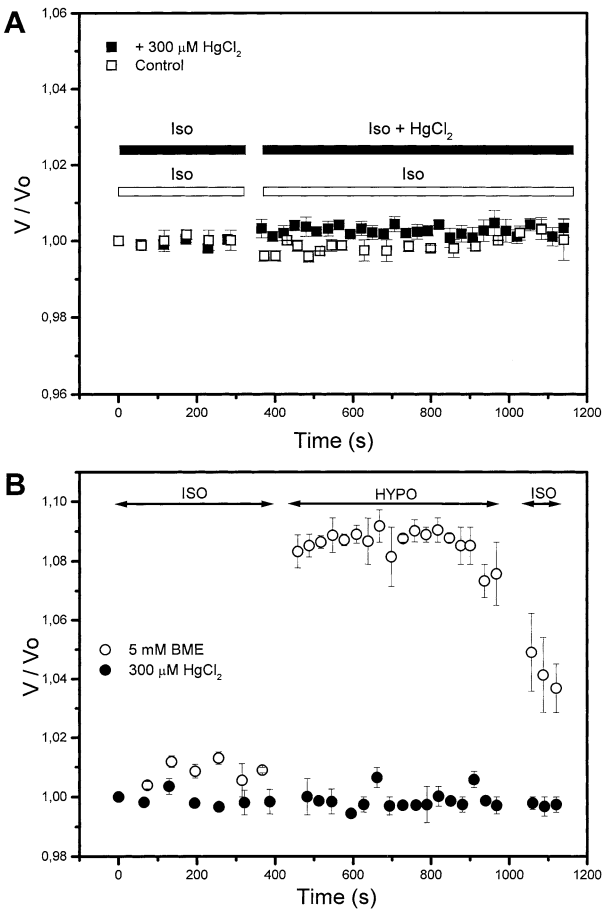

Fig. 5. (*A*) Effect of 300 μM $HgCl_2$ on the relative volume V/V_o in iso-osmolar conditions. First vacuoles were perfused with a control solution and then the solution was switched to one containing 300 μM $HgCl_2$, keeping the same osmolarity (n = 12 vacuoles, 5 independent experiments). Control experiments without $HgCl_2$ are also shown, (n = 4 vacuoles, 2 independent extractions). (*B*) Reversibility was tested by preincubating with 300 μM $HgCl_2$ during 5 min and then perfusing with 5 mM β-mercaptoethanol before and during the osmotic challenge. Mean ± SEM for 4 vacuoles obtained from 2 independent extractions.

perfusion bath contained 5 mM β-mercaptoethanol. These results proved the maintenance of membrane integrity (Fig. 5*A* and *B*).

Vacuole Volume Changes Observed when Urea Partially and Iso-Osmotically Replaced Mannitol Were also Sensitive to $HgCl_2$

Figure 6*A* shows experiments in which, after the initial equilibration period in the control solution, the perfusion solution was switched to one in which mannitol was partially and iso-osmotically replaced by 100 mOsm kg^{-1} urea (final osmolarity of the perfusion solution: 490 mOsm kg^{-1}). The observed volume change was completely reversed after coming back to the control solution. This "urea-associated" increase in volume was partially blocked when vacuoles were pre-incubated in 300 μM $HgCl_2$ (Fig. 6*B*). Vacuole swelling indicated that the urea σ coefficient was lower than the mannitol one.

Volume Adjustments at Different Medium pH

A strong pH gradient normally exists between the vacuole inside (pH about 5) and outside (pH about 7.6). When vacuoles were pre-incubated at pH 6.6 (iso-osmotic conditions) and then a step hypo-osmotic challenge was applied, a 55% reduction in the calculated $P_{os\text{-}ap}$ was observed (control, 19.87 ± 2.21 μm sec^{-1}; experimental, 8.89 ± 3.35 μm sec^{-1}, $p < 0.05$, Fig. 3). This effect of medium pH on the water permeability of plant vacuoles is a novel and important observation.

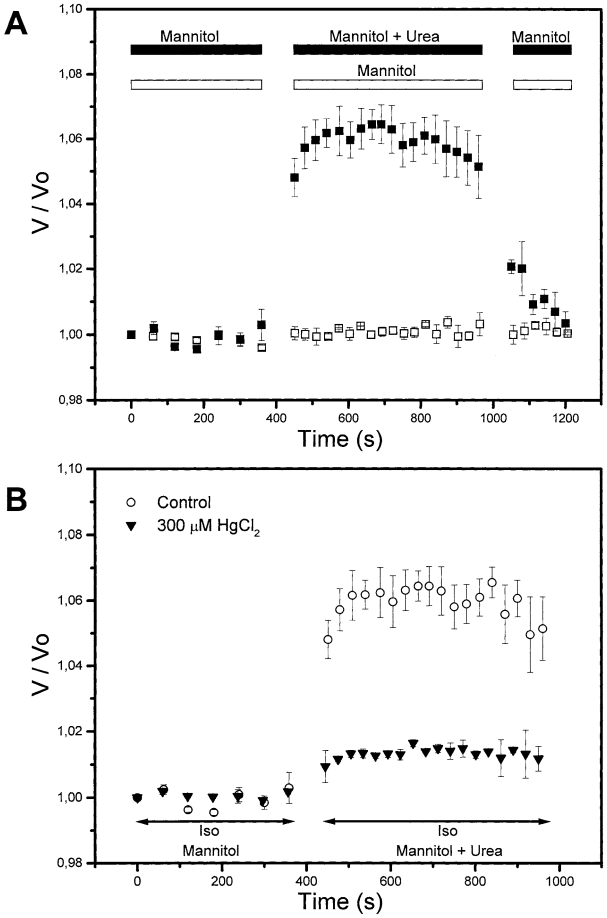

Fig. 6. Relative volume changes V/Vo in isolated vacuoles. (*A*) After an initial equilibration period in the iso-osmotic mannitol solution (490 mOsm kg^{-1}), this solute was partially replaced by 100 mM urea (final osmolarity 490 mOsm kg^{-1}). Finally, the vacuoles were again perfused with mannitol solution (*black squares*). Control vacuoles were perfused with mannitol solution throughout all the experiment (*white squares*). Mean ± SEM for 12 vacuoles obtained from 3 independent extractions. (*B*) Vacuoles pre-incubated for 5 min with 300 μM $HgCl_2$ were perfused with the iso-osmotic mannitol solution and then with an iso-mannitol-urea solution like the one described in *A*. Mean ± SEM for 10 vacuoles obtained from 2 independent extractions.

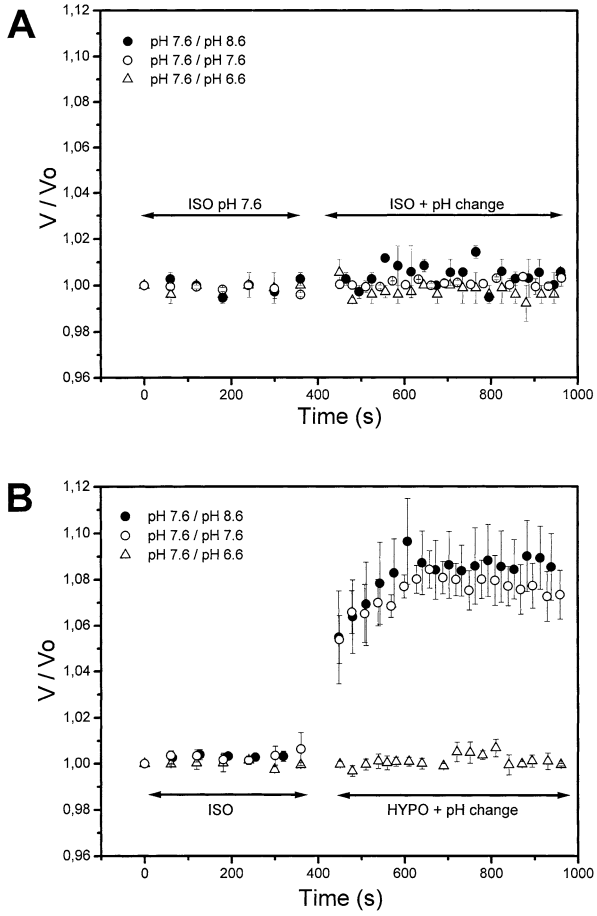

Fig. 7. Effects of the external pH on the osmotic properties of isolated vacuoles. (*A*) After an initial perfusion in iso-osmotic condition (pH 7.6), different final pHs (6.6, 7.6 and 8.6) were tested without affecting the isotonicity of the medium. (*B*) Relative volume changes V/V_o observed after the simultaneous change in both pH and osmolarity of the medium (final osmolarity 290 mOsm kg^{-1}). Mean ± SEM for 6 to 14 vacuoles obtained from 3 to 4 independent extractions.

Using the perfusion system, external pH (which here represents cytosolic pH) could be reduced to 6.6 or increased to 8.6 without affecting the vacuole volume in iso-osmotic conditions (Fig. 7*A*). Nevertheless, the volume changes induced by a hypo-osmotic challenge were strongly dependent on medium pH (Fig. 7*B*). The response to medium hypo-osmolarity was, as in the case of 300 μM $HgCl_2$, completely abolished at pH 6.6. The reversibility of this effect is shown in Fig. 8. Vacuoles were initially equilibrated in iso-osmotic conditions at pH 7.6. Then the hypo-osmotic gradient was imposed at pH 6.6. No change in the vacuole volume was observed. Finally, the bath pH was switched again to 7.6. The volume increase that developed was indistinguishable from the one observed in control conditions (Fig. 7*B*) showing that no irreversible alteration of the vacuole structure or its internal solute composition were induced during medium acidification. The same comments made when describing $HgCl_2$ effects on "plateau value" in perfusion experiments can be repeated here: the observed results can not be explained by a simple change in water permeability (*see* the Discussion section).

Finally, pH was also modified when vacuoles where exposed to iso-osmotic solutions prepared with urea and mannitol as osmolytes. Figure 9 shows that the increase in volume observed when urea partially and isotonically replaced mannitol in the bath solution (Fig. 6*A*) was completely abolished at pH 6.6. This inhibitory effect disappeared when the pH of the medium was switched again to 7.6.

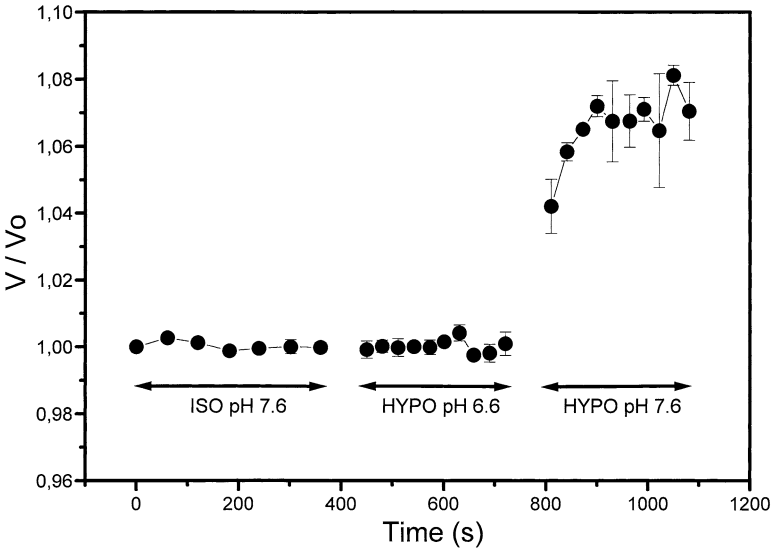

Fig. 8. Vacuoles were initially incubated under iso-osmotic conditions (ISO, pH 7.6). Then, both an osmotic challenge (final osmolarity 290 mOsm kg^{-1}) and a change of pH (to 6.6) were applied. Finally, the vacuoles were perfused again at pH 7.6, but keeping the osmotic gradient. Relative volume changes; mean ± SEM for 6 vacuoles obtained from 3 independent extractions.

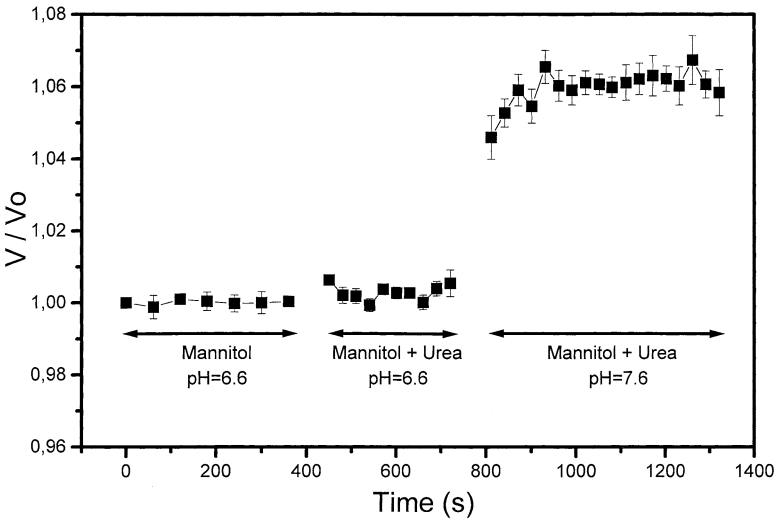

Fig. 9. After an initial equilibration period in the iso-osmotic mannitol solution (490 mOsm kg^{-1} pH 6.6), mannitol was partially replaced by 100 mM urea maintaining the final osmolarity and pH. Finally the medium pH was returned to 7.6. Data are expressed as mean relative volume changes ± SEM for 10 vacuoles obtained from 4 independent extractions.

Discussion

Water- and solute-related permeabilities and their regulation have been extensively studied, both in animal (Parisi & Bourguet, 1985, Agre et al., 1995) and plant tissues (Maurel et al., 1995; Maurel, 1997; Schäffner, 1998, Eckert et al., 1999). Results presented in this paper show, for the first time to our knowledge, that proton concentration modifies the osmotic properties of isolated plant vacuoles, in a process in which mercury-sensitive water channels are involved. Furthermore, modification of water permeability seems to favor compensatory volume changes in *Beta vulgaris* root vacuoles.

Water Permeability in Plant Vacuoles: Effects of $HgCl_2$ and Low pH

As mentioned, we measured the "apparent" osmotic water permeability coefficient. This value is probably affected, in our experimental conditions, by the presence of an "adherent water layer" transferred together with the vacuole into the hypotonic drop. The presence of this adherent water film implies a "mixing time" before the osmotic gradient was applied to the vacuole membrane. To evaluate the effects of this situation, the iso-osmotic volume transferred together with the vacuole into the hypotonic solution can be considered, as a first approximation, as having a spherical shape. From the measured volume (0.11 ± 0.02 µl) a 288-µm radius can be calculated, corresponding to a maximal possible thickness for the "adherent water layer". If we consider this layer as an "in-series unstirred layer" the corresponding water permeability coefficient would be 10.4 µm sec^{-1}, which is lower than the experimentally observed one for the $P_{os\text{-}ap}$. This implies that the "adherent layer effect" was less important than the one corresponding, in diffusion conditions, to an unstirred layer of similar thickness.

The here determined $P_{os\text{-}ap}$ values (19.87 ± 2.21 μm sec^{-1} in osmotic step gradients) are significantly lower than the previously reported P_{os} values, which had a very large dispersion range (270 ±104 μm sec^{-1}; Morillon & Lasalles, 1999). This probably indicates that, with our experimental approach, we underestimated the control P_{os} values. Nevertheless, both, under the action of a mercury compound as well as when medium pH was set to 6.6 (Fig. 3), we observed a significative reduction in the $P_{os\text{-}ap}$. If we are underestimating P_{os} control values, it is evident that we are also underestimating the inhibitory effects of both low pH or $HgCl_2$. The central goal of this work is to report this inhibitory effect of acidification of the medium on the water permeability of plant vacuoles.

It has been reported recently that medium pH modifies (in opposite ways) the permeability to water and solutes of cloned aquaporins expressed in *Xenopus* oocytes (Zeuthen & Klaerke, 1999; Yasui et al., 1999). It has been also proposed, in animal cells, that the pH of the medium can regulate the permeability of certain water channels in an on-off process (Parisi et al., 1983; Parisi & Bourguet, 1984; Parisi & Wietzerbin, 1984).

Osmotic Equilibrium in *Beta vulgaris* Root Vacuoles: the Effects of $HgCl_2$

In perfusion experiments in control conditions, vacuoles arrived at an osmotic steady state, as indicated by the initial constant volume (Figs. 2 and 4*A*). Furthermore, mannitol permeability is generally considered as being relatively low (i.e., σ for mannitol is near to one), as previously reported in experiments with tonoplast vesicles (Gerbeau et al., 1999). Nevertheless, when an osmotic gradient was imposed in perfusion experiments that reduced mannitol concentration, the new observed osmotic equilibrium volume ("plateau value") was smaller than the predicted one and also significantly lower than the volume change observed 20 seconds after the osmotic challenge in step experiments. This indicates that modification of water permeability prevents excessive swelling during the osmotic shock.

The reversible and dose-dependent reduction in the "plateau value" of vacuoles, observed in the presence of $HgCl_2$ and under a similar osmotic challenge, can be partially attributed to an "aquaporin blocking effect," especially considering that functional TIP channels are present in *Beta vulgaris* vacuoles. However, the observed results can not be explained as being due to a single effect on water permeability, because in this situation all the equilibrium volumes should have been the same, being affected only by the equilibration rate. In the experiments presented here, there was a concentration-dependent reduction of the "plateau value" and, furthermore, at 300 μM $HgCl_2$, the osmotic response was completely abolished.

Water Pathways and the Urea Reflection Coefficient in *Beta vulgaris* Root Vacuoles

The experiment presented in Fig. 6*A*, in which urea isotonically replaced mannitol, is a clear and classical evidence that the reflection coefficient for urea is lower than for mannitol. In other experiments, in which the vacuoles were preincubated with $HgCl_2$, (Fig. 6*B*), the osmotic response to urea was reduced. One can conclude that under the effect of the mercurial compounds, the reflection coefficient for urea approached the one for mannitol.

A straightforward interpretation of these results is that urea and water move through the same pathway. Literature reports plant aquaporins that are not only permeable to water but also to small solutes. Nt-TIPa is a tonoplast water channel with a high permeability to urea and glycerol, which is expressed in tobacco cells (Gerbeau et al., 1999). It has also been reported that an animal aquaporin (AQP9) is simultaneously permeable to mannitol, urea and water (Ishibashi et al., 1998).

The Effect of Medium pH on the Vacuole Water and Solute Movement

Results presented in Figs. 7 to 9 are the most original contribution of our report. First, a change in medium pH by itself had no consequence in the vacuole osmotic steady-state equilibrium (Fig. 7*A*). Second, the complete reversible disappearance of the response to an important hypotonic gradient (200 mOsm, perfusion experiments) when the pH of the medium was decreased from 7.6 to 6.6 was a striking result. Our interpretation is that an "aquaporin-blocking effect" similar to the one induced by 300μM $HgCl_2$ is the main reason for this result.

To suppose that water permeability was completely blocked would seem rather unreal. Nevertheless, the experiments presented in Fig. 8 demonstrated that, at pH 6.6, the osmotic gradient was not reduced after more than 10 minutes of application. This implied: 1) at low pH, water permeability was strongly reduced (as confirmed by P_{os} measurements, Fig. 3), and 2) the transmembrane osmotic gradient did not change significantly, because the osmotic response was completely preserved when the pH of the medium was again switched to control values. Figure 8 also shows that the pH effect was completely reversible, also indicating that the tonoplast integrity was fully preserved.

At this point we can speculate on the existence of a compensatory mechanism, modifying the volume response of the vacuole to an osmotic gradient. This

mechanism, the underlying process of which must be the subject of future studies, is probably the same one that is responsible for the unexpected plateau volume values observed after applying the osmotic gradient (Fig. 2). Furthermore, if the osmotic gradient is progressively applied at low pH or under action of $HgCl_2$, the efficiency of this compensatory process can be 100%. In this situation a steady state with no volume change is achieved even when the osmotic gradient is still present.

We are then concluding that both, under low-pH conditions as well as after $HgCl_2$ addition, water permeability is so low that permeable solute movements could compensate the osmotic flux. From a theoretical point of view this is equivalent to a strong reduction in the permeable solute reflection coefficient that, in certain circumstances, can be considered as near to zero. It has been reported previously that the concentration of a TIP protein in the vacuole membrane is extraordinarily high (Higuchi et al., 1998). As a consequence, the "free lipid bilayer" surface would be relatively low, as compared with other cell membranes. This could explain why the remaining water permeability after blocking the aquaporin pathway would be very low.

The effects of low pH on the osmotic response were also observed when urea partially replaced mannitol. In the experiment presented in Fig. 9, medium pH was initially 6.6 (no change in the vacuole volume was observed when the pH of the medium was shifted from 7.6 to 6.6, Fig. 7*A*). When urea partially and iso-osmotically replaced mannitol no change in the vacuole volume was observed. This phenomenon can be explained, in the frame of the previously described hypothesis, if the reflection coefficients for urea and mannitol are similar.

The last part of this experiment is also a crucial one. Always in the presence of the iso-osmotic mannitol-urea mixture, the pH of the perfusion bath was increased from 6.6 to 7.6 and the osmotic response was present again, confirming that the solute gradients were not affected. This demonstrates that this is a fully reversible phenomenon that does not affect the vacuole structure or its osmotic properties.

Regulation of aquaporins have been reported at different levels: 1) genes, where different patterns of transcription and protein expression have been observed (Ludevid et al., 1992; Kaldenhoff et al., 1995); 2) cytoplasmic-cell membrane traffic (Knepper, 1997) and 3) a short-term regulation level based on phosphorylation and dephosphorylation of the channels (Maurel et al., 1995, Johansson et al., 1996, 1998), or based on the recently described pH effect in cloned aquaporins expressed in *Xenopus* oocytes (Zeuthen & Klaerke, 1999; Yasui et al., 1999, Nemeth-Cahalan & Hall, 2000).

High water permeability of the tonoplast allows the vacuole to effectively buffer the cytoplasm water content, minimizing short-term volume transients (Zhang & Tyerman, 1999). Recently it was found that under osmotic stress there is an increase in the mRNA content of a tonoplast aquaporin (Barrieu et al., 1999). On the other hand, it has been reported that cytoplasmic acidification can be induced by salt stress (Katsuhara et al., 1989) and also during hypoxia conditions (Roberts et al., 1992; Edwards et al., 1998). It is possible, therefore, to suggest that modifications of the cytoplasmic pH would play an important role in the vacuole volume regulatory process.

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