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Control of Volume and Turgor in Stomatal Guard Cells

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Abstract. Water loss from plants is determined by the aperture of stomatal pores in the leaf epidermis, set by the level of vacuolar accumulation of potassium salt, and hence volume and turgor, of a pair of guard cells. Regulation of ion fluxes across the tonoplast, the key to regulation of stomatal aperture, can only be studied by tracer flux measurements. There are two transport systems in the tonoplast. The first is a Ca^{2+}-activated channel, inhibited by phenylarsine oxide (PAO), responsible for the release of vacuolar K^+(Rb^+) in response to the "drought" hormone, abscisic acid (ABA). This channel is sensitive to pressure, down-regulated at low turgor and up-regulated at high turgor, providing a system for turgor regulation. ABA induces a transient stimulation of vacuolar ion efflux, during which the flux tracks the ion content (volume, turgor), suggesting ABA reduces the set-point of a control system. The second system, which is PAO-insensitive, is responsible for an ion flux from vacuole to cytoplasm associated with inward water flow following a hypo-osmotic transfer. It is suggested that this involves an aquaporin as sensor, and perhaps also as responder; deformation of the aquaporin may render it ion-permeable, or, alternatively, the deformed aquaporin may signal to an associated ion channel, activating it. Treatment with inhibitors of aquaporins, $HgCl_2$ or silver sulfadiazine, produces a large transient increase in ion release from the vacuole, also PAO-insensitive. It is suggested that this involves the same aquaporin, either rendered directly ion-permeable, or signalling to activate an associated ion channel.

Key words: Aquaporin — Guard cell — Osmoregulation — Turgor regulation — Tonoplast ion channels

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Introduction

Many plant cells regulate their turgor pressure to a constant level in the face of changing external osmotic conditions. This is particularly important in marine algae in the intertidal zone, which face large changes in external osmotic conditions during air exposure or following rain. After the initial water flow induced by an external osmotic change, leading to a new steady state of internal osmotic pressure, cell volume and turgor, in water equilibrium with the new external conditions, many algae show altered ion fluxes, leading to changes of internal ion concentration in the direction to restore the original turgor. After a hypo-osmotic shock producing abnormally high turgor, net ion efflux is observed and turgor is reduced; in the low turgor state following a hyper-osmotic shock, net ion influx is observed, again restoring the original turgor. This has been recognized for many years, but the mechanisms are most recently reviewed by Findlay (2001).

The situation in stomatal guard cells is somewhat different. Water loss from leaves is determined by the aperture of stomatal pores in the leaf surface, set by the turgor of a pair of stomatal guard cells bounding the pore, determined in turn by the level of solute accumulation, largely potassium salt. in the guard cells. Stomatal closure to restrict water loss, essential for plant survival in dry conditions, requires net loss of potassium salt from guard cells, resulting in cell shrinkage, loss of turgor and reduction in stomatal aperture. Closure occurs in response to water stress conditions, signalled by production in the roots and transfer to the leaves of the "drought" hormone, abscisic acid (ABA). Closure also occurs on transfer from light to dark, or in response to high CO_2, ensuring that stomata are open, with consequent water loss from the leaves, only in conditions suitable for net carbon fixation requiring input of CO_2. Thus guard cells show very

large changes in cell turgor in response to environmental signals, and in the normal diurnal cycle. The great bulk of the cell salt content is vacuolar, and closure must involve net loss of potassium salt at both plasma membrane and tonoplast of the guard cell. Regulation of ion release from vacuole to cytoplasm, across the tonoplast, is absolutely essential in the process of closure, and regulation of vacuolar ion content and volume, in response to a range of signals and in the normal diurnal cycle, is therefore central to guard cell function. Such large changes in vacuolar ion content and volume are uncommon in mature plant cells in constant external osmotic conditions, and are one of the peculiar features of guard cells.

In spite of the importance and scale of changes in tonoplast fluxes in the regulation of stomatal aperture, we know comparatively little about the mechanisms of their control, or about the nature of the associated signalling chains. Much more is known about regulation and responses of ion channels in the plasma membrane, and the role of the vacuole has been relatively neglected. Part of the reason for this is the relative inaccessibility of the tonoplast in the intact cell. The plasma membrane is accessible for electrophysiological measurements, by impalement of intact guard cells or by patch-clamp measurements in guard cell protoplasts. The tonoplast is not electrophysiologically accessible in its normal physiological state, and patching of isolated vacuoles investigates a system lacking most of its signalling components. Tracer flux measurements on isolated guard cells offer a partial remedy; detailed analysis of the kinetics of tracer exchange allows measurement of tonoplast fluxes in the steady state, and provides a semi-quantitative assessment of the response of tonoplast efflux to appropriate signals, although with much poorer time resolution than is offered by electrical measurements. Such studies have provided evidence for regulation of vacuolar ion content in stomatal guard cells, and identified two different tonoplast transport systems responsible for vacuolar ion release, with different roles and properties. The process which has been most studied, and where the changes are best understood, is that of ABA-induced stomatal closure, where tracer efflux experiments allow the large net efflux of vacuolar K^+/ Rb^+ produced by application of ABA to isolated guard cells to be followed. The signalling chains are partially identified, and the experiments also produce evidence for the operation of regulatory systems in the control of vacuolar ion content.

This paper aims first to summarize our understanding of the process of vacuolar ion transfer, and of the properties of the two transport systems, and then to present some new flux data extending our understanding.

Background

Evidence for Regulation of Vacuolar Ion Content in Guard Cells

Figure 1 shows results from a washout experiment (MacRobbie, 2006), in which rate of loss of tracer $^{86}Rb^+$ was followed with time, before and after the addition of ABA. Figure 1*a* shows the rate of loss, in a plot of the rate constant for exchange, equal to (rate of tracer loss) / (tracer content), against time, while Fig. 1*b* shows the tracer content Q^* against time; each of these plots shows the results for 4 replicate strips. After an initial period of faster cytoplasmic exchange the rate constant falls to a low value, characteristic of vacuolar exchange; in this state the tracer remaining is essentially vacuolar, and after 80 minutes of washout 10 μM ABA was added to the bathing solution. The effect of ABA is to stimulate a transient increase in the rate of loss of tracer from the vacuole, but after the efflux transient the rate falls to a value very close to that before ABA was added. The effect of ABA does not appear to be a permanent increase in ion permeability, but looks more like modulation of a regulatory system, in which ABA has changed the regulatory 'set-point'. The experiment shown is one in which the tracer content after overnight loading was unusually variable, and in which the efflux transient was particularly large, but the same pattern was seen in all such experiments. It is very clear that the wide variability in ion content before ABA was added is not seen in the vacuolar content after treatment with ABA. For each of the 12 replicate strips in this experiment the washout curves of Q^* against time were fitted to two exponentials, to allow estimation of cytoplasmic content (Q_c) and vacuolar content (Q_v) separately. Figure 1*c* shows the vacuolar content before and after the ABA-induced efflux transient. It appears that after ABA treatment the vacuolar tracer content regulates to the same reduced set-point, irrespective of the variability before ABA treatment. Comparison of cytoplasmic and vacuolar contents in the 12 replicate strips (Fig. 1*d*) shows the variability reflects differences in vacuolar content, with the same cytoplasmic content over a wide range of initial vacuolar contents. The same conclusion was reached in other experiments in which washout curves were compared in control and ABA-treated tissue, by adding ABA for the final period of loading (longer than the duration of the ABA-induced efflux transient), as well as for the whole of the washout. The content curves were then used to estimate values of Q_c and Q_v, in the presence and absence of ABA, and it was found that ABA reduced vacuolar content but with no change in cytoplasmic content. The results suggest a regulatory system controlling vacuolar content (volume), whose

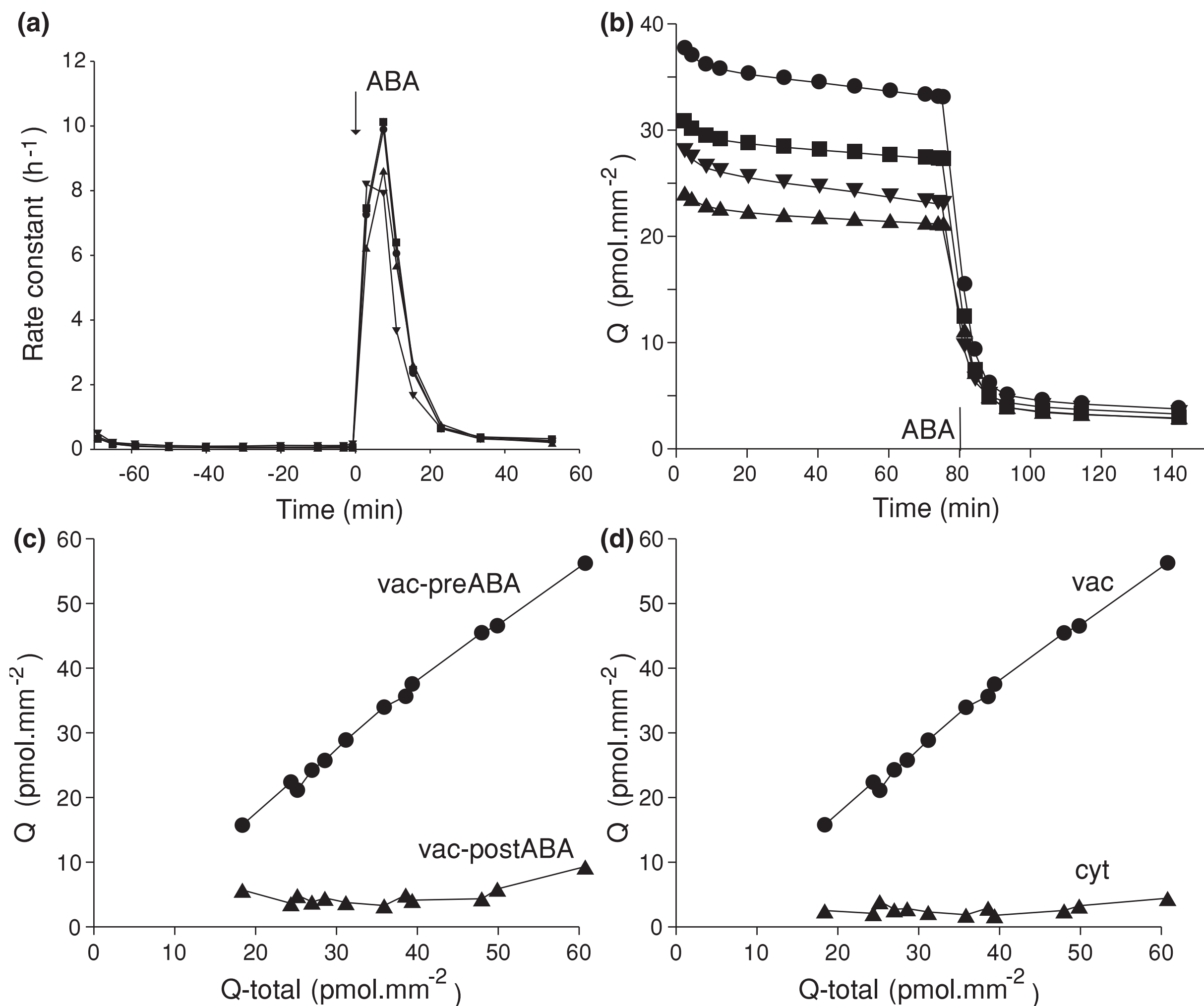

Fig. 1. Regulation of vacuolar content. ABA resets the vacuolar content to a reduced set-point. (*a*) Washout of 4 replicate strips, after overnight loading in $^{86}Rb^+$. Rate constant for exchange (rate of tracer loss / tracer content), plotted against time. 10 μM ABA added after 80 minutes of washout. (*b*) Tracer content (Q^*) plotted against time. (*c*) Washout curves of Q^* to 80 minutes were fitted to two exponentials, allowing calculation of cytoplasmic and vacuolar content at the start of the washout. Vacuolar content before ABA treatment (at the start of the washout), and after ABA treatment, plotted against *Q*-total, the total tracer content at the start of the washout, for 12 replicate strips. (*d*) Cytoplasmic (*cyt*) and vacuolar (*vac*) contents plotted against *Q*-total, the total tracer content at the start of the washout (12 replicate strips).

set-point is reset by ABA to a much lower value. Since cells of different vacuolar volume will also have different turgor pressures, the apparent regulation of vacuolar volume may reflect a regulatory system which is sensing and controlling cell turgor, via a pressure-sensitive vacuolar efflux.

Evidence that the Vacuolar Ion Efflux Tracks the Ion Content

Further evidence for involvement of a regulatory system in the control of vacuolar efflux comes from experiments in which ABA-induced efflux transients were compared at different concentrations of ABA (MacRobbie, 1995). Figure 2 presents the results of one such experiment, in which the effect of 0.1 and 10 μM ABA, added at 40 minutes of washout in the absence of ABA, were compared. Figure 2*a* (tracer content against time), and Fig. 2*b* (rate constant for loss against time), show that the efflux transient is delayed and reduced at low ABA, with the introduction of a significant lag period before its initiation, but that the same end-point is reached after the transient. The hypothesis that ABA alters the set-point of a regulatory system suggests that the guard cell can sense the ion content of the cell (or volume or turgor), and that the ion efflux at the tonoplast responds to this. The results of plotting the changing rate constant for exchange during the transient against the changing ion content, rather than against time, bear this out. Figure 2*c* shows a plot of rate constant for efflux against the reduction in ion

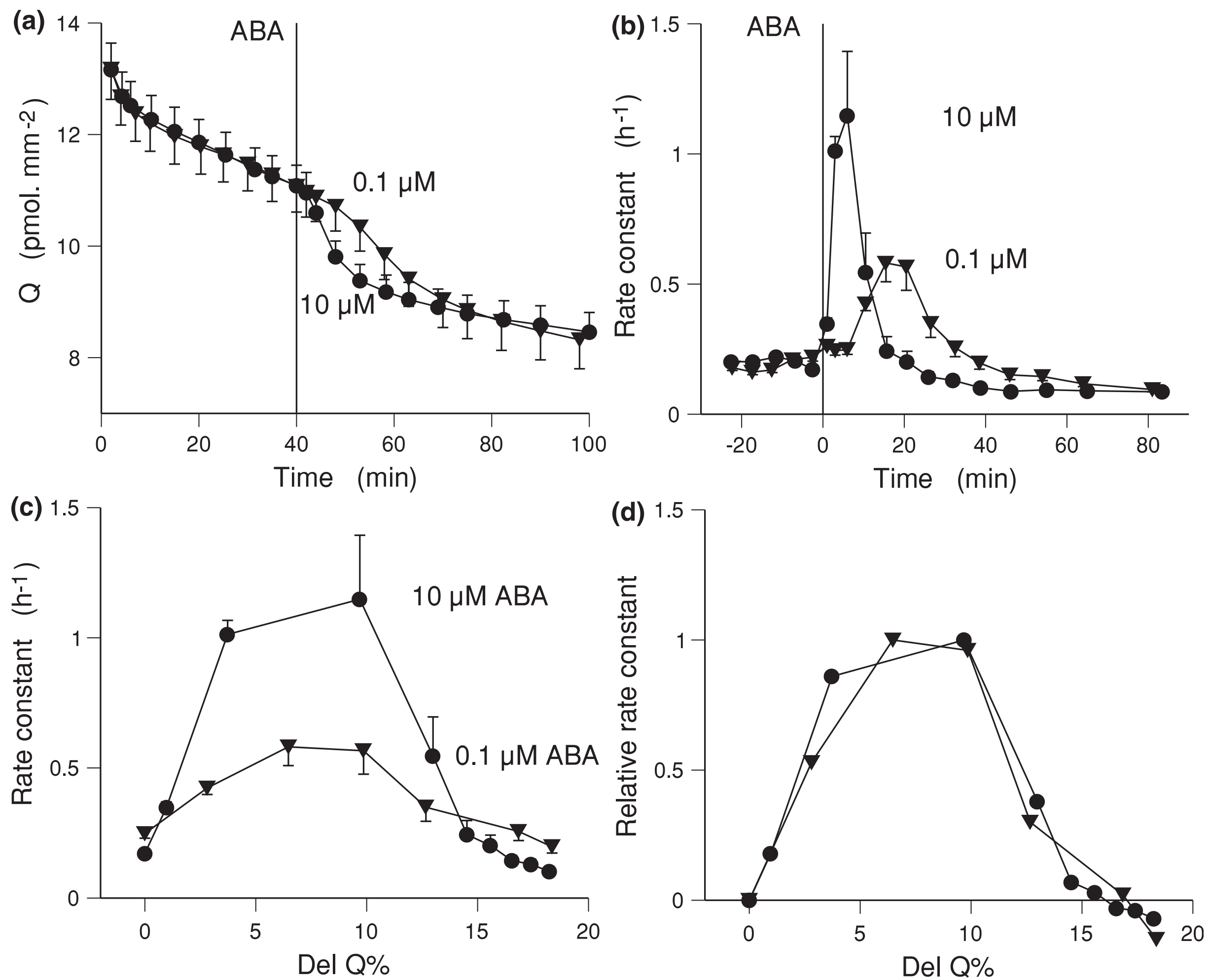

Fig. 2. Comparison of vacuolar efflux transients induced by 0.1 and 10 μM ABA. (*a*) Tracer content plotted against time of washout. ABA added after 40 minutes of washout in the absence of ABA. (*b*) Rate constant (rate of tracer loss / tracer content), plotted against time. ABA added at zero time on the graph, after 40 minutes washout in the absence of ABA. (*c*) Rate constant plotted against Del Q%, the amount lost during the transient (as the percentage of the tracer content at the start of the rise). (*d*) Relative excess rate constant, the excess rate constant as fraction of its peak value, plotted against Del Q%, the amount lost during the transient (as percentage of the tracer content at the start of the rise). The excess rate constant is the rate constant during the ABA transient minus the rate constant before the start of the rise. Each graph shows the mean of 4 replicate strips, with SEM in (*a*), (*b*) and (*c*).

content during the transient, and the two peaks are reached at the same ion content, although the peak heights still differ. If, however, the relative stimulation during the transient is plotted, the two curves coincide (Fig. 2*d*). Thus, both the peak and the end of the transient appear to be reached at specified values of content, but after different times at different ABA concentrations. The vacuolar efflux appears to track the declining ion content (or volume or turgor), during adjustment to a new set-point. However, the difference in absolute peak heights in Fig. 2*c* implies that different numbers of tonoplast channels are activated in the two conditions, but with each channel responding to ion content (or volume or turgor) in the same way.

The efflux transient can also be delayed and reduced by adding 1 mM Ba^{2+} to the bathing solution, at both 0.1 and 10 μM ABA. Comparison of the lag periods and rates of rise in a large number of transients in six different experiments showed that the ability to develop an ABA-induced efflux transient developed in two stages, with indications of two signalling thresholds. On reaching the first threshold a transient is initiated after a lag period, but with a low rate of rise (MacRobbie, 2000). As the ABA concentration increased (or Ba^{2+} was removed) more effective transients developed, in which first the length of the lag was reduced, but the rate of rise remained unchanged. In the full transient at 10 μM ABA or above, a second threshold appears to be passed, the lag disappears and the rate of rise increases significantly. Inhibitor studies suggested that the ABA-induced efflux transient is triggered by increase in cytoplasmic Ca^{2+}, but that both influx

from outside and release of Ca^{2+} stores can contribute to the increase. The smaller transient is sensitive to inhibitors of internal Ca^{2+} release, but in conditions in which the second threshold has been reached, the efflux transient seems to be sensitive to removal of external Ca^{2+}, suggesting influx of Ca^{2+} from outside makes the major contribution to triggering the large efflux transient. The results shown in Fig. 2 therefore suggest that at higher concentrations of ABA, where Ca^{2+} influx is activated, a larger number of tonoplast channels are altered by ABA, having their set-points reduced, to the new pattern of dependence on ion content or turgor.

Evidence for Pressure Sensitivity of Vacuolar Ion Release

An obvious way in which regulation of vacuolar volume could be achieved would be to have vacuolar ion fluxes sensitive to changes in cell turgor, if pressure changes (presumably perceived at the plasma membrane) were translated into a signal that modulates ion fluxes at the tonoplast. The effect of changes in cell turgor produced by changes in external osmotic pressure were therefore investigated, by adding or removing mannitol from the external solution, while following efflux of vacuolar tracer (MacRobbie, 2006). Figure 3 shows the results of such an experiment. Following a change in external osmotic pressure there will be an initial period of water flow into or out of the cell, before a new steady state of altered volume and turgor is established. Since the tonoplast is much more permeable to water than is the plasma membrane (Niemietz & Tyerman, 1997) the rate of water flow at tonoplast and plasma membrane will be nearly equal during the swelling process (Tyerman et al., 1999). Two effects can be distinguished. There is a slower response, corresponding to the new steady state with the cell in water equilibrium with the new external solution, and here the vacuolar ion efflux is reduced at low turgor on adding mannitol externally, and settles to a new higher rate after increase in turgor on removing mannitol. In 5 different experiments, the vacuolar efflux was reduced on adding 150 mM mannitol, to 0.52 ± 0.06 of its value before the addition. Thus the vacuolar ion efflux in the steady state is sensitive to turgor, providing a mechanism for osmoregulation, restoring turgor or volume towards its initial state. These changes are abolished by phenylarsine oxide (PAO), a powerful inhibitor of the resting tonoplast efflux and of the ABA-induced efflux transient, suggesting that the same tonoplast channel is involved in the resting efflux, the ABA response and regulatory system arising from pressure-sensitivity. It is likely that changes in cytoplasmic Ca^{2+} are involved in both ABA and pressure responses.

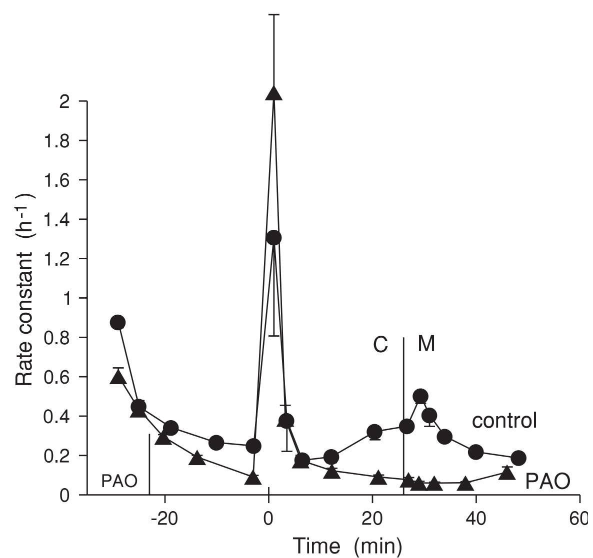

Fig. 3. Effect of hypo-osmotic and hyper-osmotic transfers on tracer efflux, in the presence and absence of 25 μM PAO. Tissue was loaded in the presence of 150 mM mannitol in the bathing solution. After washout for 35 minutes in the presence of mannitol (M) tissue was transferred, at zero time on the graph, to control solution (*C*) without mannitol for 26–28 minutes, before return to mannitol. In the PAO set, tissue was pre-treated with PAO for 18 minutes before the hypo-osmotic shock. Rate constant for exchange (rate of loss of tracer / tracer content) (in h^{-1}) plotted against time. Each point shows the mean ± SEM of 4 replicate strips.

A number of studies in other plant systems show transient increases in cytoplasmic Ca^{2+} in response to hypo-osmotic shock. The first such demonstration was in the brackish water giant alga, *Lamprothamnium*, where Ca^{2+} was involved in the hypotonic regulation in that cell (Okazaki & Tazawa, 1990). Ca^{2+} transients have also been observed in *Fucus* rhizoids (Taylor et al., 1996; Goddard et al., 2000), and tobacco cells in culture (Takahashi et al., 1997; Cessna, Chandra & Low, 1998). The Ca^{2+} transient is usually biphasic, with an initial rise attributed to Ca^{2+} influx, followed by a more prolonged second phase attributed to release of Ca^{2+} from internal stores. The most detailed description is given by Goddard et al., 2000), in *Fucus* rhizoids, identifying different spatial and temporal patterns of change in response to different size osmotic shock. Large shocks produce fast rises of Ca^{2+} in the apical region, followed by an explosive propagating Ca^{2+} wave, involving release from internal stores, initiating in the nuclear region, whereas small shocks produced delayed rises, which were more prolonged but did not propagate. Increase in Ca^{2+} in the subapical region was required for osmoregulation. A stretch-activated Ca^{2+}-permeable channel in the plasmalemma was identified as the source of the initial Ca^{2+} influx in *Fucus* (Taylor et al., 1996). There is also evidence of osmotically sensitive changes in cytoplasmic Ca^{2+} in guard cells, from the effect of external

osmotic changes on the pattern of Ca^{2+} oscillations (Hetherington et al., 1998); oscillations were suppressed on the addition of 350 mM mannitol to the bathing medium, but restored on removal of mannitol, while 150 mM mannitol generated slower oscillations of smaller amplitude. The concentration of Ca^{2+}, averaged over the cycle, appeared to increase with turgor. Several stretch-activated channels have also been identified in guard cells, permeable to K^+, Ca^{2+}, and Cl^- (Cosgrove & Hedrich, 1991). Thus it is plausible that mechanisms for osmoregulation in guard cells are similar to those identified in *Fucus*, involving Ca^{2+}-based signalling. Increase in cytoplasmic Ca^{2+} could activate a Ca^{2+}-sensitive tonoplast channel, and it should be noted that, although efflux of Rb^+ is measured here, the response could be initiated by activation of a Cl^- channel. Efflux of salt from the vacuole could be triggered by activation of either the anion or the cation flux; once the primary channel is activated, the membrane potential will change until the electroneutral position of balanced flux of anion and cation is achieved.

In addition to the slow effect of osmotic change on vacuolar ion efflux, reflecting pressure sensitivity in the new steady state, a second effect is also evident in Fig. 3, a large fast transient associated with the first few minutes after a hypo-osmotic shock, the period when water is flowing into the cell. There is no corresponding fast transient after a hyper-osmotic transfer. The fast hypo-osmotic transient is not inhibited by PAO, suggesting it involves a different tonoplast channel, whose properties are discussed in the next section.

Initial Fast Hypo-osmotic Efflux Transient

Hypo-osmotic transients were measured for different-size downshifts, in different mannitol concentrations. The peak height of the fast hypo-osmotic transient increases with the size of the shock, and thus with the rate of water flow, but is independent of the absolute value of turgor; the same transient is produced by a 50 mM decrease in external mannitol, whether starting from 150, 100 or 50 mM mannitol. The transient must represent activation of a tonoplast ion channel, since the ion efflux is moving against the direction of water flow. The effect of the increased ion flux will be to minimize cytoplasmic dilution following the hypo-osmotic shock, and this is likely to be its physiological function. The fast hypo-peak implies that the tonoplast membrane has a mechanism for sensing and responding to an osmotic gradient producing an inward water flow. The strongest candidate for the sensor is an aquaporin, but the associated signalling processes are not known. Aquaporins in plants and their functions have been reviewed (Tyerman et al., 1999; Luu & Maurel, 2005). The *Arabidopsis* genome has 10–11 homologues of the sub-group TIP, localized to the tonoplast. Two TIPS are expressed in sunflower guard cells, and conditions inducing stomatal closure, either in the diurnal cycle or in water stress, produce significantly higher levels of mRNA for one of these, *SunTIP7* (Sarda et al., 1997). The most plausible interpretation of the fast hypo-transient is that it represents an example of what may be termed the Hill hypothesis, that an important function of aquaporins is to act as sensors for gradients of osmotic or turgor pressure, and to relay such information to signaling pathways associated with control systems (Hill, Shachar-Hill & Shachar-Hill, 2004). Their proposal is that the ability to sense osmotic or turgor pressure gradients is inherent in the structure of each monomer in the tetrameric aquaporin. Each monomer is hour-glass shaped, with a narrow channel in the centre of the membrane, separating inner and outer atria. Exclusion of solutes from the atrium will create a negative pressure within the atrium, producing deformation of the protein; in the presence of either a pressure gradient or an osmotic gradient across the membrane there will be a pressure gradient between the two atria, driving the water flow though the central channel, but also producing an asymmetric deformation of the protein. Changes in pressure or osmotic conditions produce changes in water permeability, in *Chara*, in root cortical cells or in the aquaporin NOD26 (Wan, Steudle & Hartung, 2004; Ye, Wiera and Steudle, 2004; Ye, Muhr & Steudle, 2005; Niemietz & Tyerman, 1997; Vandeleur et al., 2005), thus demonstrating protein deformation in response to pressure in the atria. This implies that aquaporins can register osmotic or pressure gradients by asymmetric protein deformation, and are thus capable of acting as sensors of such gradients. The Hill hypothesis suggests aquaporins may also be involved in generating the response, by interaction with downstream signaling elements, in unspecified ways. They suggest this could be achieved by gross changes in conformation of the tetramer in response to strain and deformation in the monomers, involving switching between two configurations, of the kind seen in other tetrameric proteins, such as hemoglobin. If one or another configuration interacts with cell signaling elements, then a cell response to a changing osmotic or pressure gradient could be generated. If this hypothesis is to be invoked to explain the hypo-transient in vacuolar ion fluxes, then the asymmetry in response implies that the protein conformation produced by higher pressure in the cytoplasmic atrium is required for the response, but there are two ways in which an ion flux could be generated. One possibility is that in this particular stressed protein conformation the aquaporin becomes permeable to ions. Although aquaporins are in general impermeable to ions, there are exceptions; NOD26 (Weaver et al., 1994; Lee et al., 1995),

bovine lens MIP (Ehring et al., 1990), and Hg^{2+}-treated AQP6 in intracellular vesicles (Yasui et al., 1999; Hazama et al., 2002) have all been shown to be ion-permeable. Thus it is possible that an aquaporin is both sensor and responder. Alternatively the aquaporin could exist in a tonoplast membrane complex, associated with an ion channel, in which a conformational change in the aquaporin induced a conformational change in the ion channel. Thus, although it is very likely that an aquaporin is acting as sensor, the mechanism for generating an ion flux in response to a hypo-osmotic transfer is unknown.

Further Experiments

In view of the evidence for involvement of an aquaporin in regulation of tonoplast ion fluxes, the effect of inhibitors of aquaporins on vacuolar ion efflux was investigated, with the results reported below. Two such inhibitors were used, Hg^{2+}, and silver sulfadiazine (Niemietz & Tyerman, 2002), and both proved effective. The results are most easily explained if treatment with either inhibitor induces ion permeability in an aquaporin in the tonoplast.

Materials and Methods

Flux measurements were as described previously (MacRobbie, 2002). Isolated guard cells of *Commelina communis* L. were prepared by treatment of epidermal strips at low pH, to kill all cells other than guard cells. Cells were loaded by incubation overnight (about 16 h) in solutions containing 2 mM RbCl, 0.1 mM $CaCl_2$, 10 mM PIPES pH 6, labelled with $^{86}Rb^+$ (Amersham Pharmacia). Thus Rb^+ is used as an analogue for K^+, not as a tracer. Effluxes were measured by transferring individual strips to successive 0.75-ml portions of non-radioactive solutions, in the wells of plastic culture chambers on a vibrating shaker, and counting both these washout solutions and the residue left in the tissue at the end of the efflux.

Tracer was expressed as pmol·mm^{-2}, on the basis of area of each individual strip, and the rate of loss was calculated for each time interval. During the washout the total tracer content in the tissue (Q^*) will be represented by the sum of two exponentials, reflecting exchange in two compartments, the cytoplasm (fast component) and vacuole (slow component). The rate constant for exchange (h^{-1}) was calculated as (rate of loss of tracer) / (tracer content), and plotted against time. In constant conditions, the rate constant falls with time as the cytoplasmic efflux proceeds, to reach a steady value equal to the rate constant for vacuolar exchange when the slow phase is reached. When conditions are changed during the slow phase of exchange, the effect on the vacuolar flux can be assessed.

Curves of tracer content against time were fitted to two exponentials, and cytoplasmic and vacuolar contents (Q_c and Q_v) calculated from the intercepts and rate constants of this fitting (MacRobbie, 1981). Q_c and Q_v are close to, but not equal to, the fast and slow intercepts, since some tracer is transferred to the vacuole during the period of fast cytoplasmic exchange during the washout.

Results

Effects of Aquaporin Inhibitors on Vacuolar Efflux

Figure 4*a* shows the effect of adding 2, 5 or 25 μM $HgCl_2$ to the bathing medium after 40 minutes of washout, during the period of vacuolar exchange, as a plot of the rate constant for exchange against time. At each concentration the rate constant falls to a minimum before starting to rise; at 25 μM Hg^{2+} there is then a large efflux transient with a peak at 18 minutes, but at the lower concentrations the rise is delayed and less steep, and no peak is reached in the 80 minutes of washout in the presence of Hg^{2+}. Figure 4*b* shows that 25 μM PAO, which inhibits the resting vacuolar efflux and completely blocks the ABA-induced efflux transient, does not abolish the peak induced by Hg^{2+}. In this experiment the peak in the presence of PAO was 75 ± 18 % of the control, but in a second experiment the Hg^{2+}-induced peak was 136 ± 20 % of the control. This suggests that the stimulation of vacuolar efflux on adding Hg^{2+} involves a separate channel from that involved in the ABA response.

The effect of silver sulfadiazine (AgS), another aquaporin inhibitor (Niemietz & Tyerman, 2002), was also tested, as shown in Fig. 5, in which roughly similar peaks were produced by 25 μM Hg^{2+} or 10 μM silver sulfadiazine. Gold, in the form of $HAuCl_4$ was also tested, but was ineffective.

Effects of Aquaporin Inhibitors on Cytoplasmic and Vacuolar Contents and Rate Constants

The addition of either Hg^{2+} or silver sulfadiazine produces a marked transient increase in vacuolar efflux, but as with ABA the rate constant then falls again to a value similar to that before the inhibitor was added, but at a much reduced ion content. The contents and rate constants for exchange of vacuolar and cytoplasmic compartments were compared in washout curves after varying periods of treatment with AgS or Hg^{2+}, by comparison of efflux curves after varying time of exposure to the inhibitor. In the treated tissue the inhibitor was added for the final period of the loading, and was present for all of the washout time. Figure 6*a* shows the washout curves for the control, in which 10 μM AgS was added after 40 minutes of washout, and for 4 sets of tissue pretreated in AgS for 1.5, 3.6, 4.2 and 5 h before the start of the washout. The early stages of the curves of total tracer content shown in Fig. 6*a* were fitted to two exponentials, and Fig. 6*b* shows values for the rate constants for cytoplasmic and vacuolar exchange, for these treatments. The vacuolar rate constant is high over a few hours of treatment, but then falls again, while there is no effect on the cytoplasmic rate

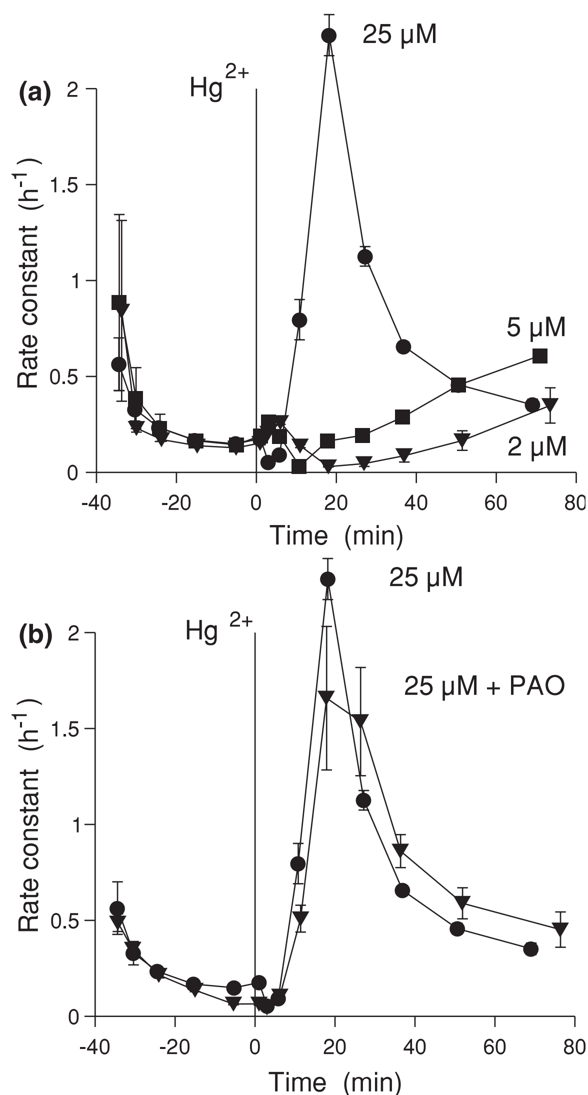

Fig. 4. Effect of Hg^{2+} on the vacuolar efflux. Tissue was loaded overnight with $^{86}Rb^{+}$, before start of the washout. After 40 minutes of washout $HgCl_2$ was added at 2, 5 or 25 μM, or at 25 μM in the presence of 25 μM PAO (added 20 minutes before treatment with Hg^{2+}). Rate constant for exchange plotted against time; mean ± SEM of 4 strips. (*a*) 2, 5 or 25 μM AgS (*b*) 25 μM AgS in the presence and absence of 25 μM PAO.

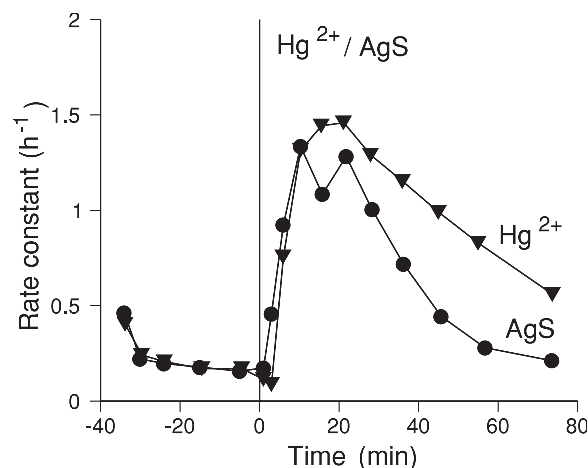

Fig. 5. Comparison of the effect of Hg^{2+} and AgS on the vacuolar efflux. Rate constant for exchange plotted against time, with $HgCl_2$ (25 μM) or AgS (10 μM) added after 40 minutes of washout.

constant. Thus the effect is on a channel in the tonoplast, and there appears to be no effect at the plasmalemma.

This experiment also gave values for cytoplasmic and vacuolar content after varying time of exposure to the inhibitor, estimated from the intercepts and rate constants, with the results shown in Fig. 6*c*. It is clear that there is a marked decrease in vacuolar content after AgS treatment with no associated change in cytoplasmic content.

Figure 7 shows a similar experiment with 25 μM Hg^{2+}, over 4 hours of treatment. In this experiment the vacuolar content fell over about 2 hours from 50 ± 1.6 pmol·mm^{-2} to 20 ± 2 pmol·mm^{-2}; apertures in treated and untreated tissue were, as expected, very different, falling from 10.5 ± 0.4 (n = 32) μm in the untreated tissue to 2.7 ± 0.2 (n = 48) μm after 4 hours treatment. Comparisons were also made in two other experiments, at 10 and 25 μM Hg^{2+} over 6 h, and at 5 and 25 μM Hg^{2+} over 4.3 h. In every case the cytoplasmic content was unaffected, but the vacuolar content fell markedly over the first 2 hours of treatment, but remained constant thereafter.

These experiments show that the plasmalemma flux over the period 1–5 hours of treatment with inhibitor is not different from the untreated control. It is difficult to assess the effect on the plasmalemma flux at shorter times. The peak of the vacuolar transient is around 20 to 30 minutes of treatment, and since fluxes are changing during this period it is not easy to assess the effect on the plasmalemma. The best indication that there is no effect at the plasmalemma comes from an experiment in which efflux curves were measured for the addition of 25 μM Hg^{2+} at 40 minutes of washout, which showed a transient with a peak at 20 minutes of treatment, and for a 20-minute pre-treatment with Hg^{2+} in the loading solution. The initial rates of loss were 30 ± 3 (n = 8) pmol·mm^{-2}·h^{-1} in the control, and 66 ± 3 (n = 4) pmol·mm^{-2}·h^{-1} in the Hg^{2+}-pre-treated tissue, a difference of 36 ± 4. In the efflux transient measured when Hg^{2+} was added after 40 minutes of washout the increase in vacuolar rate to the peak at 20 minutes was 24 ± 8 pmol·mm^{-2}·h^{-1}, and thus the extra vacuolar contribution accounts for most of the difference in the two initial rates. There does not appear to be any extra effect of these inhibitors on the efflux at the plasmalemma, in spite of a marked increase in vacuolar efflux during the transient, suggesting a specific

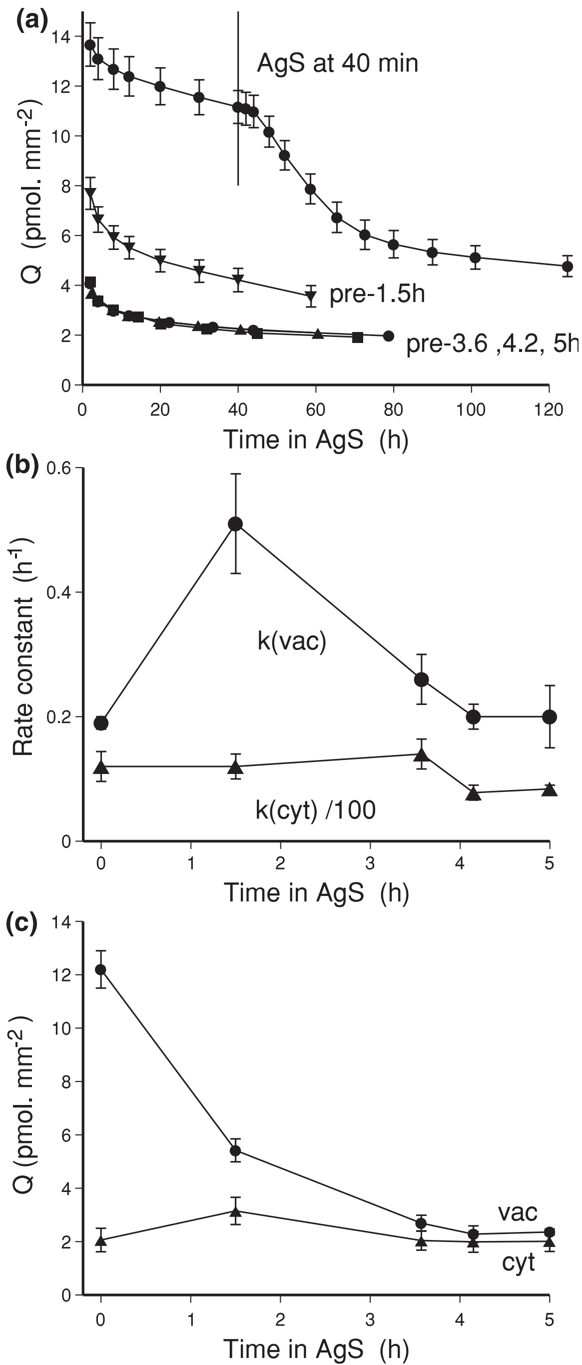

Fig. 6. Changes in cytoplasmic fluxes and content with time of treatment with AgS. Tissue was loaded overnight with $^{86}Rb^{+}$. In the control, 10 μM AgS was added after 40 minutes of washout in the absence of AgS. In the other sets, AgS was added for the final loading period, for 1.5 to 5 hours, and was present throughout the washout. The early stages of the washout curves were fitted to two exponentials for calculation of cytoplasmic and vacuolar rate constants, fluxes and contents. (*a*) Plot of tracer content against time of washout. (*b*) Cytoplasmic and vacuolar rate constants, plotted against time of exposure to AgS. (*c*) Cytoplasmic and vacuolar contents, plotted against time of exposure to AgS. Each plot shows mean and SEM of 4 replicate strips.

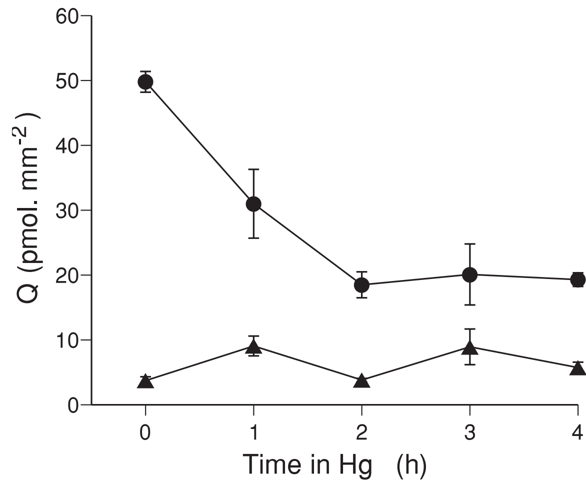

Fig. 7. Changes in cytoplasmic and vacuolar contents with time of treatment with Hg^{2+}. Tissue was loaded overnight with $^{86}Rb^{+}$. In the control, 25 μM Hg^{2+} was added after 40 minutes of washout in the absence of Hg^{2+}. In the other sets, Hg^{2+} was added for the final loading period, for 1 to 4 hours, and was present throughout the washout. The early stages of the washout curves were fitted to two exponentials for calculation of cytoplasmic and vacuolar contents, shown plotted against time of exposure to Hg^{2+}. Each plot shows mean and SEM of 4 replicate strips.

effect on a tonoplast channel. Once the vacuolar content has reached its new reduced level, the rate constant for vacuolar exchange is equal to the value before the inhibitor was added, and thus the tonoplast flux is reduced after the transient, but only in proportion to the vacuolar content.

The effect of AgS on the hypo-osmotic transient was also tested, and there was still a clear response to the removal of mannitol, a fast hypo-osmotic transient. It is difficult to compare the two transients, in the presence and absence of AgS, for two reasons. The first is that the vacuolar contents at the time of the hypo-osmotic shock were very different, after loss of a very large fraction of vacuolar content during the AgS transient. The second is that if AgS inhibits the water permeability of the aquaporins, reducing the water flow in response to the osmotic gradient, the two transients will be generated by different rates of water flow. The fact that there is still a marked response shows that AgS does not abolish the hypo-osmotic efflux transient, but also suggests that the water permeability is not strongly inhibited by AgS.

Discussion

There are two different systems involved in the flux of ions from vacuole to cytoplasm, with different inhibitor sensitivities, and presumably different roles in the regulation of vacuolar volume and turgor. There is first the Ca^{2+}- sensitive channel involved in the response to ABA, and in the pressure-sensitivity.

This is strongly inhibited by PAO. The flux through this channel is measured in the new steady state established after the volume change following an osmotic change, with a new turgor and internal osmotic pressure, in water equilibrium with the changed external osmotic pressure. The rate of vacuolar ion release reduces as turgor is reduced by adding mannitol outside, and is increased at the increased turgor following removal of mannitol. This provides a system of osmoregulation, which will tend to restore the original turgor. A similar process in algae has been recognized for many years. In the guard cell this appears to involve a system for regulating tonoplast efflux in response to turgor changes, regulating turgor via control of vacuolar volume.

The details of the ABA response are difficult to interpret in signaling terms. PAO strongly inhibits the resting tonoplast efflux, and abolishes both the ABA-induced efflux transient, and the responses to turgor, suggesting that all are mediated by the same channel. The ABA effect on tonoplast flux is transient, suggesting that ABA resets the set-point of a pressure-sensitive control system, and this view is reinforced by the fact that during the transient the efflux tracks the ion content, but over a different time scale in optimal and sub-optimal conditions. It would appear that different numbers of tonoplast channels are altered by different concentrations of ABA, in having their set-point reduced, but with each channel showing the same dependence on turgor, responding to the new error signal, the difference between the ion content (volume, turgor) and the new set-point. It has been argued that the ABA response involves activation of a Ca^{2+}- triggered channel, with different relative contributions from Ca^{2+} influx and internal Ca^{2+} release at different concentrations of ABA. At 10 μM ABA, Ca^{2+} influx plays the major role, whereas at 0.1 μM ABA, Ca^{2+} release makes the major contribution. If these conditions alter the state of different numbers of channels then it implies that the number of channels affected is sensitive to the source of Ca^{2+}, and the site of its production. The signalling intermediate by which the channel senses turgor, perceived at the plasma membrane, is uncertain. There is evidence (already discussed) that in several plant systems a hypo-osmotic shock induces a transient increase in cytoplasmic Ca^{2+}, and a stretch-activated, Ca^{2+}-permable channel has been suggested (Taylor et al., 1996), providing a role for Ca^{2+} in the pressure sensitivity. However, it is difficult to reconcile the different postulated roles for Ca^{2+} in the overall turgor regulatory system and in the response to ABA. The detailed signalling pathways are far from resolved.

The second ion transport pathway in the tonoplast is not inhibited by PAO, and is responsible for the fast hypo-osmotic transient, responding to inward water flow by increase in ion efflux, dependent on the size of the osmotic gradient, the rate of water flow, and not on the absolute osmotic conditions. A tonoplast channel sensitive to osmotic gradients has been observed in beet vacuoles (Alexandre & Lassalles, 1991), but this responded symmetrically to hyper-osmotic and hypo-osmotic gradients, whereas the present response is strictly asymmetric, responding only to inward water flow. It is suggested that an aquaporin is the most likely sensor of water flow, with the ionic response initiated by deformation of the aquaporin protein, as suggested by Hill et al. (2004). The deformed aquaporin might then become ion-permeable, or might signal to an associated ion channel in a membrane complex. In either case the aquaporin is involved in regulation of vacuolar volume, and the process will minimize cytoplasmic dilution following a hypo-osmotic shock. It will certainly affect cytoplasmic osmotic conditions. The amount of extra ion transfer is only a small fraction of the vacuolar content, but is large in relation to the cytoplasmic content. The extra loss of vacuolar Rb^+ during the hypo-transient generated by removal of 150 mM mannitol was 3.9 ± 0.8 (8)% of the vacuolar content, but since the cytoplasmic content is only about 5–10% of the vacuolar content this represents a large amount in cytoplasmic terms, transferred from vacuole to outside through the cytoplasm. The question arises whether this system has any other physiological role in the guard cell. The system would prevent the generation of large osmotic gradients across the tonoplast during salt accumulation, regulating the rate of swelling. If the water permeability of the tonoplast was down-regulated for some reason, then, as salt transfer generated a larger osmotic gradient across the tonoplast, ion release would be initiated, reducing the net influx to the vacuole. Net salt accumulation would be regulated by the ability of the water flux to follow. There is evidence that plasma membrane aquaporins can exist in a closed state and be subsequently activated during a hypo-osmotic treatment (Moshelion, Moran & Chaumont, 2004), and that in other instances the water permeability of aquaporins can be inhibited by low temperature or by mechanical stress (Lee, Chung & Steudle, 2005). Thus it is possible that tonoplast water permeability might be restricted in some conditions, and the mechanism proposed would prevent the generation of large osmotic gradients across the tonoplast if salt accumulation was still active. The amounts of ion movement will not have a large effect on vacuolar content, but will have a very large effect on cytoplasmic ion content and osmotic pressure.

The effect of inhibitors of aquaporins, Hg^{2+} and AgS, on vacuolar efflux was unexpected. The marked increase in efflux at the tonoplast over the first 1–2 hours of treatment led to a large reduction in vacuolar ion content, to 20–40% of the initial value. However, the vacuolar rate constant then

decreases again, and the vacuolar content stabilizes at this low value. This flux is not sensitive to PAO, suggesting it involves the same channel as is responsible for the fast hypo-osmotic efflux transient, and the simplest explanation is that both processes involve an aquaporin. As with the hypo-osmotic transient, there are two ways in which an aquaporin could induce increased vacuolar efflux. It could be that treatment with these inhibitors renders the aquaporin permeable to ions, but it could also involve an aquaporin signaling to an associated ion channel and activating it. It is interesting that the established example of an aquaporin rendered ion-permeable by Hg^{2+} treatment is in an intracellular membrane, AQP6 in intracellular vesicles from renal epithelia (Yasui et al., 1999; Hazama et al., 2002). There are two suggestions of a role for aquaporins in internal osmoregulation in animal systems. AQP1 in rat pancreatic secretory vesicles has been shown to be involved in a GTP-driven swelling response (Cho et al., 2002), and there is evidence for a role for an aquaporin (AQP5) in osmoregulation in parotid salivary cell secretory granules (Matsuki et al., 2005). The simplest explanation of the present results is that deformation of a tonoplast aquaporin by an osmotic gradient driving an inward water flow, or treatment with Hg^{2+} or AgS, both produce a protein conformation that is ion-permeable. Such a system would play a role in internal osmoregulation, in regulation of vacuolar volume, and it may well be that involvement of aquaporins in osmoregulation in internal membrane compartments is widespread and an important unrecognized function of these proteins. The wider role of aquaporins as osmotic sensors and regulators in many systems was proposed by Hill et al. (2004), and regulation of vacuolar volume in guard cells may be an example of their hypothesis in operation.

In summary, there appear to be two independent systems involved in osmoregulation in guard cells. Turgor appears to be regulated by a PAO-inhibited transport system in the tonoplast, in which ion efflux from the vacuole responds to turgor, regulating vacuolar volume and hence cell turgor; the signaling mechanisms by which pressure, perceived at the plasma membrane, can modulate the tonoplast flux remain uncertain. The second system is PAO-insensitive, and most likely involves an aquaporin. Ion efflux from the tonoplast is activated by inward water flow, providing internal osmoregulation between cytoplasm and vacuole. Whether the aquaporin is both sensor and responder, acting directly as an ion channel, or whether it signals to an associated ion channel in a membrane complex is not known. Efflux at the tonoplast is also stimulated by inhibitors of aquaporins, Hg^{2+} and AgS, probably also involving the aquaporin, rendered ion-permeable by these inhibitors.

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