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An electrogenic pump in the xylem parenchyma of barley roots

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Analysis of an electrogenic pump in the plasma membrane of xylem-parenchyma protoplasts from barley roots was performed using the patch-clamp technique in the whole-cell configuration. Particularly with regard to understanding xylem loading and unloading, the study of the electrogenic pump from this cell type is important; its functional confirmation was lacking to date. About one-half of the investigated protoplasts displayed current responses with reversal potentials between −80 and −200 mV. The application of fusicoccin, an H^+-pump stimulator, caused an increase in currents recorded at a membrane potential of 0 mV and a shift of the reversal potential by about −50 mV. Treatment with dicylohexylcarbodiimid, an H^+-pump inhibitor, resulted in the reduction of the current at 0 mV. The Ca^{2+}-pump inhibitor, erythrosin B, showed no effect on current density at 0 mV and on the polarisation of the membrane potential. Enlarging the transmembrane pH gradient by raising the pH of the extracellular solution from 5.8 to 8.8 stimulated the currents. These are strong indications that the electrogenic pump was an H^+-pump. Neither intracellular pH nor the intracellular Ca^{2+} concentration affected its activity. Simultaneous activity of the electrogenic pump and anion conductances could produce states in which protoplasts exhibited 'intermediate' reversal potentials. It was concluded that the electrogenic pump was not directly involved in the loading of KCl and KNO_3 into the xylem but, in combination with anion channel activities, contributed to the establishment of membrane potentials at which electroneutral salt transport and acid release can proceed.

Introduction

The transport of nutrient ions from the soil to the shoot of a plant proceeds in two stages: (1) by absorption into the root symplast and (2) by transfer from the symplast to the stelar apoplast, in which the xylem vessels function as conduits for the flow of water and ions to the shoot. Electrogenic mechanisms, in general proton pumps, produce electrical potential differences and differences in pH across the plasmalemma of the cells which absorb ions from the soil solution, and this electrochemical potential difference is large enough to extract ions like K^+, NO_3^- or Cl^- from the soil. Fisher et al. (1970)

Abbreviations – DCCD, dicylohexylcarbodiimid; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid; FC, fusicoccin; Glc, gluconate; TEA^+, tetraethylammonium; X-IRAC, inwardly rectifying anion channel; X-QUAC, quickly activating anion conductance; X-SLAC, slowly activating anion conductance (X stands for xylem parenchyma).

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demonstrated that uptake of K^+ correlated with hydrolysis of adenosine 5′-triphosphate (ATP). The latter process is electrogenic; H^+ is being released. By titration it was determined that H^+ release and K^+ uptake of barley roots occurred at a charge ratio of 1:1 (Behl and Raschke 1987). The proton gradient produced by the electrogenic H^+-pump provides also the driving force for proton–anion cotransport during anion uptake (Dunlop 1989, McClure et al. 1990, Ullrich and Novacky 1990). High- and low-affinity K^+- and NO_3^- transporters have been identified on the molecular level, although, with regard to K^+ uptake, their mode of action in the plant is not yet clear (Forde 2000, Rodrígez-Navarro 2000, Véry and Sentenac 2003).

Opposing views exist on whether the transfer of ions into the xylem is an active process. Pitman (1972) reported that uptake and transfer of Cl^- by barley roots were inhibited by the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). He concluded that it is also necessary to have a second, active transport prior to entry to the xylem. The evidence for this two-pump hypothesis of transport of KCl was convincing. On the other hand, stelar cells are equipped with K^+- and anion channels (Gaymard et al. 1998, Gilliham and Tester 2005, Köhler and Raschke 2000, Roberts and Tester 1995, Wegner and Raschke 1994). The K^+ content of the shoot was reduced significantly in knockout mutants of *Arabidopsis* lacking the K^+ channel SKOR (Gaymard et al. 1998), as was the K^+ concentration in the xylem sap after inhibition of the K^+-selective outwardly rectifying conductance in a xylem-perfusion experiment by Wegner and De Boer (1997). These results indicate strongly that K^+ channels serve as major pathways for the release of K^+ into the xylem. An estimation of salt fluxes into the xylem based on patch-clamp measurements from the xylem parenchyma of barley roots and the comparison with transport rates from barley roots reported by Pitman (1971) led to the inference, that K^+ and anion conductances allowed an electroneutral, thermodynamically passive release of KCl and KNO_3 into the xylem (Köhler and Raschke 2000, Wegner and Raschke 1994). No need appeared for a pump energising the transfer of salts to the xylem, supporting the view of Dunlop and Bowling (1971) that radial transport is driven by an active step at the outer surface of the root whilst movement from the living cells into the vessels is passive. However, H^+-pumps are ubiquitous and important for membrane energisation (Palmgren 1994, 2001, Sondergaard et al. 2004, Sze et al. 1999). In stelar cells, a number of transporters of which most are very likely driven by the pH gradient were discovered: two types of Na^+ transporters, which might function in retrieving Na^+ from the xylem (Shi et al. 2002) and loading Na^+ into the xylem (Hall et al. 2006), sulphate transporters, most likely reabsorbing sulphate from intercellular spaces (Kataoka et al. 2004, Takahashi et al. 2000), an amino acid symporter, which may function in uptake of amino acids from the xylem sap (Okumoto et al. 2002) and a boron transporter, involved in boron transport into the xylem (Frommer and von Wirén 2002, Takano et al. 2002). Xylem-parenchyma cells of barley roots were strongly labelled by antibodies against the plasma membrane H^+-ATPase (Samuels et al. 1992). Activity of an electrogenic pump appeared frequently in xylem-parenchyma cells of barley roots (Köhler and Raschke 1998). This observation prompted a study of its characteristics and a consideration of its possible function in xylem-parenchyma cells. Here we report results of this work and address the function of the electrogenic pump in combination with anion conductances. So far, mostly the concerted action of the H^+-pump and cation conductances has been considered (De Boer and Volkov 2003, Tyerman et al. 2001) but not its importance for anion and acid release.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Apex; Cebeco Zaden BV, Lelystad, Netherlands) was grown hydroponically on constantly aerated full-strength Long–Ashton nitrate-type solution (Hewitt and Smith 1975) with a day/night rhythm of 12/12 h and 20/18°C. Quantum flux from fluorescent tubes (L65W/25S, Osram, München, Germany) was 300 μmol m^{-2} s^{-1}. Protoplasts of xylem-parenchyma cells were isolated from roots of 3- to 5-week-old plants as described by Köhler and Raschke (2000) and Wegner and Raschke (1994).

Electrical recording and solutions

The properties of the electrogenic pump in the plasma membrane were investigated by the patch-clamp technique in the whole-cell configuration (Hamill et al. 1981) by the use of an EPC-7 amplifier (List Electronic, Darmstadt, Germany). Pipettes were prepared with a glass capillary puller (L/M-3P-A, List Electronic) from borosilicate glass capillaries (Kimax-Glass 34500-99, Witz Scientific, Maumee, OH) coated with Sylgard (Dow Corning, Midland, MI), and fire polished (L/M-CPZ-101, List Electronic). Electrode tip potentials were nulled during the patch-clamp procedure. All recordings were made at temperatures between 22 and 24°C. Liquid junction potentials between pipette and bath solutions were measured and corrected for according to the method of Neher (1992).

The Pulse and Pulse Fit programs of Heka Elektronik, Lambrecht, Germany, were used for pulse generation and

data analysis. Voltage ramps, usually extending from −200 to 100 mV within 1 s, were applied to obtain current–voltage curves. Holding potentials were typically −50 or −90 mV. Frequently, reverse voltage ramps were interspersed to verify that effects of a minor slow capacitance, which could not be completely compensated, did not affect significantly the recorded current–voltage relationships. Current–voltage curves obtained from voltage ramps and voltage pulses were identical (not shown). Data were filtered at 300 Hz. For correct data acquisition, the sampling frequency was at least four times the filter frequency.

Each parenchyma cell disintegrated into an average of six protoplasts during preparation (Wegner and Raschke 1994). To compare measurements obtained with different protoplasts, currents were normalised to current density, j (μA cm^{-2}) and plotted against membrane potential, U (mV). The specific plasma membrane capacity of xylem-parenchyma protoplasts was 0.9 μF cm^{-2} (Wegner and Raschke 1994). In case of stimulation or inhibition, the current measured at the end of each experiment was related to the current before the application of the substance. Mean values of measured data are given with standard error.

For the investigation of the electrogenic pump, solutions were designed which aimed at the repression of ion conductances which would disturb the detection of the pump current. The bath solution representing the apoplastic space was (m*M*) 5 $Ca(Glc)_2$ (or $Ca(OH)_2$), 8 $MgCl_2$, 10 Mes, pH 5.8 (adjusted with Tris). The intracellular solution is filled into the pipette and equilibrates with the cytoplasm. The standard intracellular solution was (m*M*) 10 Mg^{2+}-ATP, 5 μ*M* free Ca^{2+} (added as $Ca(Glc)_2$ or as $Ca(OH)_2$), 10 m*M* $MgCl_2$, 10 *N*-hydroxyethyl-ethylenediamine-tri-acetic acid, 10 Tris, pH = 7.2 (adjusted with Mes). These solutions are referred to as pump solutions. Anion solutions were designed for the investigation of anion currents (see Köhler and Raschke 2000, Köhler et al. 2002) and were (m*M*) 30 tetraethylammonium chloride (TEACl), 5 $Ca(Glc)_2$, 2 $MgCl_2$, 10 Mes, pH 5.8 (adjusted with Tris) (bath solution) and 120 TEACl, 0.15 μ*M* free Ca^{2+} (added as $Ca(Glc)_2$), 10 EGTA, 2 ATP, 2 $MgCl_2$, 10 Tris, pH 7.2 (adjusted with Mes) (pipette solution). The osmolality of all bath and pipette solutions was adjusted with mannitol to 500 and 530 mosmol kg^{-1}, respectively, using a vapour pressure osmometer (5100 C, Wescor, Logan, UT). Total and free concentrations of divalent cations were calculated using the program 'Calcium' (Führ et al. 1993). Stock solutions of fusicoccin (FC), dicylohexylcarbodiimid (DCCD), 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) and erythrosin B were prepared and added to obtain the final concentrations given. Final ethanol (in case of FC) or acetone content (in case of DCCD) was below 1% (v/v) and had no effect on ion currents or membrane voltages.

Equilibrium potentials were calculated according to the Nernst equation using ion activities. Using pump solutions they were 0 mV (Cl^-), 96 mV (Ca^{2+}), 81 mV (H^+) and 25 mV (Mg^{2+}). Using anion solutions, equilibrium potentials were 30 mV (Cl^-), 143 mV (Ca^{2+}), 81 mV (H^+) and 12 mV (Mg^{2+}). It is unlikely that TEA^+ and Glc^- (gluconate) contributed to the membrane potential but if TEA^+ and Glc^- would pass non-specific ion channels, the activity of such channels would drive the membrane potential to 0 mV.

Sign convention

Membrane potentials are defined as the voltage on the cytoplasmic side of the membrane with respect to the physiological outside. In the whole-cell configuration the voltage between the pipette and the reference electrode corresponds to the membrane potential. A positive current corresponds to a cation efflux from the protoplast or to an anion influx into the protoplast, and vice versa with regard to a negative current.

Results

Identification of an electrogenic pump

In xylem-parenchyma protoplasts prepared from barley roots, the current response to voltage ramps displayed reversal at negative potentials and a saturating current in 77 out of 170 experiments (=45%) in the whole-cell configuration (Fig. 1) under conditions when other ion conductances were repressed. This response is typical for electrogenic pumps (Läuger 1991). Currents reversed between −80 and −200 mV. The mean value of membrane potentials measured in the current clamp modus was −140 ± 5 mV. Nernst potentials of all ions present in

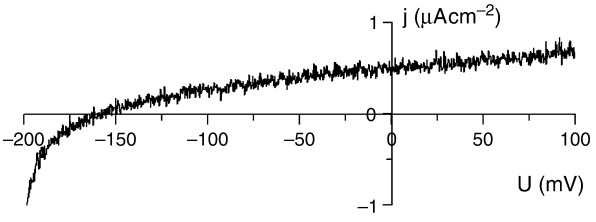

Fig. 1. Current response of an electrogenic pump with a reversal potential around −160 mV and a current approaching saturation. Currents across the plasma membrane of xylem-parenchyma protoplasts were measured in response to a voltage ramp (see Materials and methods). Pump solutions with $Ca(Glc)_2$ were used.

the solutions were much more positive than this range (see Material and methods).

Effects of FC, DCCD and erythrosin B

For the identification of the electrogenic pump the H^+-pump stimulator FC and the inhibitor DCCD were applied. FC and DCCD are commonly used to assign measured currents to H^+-pump activity (Findlay et al. 1994, Tyerman et al. 2001). After an external application of 1 μM FC, both the current at 0 mV increased and the membrane potential hyperpolarised indicating an increase in pump activity (Fig. 2A). On average the shift of the reversal potential was around −50 mV and currents at 0 mV were stimulated 2.3-fold (n = 11). After the removal of FC, the current returned to its original level. In contrast, the addition of DCCD to the extracellular solution (resulting in a concentration of 100 μM) caused a strong inhibition of the current at 0 mV (n = 8, Fig. 2B). On average, currents at 0 mV decreased 10.6-fold, which resulted in (more or less) their disappearance. The membrane potential was shifted towards 0 mV, on average, by around 50 mV. The inhibition by DCCD was irreversible.

Erythrosin B was applied at the high concentration of 1 mM to suppress a possible activity of Ca^{2+}-ATPases. No effect appeared on current density at 0 mV, nor on the polarisation of the membrane potential (n = 3, Fig. 3).

Effects of changes in the transmembrane pH gradient

An increase in extracellular pH increases the driving force for proton pumping. As it would be expected for an H^+-pump, the current was stimulated by an enlarged transmembrane pH gradient and the membrane became hyperpolarised (n = 5, Fig. 4). After raising the extracellular pH from 5.8 to 8.8 (corresponding to a potential increase by about 177 mV), the current at 0 mV increased by up to five-fold. On average, current density at 0 mV increased two-fold and the membrane potential shifted about −60 mV.

Effects of changes in intracellular proton concentrations by factors of 2 were measured by comparing whole-cell currents at a pH in the pipette of 6.9 with those of 7.2 and 7.5. The small differences in reversal potentials were not statistically significant (Table 1). Apparently, the activity of the electrogenic pump was not affected by changes in intracellular pH.

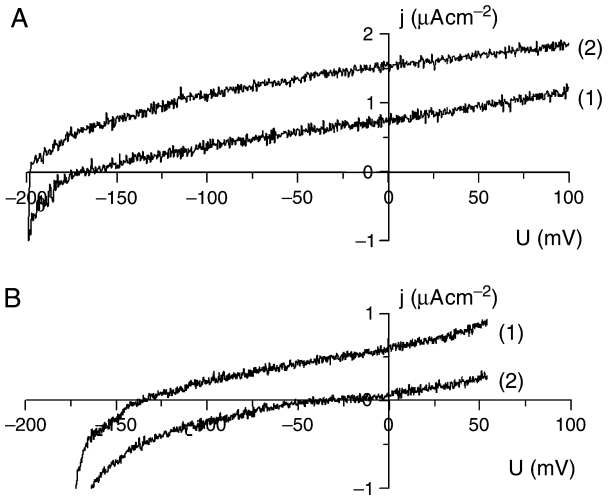

Fig. 2. (A) Stimulation of the current by FC. (B) Inhibition of the current by dicylohexylcarbodiimid (DCCD). Representative current responses to voltage ramps (1) before and (2) after the addition of 1 μM FC or 100 μM DCCD to the bath are shown. Pump solutions with $Ca(Glc)_2$ were used.

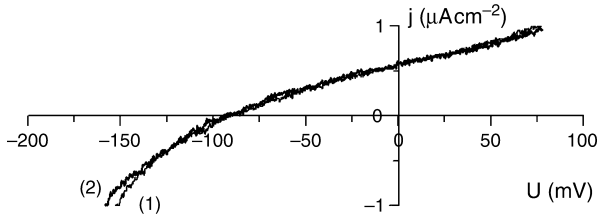

Fig. 3. No effect of erythrosin B. A representative current response to a voltage ramp (1) before and (2) after the addition of 1 mM erythrosin B to the bath is shown. Pump solutions with $Ca(OH)_2$ were used.

Characteristics of the pump current

Average current density at U = 0 mV, which is taken as a measure of the pump current, was 0.57 ± 0.06 μA cm^{-2} (Table 2, column 6). To avoid erroneous pump current measurements, only those experiments were analysed in which no conspicuous Cl^- or K^+ currents were evident. In t-tests with a $P = 0.05$ a significant change of the current density with low (0.15 μM) and high (5 μM) intracellular Ca^{2+} could not be discovered (Table 2, t-tests between columns 1 and 6 and columns 1 and 4).

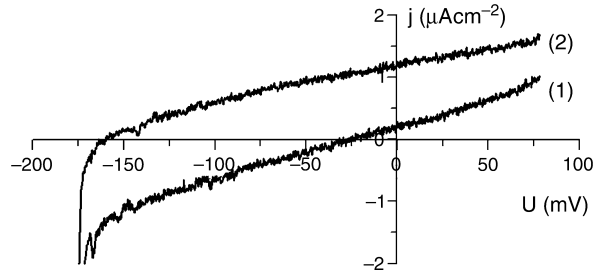

Fig. 4. Stimulation of the current by an enlarged transmembrane pH gradient. Representative current responses to a change in the bath solution from (1) pH 5.8 to (2) pH 8.8 are shown. Pump solutions with $Ca(OH)_2$ were used. The pH was raised to 8.8 with Tris.

Table 1. Reversal potentials (E_{rev}) and number of experiments (n) at three intracellular pH values, adjusted by Mes/Tris in the pipette. No significant differences were detected (*t*-test, $P = 0.05$). In rows 3 and 4 intracellular H^+ concentrations and the Nernst potentials of H^+ are given. External pH was 5.8 [H^+] = 158 × 10^{-8} mol l^{-1}. Pump solutions with $Ca(OH)_2$ were used.

	pH 6.9	pH 7.2	pH 7.5
E_{rev}, mV	−81 ± 10	−66 ± 7	−62 ± 11
n	11	27	12
[H^+], 10^{-8} mol l^{-1}	12.6	6.3	3.2
E_{H^+}, mV	65	83	100

Therefore, we assumed that the intracellular Ca^{2+} concentration did not affect pump activity. Similarly, it did not make any difference if the extracellular Ca^{2+} concentration was raised from 5 to 40 m*M* (Table 2, *t*-tests between columns 1 and 2 and columns 4 and 5), or if TEA^+ or K^+ was the counterion to Cl^- (Table 2, *t*-test between columns 1 and 3).

Balance of anion and pump currents

Currents ascribed to the electrogenic pump were comparatively small. Therefore, conductances for other ions easily short-circuit pump activity. This became obvious when membrane potentials were measured in the current clamp modus after establishing the whole-cell configuration (Fig. 5). In most measurements with pump solutions the electrogenic pump was dominating, reflected by reversal potentials negative of −80 mV. Nevertheless, frequently more depolarised membrane potentials were measured, possibly because of the activation of shunting conductances (Fig. 5A). Using solutions favoring anion currents by increasing activities of Cl^-, the membrane potential was not always set by the anion conductances but rather adjusted at more negative values (Fig. 5B). In the case of the diffusion of solely Cl^- the membrane potential was expected to be 30 mV. It is interesting that anion currents were regularly so small that they could be balanced by pump currents resulting in intermediate reversal potentials in the range from −10 to −70 mV.

Three types of anion conductances were identified in the root xylem parenchyma from barley (Köhler and Raschke 2000). Indeed, the activity of all of them in combination with the pump current resulted in intermediate reversal potentials (Fig. 6). The simultaneous activity of electrogenic pumps and quickly activating anion conductance (X-QUAC, X stands for xylem parenchyma) could, in some cases, even lead to three zero-crossings (Fig. 6C). Application of the anion channel inhibitor DIDS depressed anion currents effectively, and current fluctuations because of channel activity disappeared. It had been shown that X-QUAC is inhibited by DIDS (Köhler et al. 2002). Possibly, also pump activity was slightly inhibited after the addition of DIDS, because the current around −40 mV decreased (Fig. 6C). This is in line with the literature which reported no or only small inhibitory effect of DIDS on the plasma membrane H^+-ATPase (Churchill and Sze 1984, Varanini et al. 1995). Although we cannot exclude any effect of DIDS on the electrogenic pump from barley roots, its characteristic current response could still be measured in the presence of 100 μ*M* DIDS. Therefore, the inhibitor could be used to distinguish between activity of the electrogenic pump and anion conductances. Also in experiments using pump solutions the application of DIDS caused a large reduction of the current and large hyperpolarisation of the membrane potential from −47 ± 19 to −107 ± 17 mV (n = 6, Fig. 7). The typical current response for H^+-pumps displaying an apparent saturation of the transmembrane current emerged. Further addition of the Ca^{2+} channel inhibitor nifedipine did not have any significant effect on current or membrane potential (n = 3, Fig. 7, trace 3). A hyperpolarisation-activated Ca^{2+} conductance was active in 25% of stelar protoplasts from maize roots. Activity was particularly apparent when solutions

Table 2. Current density at 0 mV ($j_{0\ mV}$) under different conditions. No significant differences between low (0.15 μ*M*, columns 1–3) and high (5 μ*M*, columns 4–6) intracellular Ca^{2+} (*t*-test, $P = 0.05$). The range of current densities and number of experiments (n) are given in rows 2 and 3, respectively. 1, anion solutions, 2, anion solutions with 40 m*M* $Ca(Glc)_2$ in the extracellular solution, 3, anion solutions in which tetraethylammonium chloride had been replaced by KCl, 4, anion solutions with 5 μ*M* Ca^{2+} in the intracellular solution, 5, anion solutions with 5 μ*M* Ca^{2+} in the intracellular solution and 40 m*M* Ca^{2+} in the extracellular solution, 6, pump solutions with $Ca(Glc)_2$. For comparison, j_{0mV} in pump solutions with $Ca(OH)_2$ was 0.47 ± 0.08 μA cm^{-2} (n = 55). Compositions of anion and pump solutions are listed in Materials and methods.

	1 0.15 μ*M* Ca^{2+} anion	2 0.15 μ*M* Ca^{2+} anion/40 Ca^{2+}	3 0.15 μ*M* Ca^{2+} anion/KCl	4 5 μ*M* Ca^{2+} anion	5 5 μ*M* Ca^{2+} anion/40 Ca^{2+}	6 5 μ*M* Ca^{2+} pump
$j_{0\ mV}$, μA cm^{-2}	0.69 ± 0.08	0.59 ± 0.08	0.6 ± 0.08	0.53 ± 0.11	0.78 ± 0.05	0.57 ± 0.06
j range, μA cm^{-2}	0.2–1.8	0.3–1.3	0.3–0.8	0.2–0.9	0.7–0.9	0.1–2.5
n	25	13	5	6	4	53

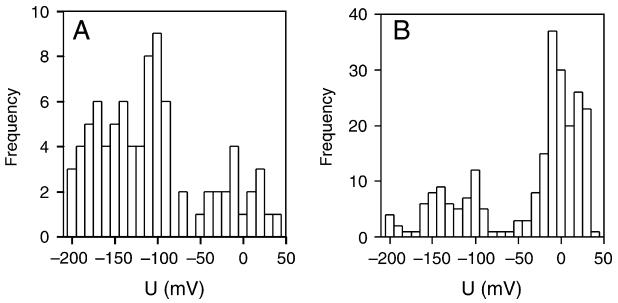

Fig. 5. Membrane potentials of xylem-parenchyma protoplasts using (A) pump solutions and (B) anion solutions (increased activities of Cl^- in both, bath and pipette). Membrane potentials were measured in the current clamp modus after the establishment of the whole-cell configuration. For histograms, values were binned into 10 mV steps.

were designed to maximise the appearance of divalent cation currents (Gilliham and Tester 2005). Under our experimental conditions, anion conductances constituted the major transmembrane shunt.

Discussion

The electrogenic pump in the xylem parenchyma displays characteristics of an H^+ pump

Current–voltage relationships of protoplasts isolated from the xylem parenchyma of barley roots displayed a negative reversal potential and an asymptotic approach to a maximum current (resembling saturation); they exhibited the behaviour of a 'rheogenic' pump (Läuger 1991): this pump appears to belong to the group of P-type ATPases (Buch-Pedersen and Palmgren 2003, Hodges 1976, Palmgren 2001, Poole 1978, Serrano 1989, 1993, Spanswick 1981). Low membrane potentials, occasionally as low as −200 mV, were recorded, which was more negative than the equilibrium potentials of all ions present in the solutions. The same range of reversal potentials has been reported for the electrogenic pumps in guard cells (Lohse and Hedrich 1992) and wheat root protoplasts (Findlay et al. 1994). Because of these characteristics and the fact that currents were stimulated by FC or by an enlarged transmembrane pH gradient and inhibited by DCCD, convincingly indicated that these currents can be associated with the activity of an H^+-pump (Figs 2 and 4). Xylem-parenchyma cells of barley roots were strongly labelled by antibodies against the plasma membrane H^+-ATPase (Samuels et al. 1992) which supports the presence of an H^+-ATPase in this cell type. However, direct evidence for ATP serving as substrate remains to be presented.

The fluorescein derivative erythrosine B is a much more effective inhibitor of Ca^{2+}-ATPases (in the n*M* range) than

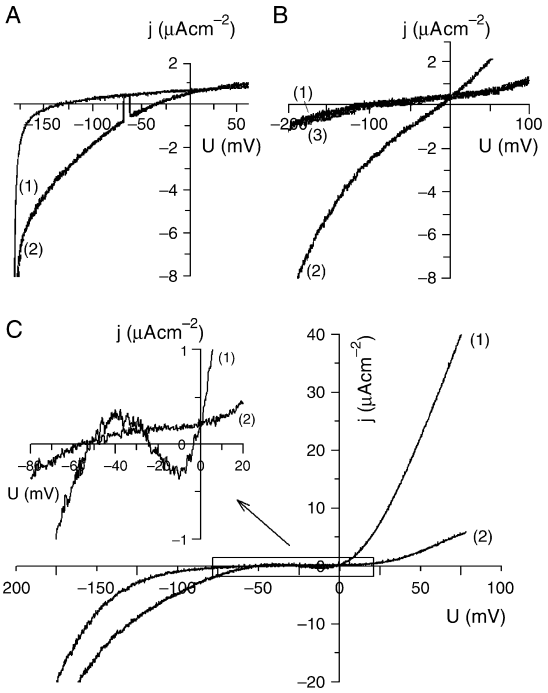

Fig. 6. Simultaneous activity of anion conductances and the electrogenic pump resulted in intermediate reversal potentials. (A) Activation of X-IRAC (=inward-rectifying anion channel, X stands for xylem parenchyma) shifted the reversal potential from around −120 to −40 mV. Initially, X-IRAC was closed and the current response showed the typical characteristics of an electrogenic pump (1). In a consecutive voltage ramp X-IRAC was open (2). The opening of a single channel was sufficient to shift the reversal potential. The brief closing of X-IRAC showed nicely that no other conductance was active because the current matched the one measured in the first ramp. (B) Activation of X-SLAC (=slowly activating anion conductance) shifted the reversal potential from around −100 to −10 mV. Clamping the voltage at −150 mV deactivated X-SLAC. The characteristic current response of an electrogenic pump dominated (1). Then X-SLAC was activated by clamping the voltage at 30 mV and the current through open channels was recorded by applying a voltage ramp. The reversal potential shifted towards more positive voltages (2). This was reversible. After deactivating X-SLAC again at −150 mV, the initial current response was restored (3). (C) Simultaneous activity of the pump and X-QUAC (=quickly activating anion conductance) could lead to multiple zero-crossings of the total current (1). After application of 100 μ*M* 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid to the bath solution, the anion current was largely suppressed and the fluctuation of the current between 0 and −60 mV disappeared (2). To see the effect in detail, the diagram was locally rescaled, as indicated by the arrow (inset). In all measurements shown here, voltage ramps were applied to obtain current–voltage relationships and anion solutions with 5 (A, B) and 40 (C) m*M* $Ca(Glc)_2$ were used. For a detailed characterisation of X-IRAC, X-SLAC and X-QUAC see Köhler and Raschke (2000).

of H^+-ATPases (in the μ*M* range) (De Michelis et al. 1993). This inhibitor had no effect on xylem-parenchyma protoplasts (Fig. 3), showing (1) that there was no Ca^{2+}-ATPase activity, and (2) that the electrogenic pump present in these cells was not inhibited by a concentration

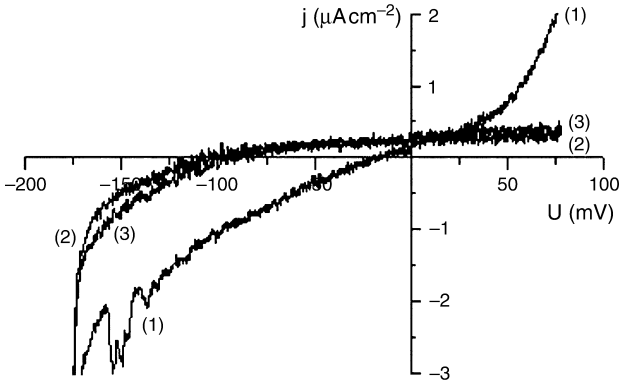

Fig. 7. Anion conductances short-circuiting pump activity. Current responses (1) before and (2) after the addition of the anion channel inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) are shown. The addition of 100 μ*M* DIDS brought out the typical current response of the electrogenic pump. Further addition of the Ca^{2+} channel inhibitor nifedipine (final concentration 100 μ*M*) was without effect on the current–voltage curve (3). Pump solutions with $Ca(OH)_2$ were used.

as high as 1 m*M*, as could have been expected according to Briskin et al. (1995) and De Michelis et al. (1993). The magnitude of the pump current was neither affected by a change in the intracellular Ca^{2+} concentration nor by an increase of the extracellular Ca^{2+} concentration (Table 2). These results argue for an H^+-pump that generated the current. If any Ca^{2+}-pumps had been active, their currents would have been too small to be discovered.

The fact that pump activity occurred only in around one-half of the investigated protoplasts may have had two causes: (1) pump currents were frequently too small to emerge in the records or were short-circuited by other ion conductances and (2) the distribution of H^+-ATPases may be polar in xylem-parenchyma cells which would result in a non-uniform allocation of the proteins to the subprotoplasts when protoplasts subdivide during preparation (Wegner and Raschke 1994). Despite the strong labelling of xylem-parenchyma cells from barley roots with antibodies against the plasma membrane H^+-ATPase the resolution was insufficient to discern differences in the subcellular distribution of the protein (Samuels et al. 1992).

Pump currents, and effects of K^+, Ca^{2+} and pH

The pump current was 0.6 μA cm^{-2} on average (Table 2). For comparison, pump activities of other cell types were 0.2 and 0.7 μA cm^{-2} in the root cortex of wheat (Findlay et al. 1994, Tyerman et al. 2001), 1.5–3.8 (Lohse and Hedrich 1992) and 13 μA cm^{-2} (Blatt 1988) in *Vicia faba* guard cells, approximately 0.6–1.4 μA cm^{-2} in mesophyll cells from *V. faba* (Lohse and Hedrich 1992), and 250 μA cm^{-2} in *Arabidopsis* root hairs (Lew 1991). Pump currents in xylem-parenchyma protoplasts from barley roots were neither significantly affected by the presence of K^+ or TEA^+ nor significantly affected by the changes in the cytosolic Ca^+ concentration from 0.15 to 5 μ*M* (Table 2). The absence of an effect of K^+ is not surprising. Although a stimulation of the H^+-ATPase by K^+ has been reported, it was very small and H^+ transport occurred also in the absence of K^+ (Leonard and Hotchkiss 1976, Nejidat et al. 1986, Vara and Serrano 1982). More remarkable is the absence of an effect of Ca^{2+}. In its insensitivity to changes in the cytosolic Ca^{2+} concentration from 0.15 to 5 μ*M* the H^+-pump of xylem-parenchyma cells differs from that of guard cells and mesophyll cells, which are inhibited by intracellular Ca^{2+} with a K_i of 0.3 μ*M* (Kinoshita et al. 1995). Interestingly, the Ca^{2+} concentration required for half inhibition of ATP hydrolysis was with 150 μ*M* much higher in plasma membranes of maize roots than in guard cells (Leonard and Hotchkiss 1976). The H^+-pump from xylem-parenchyma cells might thus be controlled in a different way than that of leaf cells. Possibly, isoforms with low sensitivity towards Ca^{2+} are expressed in roots. Other regulatory factors, which still have to be identified, appear to be crucial to modify H^+-pump activity in this tissue.

There was no clear indication of an involvement of the electrogenic pump in the regulation of cytoplasmic pH. Two doublings of the intracellular H^+ concentration had no significant effect on pump activity. The values of reversal potentials determined at pH 6.9, 7.2 and 7.5 remained within the range of statistical variation (Table 1). Usually, the pH optimum of H^+-ATPases is around 6.7. On the alkaline side, pH profiles differ. Whereas some H^+-ATPases show a sharp decline in activity above pH 7.0 (Becker et al. 1993, Luo et al. 1999), others have a much broader profile (Luo et al. 1999, Nejidat et al. 1986). The H^+-pump from xylem-parenchyma protoplasts seems to belong to the second group with a reasonable activity up to a cytoplasmic pH-value of 7.5.

As Sondergaard et al. (2004) pointed out, the analysis of each individual member of the plasma membrane H^+-ATPase family in a given organism is required before we can conclude about their physiological role in nutrient uptake and translocation. The data presented here contribute to the understanding of the physiology of root xylem-parenchyma cells. It is striking that the cytosolic Ca^{2+} concentration was not crucial for the activity of the H^+-pump but that K^+ and anion conductances from the same cell type are controlled by intracellular Ca^{2+}. The K^+ outward-rectifying conductance and X-QUAC, which could mediate passive loading of the xylem with K^+ and NO_3^-, predominated

at physiological Ca^{2+} levels (150 n*M*), whereas at elevated Ca^{2+} levels (5 μ*M*) mainly the non-selective outward-rectifying conductance and inwardly rectifying anion channel (X-IRAC) were active (Köhler and Raschke 2000, Wegner and De Boer 1997).

Control of membrane potential and nutrient transfer

Pump activity was large enough to electrically neutralise currents through anion conductances in the voltage range of low anion conductivity (Figs 5–7). Three types of anion conductance were identified in xylem-parenchyma cells: X-IRAC, X-QUAC and the slowly activating anion conductance X-SLAC (Köhler and Raschke 2000). We suggest that all three of them might be involved in electroneutral acid transfer into the xylem. So far, only X-IRAC had been considered to provide a path for counter ions for H^+ during the activity of the electrogenic pump (Köhler and Raschke 2000). Because of their different Ca^{2+} dependences (see above) X-IRAC and X-QUAC might play different roles under different conditions. The simultaneous activities of the H^+ pump and one or more of the anion channels makes the generation of a high pH gradient possible, whilst maintaining electroneutrality. Anion efflux is possible down to −200 mV through X-IRAC or X-QUAC (Köhler and Raschke 2000, Köhler et al. 2002). The established proton gradient would be available for dissipation by coupled ion transport through cotransporters and antiporters (see introduction). Proton pumping, together with chloride transport through X-QUAC, was suggested to function in the loading of borate into the xylem through the boron transporter BOR1 (Frommer and von Wirén 2002, Takano et al. 2002). Although the apoplast possesses buffering capacity because of cation exchange sites and the presence of organic acids, the stimulation of the H^+-ATPases in the stelar tissue by FC resulted in a decrease in pH in the xylem, as has been shown by De Boer and Volkov (2003) in perfusion experiments. Na^+ release into the xylem, probably via Na^+–H^+-antiporter, was increased and, simultaneously, K^+ flux into the xylem declined, because of the deactivation of the K^+ outward rectifier (De Boer and Volkov 2003). These K^+ channels are deactivated at negative membrane potentials but are also modulated by pH (Lacombe et al. 2000), linking H^+-ATPase activity to ion channel control.

The pump polarises the plasmalemma, short-circuiting by ion conductances leads to depolarisation. Correspondingly, the xylem parenchyma will switch from a state of ion uptake to one of ion loss. Negative membrane voltages will be required for reabsorption of NO_3^- by a putative NO_3^-/H^+-symporter and also for the uptake of amino acids (Okumoto et al. 2002). K^+ uptake from the xylem sap into the stelar symplast needs voltages more negative than −100 mV (Wegner and Raschke 1994), whereas passive xylem loading with salts is restricted to the voltage range in-between the equilibrium potentials for cations and anions, presumably positive of –50 mV (Köhler and Raschke 2000, Köhler et al. 2002, Wegner and De Boer 1997). Therefore, it will depend on the membrane potential whether salt release or uptake takes place. The balance between the activities of the H^+-pump and the anion conductances could affect the position between a depolarised and a hyperpolarised state of the parenchymal membrane and, in turn, this switch would determine whether there will be salt uptake or salt loss. Depolarisation by increased activity of anion conductances establishes the condition for xylem loading, and pump activity works against it. De Boer and Volkov (2003) pointed out that the Casparian strip insulates the xylem apoplast electrically from the cortical apoplast. This means that the electrical potential difference of the cells in the xylem parenchyma could be independent from the cortical potential difference but be subject to control, for instance, from the shoot. We recognise that the electrogenic pump in the xylem parenchyma does not participate directly in the transfer of KCl and KNO_3 to the xylem but, in combination with the short-circuiting conductances, plays a crucial role in controlling xylem unloading and loading through modulation of the voltage difference across the plasmalemma of the cells of the xylem parenchyma.

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