Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioelectrochemistry 60 (2003) 47–55
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

A short-term NaCl exposure increases the Na^+ conductance of outward-rectified cation currents in the pith cells of sweet pepper

Margaretha Blom-Zandstra*, Sake A. Vogelzang

Plant Research International, PO Box 16, 6700 AA Wageningen, The Netherlands

Received 25 September 2002; received in revised form 28 February 2003; accepted 26 March 2003

Abstract

The regulatory role of pith cells in the stem in Na^+ recirculation in sweet pepper was investigated by evaluating the transport characteristics of the plasma membrane of this cell type and comparison with those of root cells. Ion conductivity and Na^+ permeability of the plasma membranes of protoplasts of both cell types were studied with the patch-clamp technique in the whole-cell configuration, before and after addition of NaCl to the bath medium. Protoplasts of both pith and root cells showed outward rectifying currents with a reversal potential (V_r) near to the equilibrium potential of K^+ (EK). Addition of NaCl to the bath medium caused a stronger shift of the reversal potential, V_r, in pith protoplasts than in root protoplasts, indicating that the outward rectified currents are permeable to Na^+, especially in the pith cells.

After plant exposure to exogenous NaCl via the nutrient solution for 1 week, V_r in the root cells was closer to EK than in the control plants and hardly shifted upon addition of Na^+. This indicated that the net permeability of the OR channel complement in the plasma membrane to Na^+ was lower following exposure to Na^+. V_r in the pith protoplasts, on the other hand, shifted significantly more than in the control plants, suggesting an increase of the permeability to Na^+. Moreover, the Na^+ channel blocker amiloride blocked the currents in this cell type. It is concluded that pith cells have appropriate features of outward rectified currents to enable Na^+ accumulation or release when NaCl is present in or removed from the nutrient medium. Probably, exogenous NaCl even induced expression and formation of Na^+-permeable channels in pith cells.

Keywords: Patch clamp; Conductance; *Capsicum annuum* L.; Plasma membrane; ORC; Na^+

1. Introduction

The use of sustainable plant growth systems in greenhouses is developing. In hydroponic systems where nutrients recirculate for several weeks, emission of nutrients to surface water is reduced but NaCl concentrations in the nutrient solution may rise considerably during cultivation, which may cause salinity stress. Many plant species are tolerant to such sodium stress. Sweet pepper, an important and representative horticultural crop grown in hydroponic systems, can avoid high Na^+ concentrations in the leaves by preferential accumulation in the pith cells of the stem [1]. Sodium uptake by sweet pepper appears to be a well-regulated process: when plants are exposed to NaCl in the nutrient solution, the Na^+ concentration in the plants reaches a steady state after 1 week. Moreover, the Na^+ concentration in the xylem sap always closely correlates with the amount of Na^+ accumulated in the pith cells leading to an exponential decrease in Na^+ concentration toward the tip of the stem. This suggests that the efficacy of Na^+ transfer from the xylem to the pith depends on the concentration gradient.

Pith cells are very suitable for accumulation of Na^+ for several reasons: (1) Pith cells have large vacuoles and therefore a high accumulation capacity, (2) the number of pith cells increases proportional to plant growth, and (3) their strategic location within the stele offers the special opportunity to regulate influx into and efflux from the shoot [2,3]. Indeed, pith cells of sweet pepper have been shown to play a decisive role in Na^+ transfer from xylem to phloem and release from the roots, probably resulting from downward phloem transport [1]. In this way, the plant effectively prevents Na^+ accumulation in the leaves and minimises the adverse effects on photosynthetic activity. Probably, pith cells play a crucial role in the regulation of Na^+ levels by controlling uptake, storage and transfer through the plant.

* Corresponding author. Tel.: +31-317-475819; fax: +31-317-423110.
E-mail address: greet.blom@wur.nl (M. Blom-Zandstra).

1567-5394/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1567-5394(03)00045-8

This raises the question whether the membrane characteristics of pith cells may reveal how these cells preferentially accumulate Na^+ and how a sharp gradient between pith cells and xylem can be maintained.

As a fundamental mechanism in salt tolerance in plants, an active antiport would function to sequester Na^+ into the vacuole, resulting in avoidance of cytoplasmic Na^+ toxicity and maintenance of a high cytoplasmic K^+/Na^+ ratio [4]. The presence of a tonoplast Na^+/H^+ antiporter involved in vacuolar Na^+ sequestration has been well documented for cell suspensions from sugar beet and root cells from several plant species [4]. For sugar beet cell suspensions and barley roots, it was shown that although Na^+/H^+ antiport activity was not detected in plants grown in the absence of NaCl, antiport activity was rapidly induced upon NaCl exposure.

Although the route for Na^+ uptake into the root symplast has not been established in much detail [5], several classes of cation channels seem to be involved [6]. Concerning Na^+ transport through the plasma membrane into the cytoplasm, the Na^+ electrochemical gradient dictates that at physiological steady-state values of the membrane potential $\Delta\Psi$ (inside negative, i.e. -120 to -200 mV [7]), the influx is passive [8]. Sodium acts as a competitor for K^+ uptake [9,10], suggesting that both cations use a similar transport system and it has been assumed that non-selective (K^+) ion channels allow Na^+ entry into the cells [11–13]. Evidence is now accumulating that non-selective cation channels might even be the major pathway for Na^+ influx into root cells [14–18]. Plasma membrane depolarisation and exposure to high external NaCl increases the open probability of outward rectifying cation channels in tobacco cells [19], thereby allowing Na^+ influx following the steep electrochemical gradient.

To gain more insight into a possible regulatory role of pith cells of sweet pepper in Na^+ recirculation at the protoplast level, we compared cation transport characteristics of plasma membranes of pith cells with those of the root cells. For this purpose, isolated protoplasts of both cell types were studied using the patch-clamp technique in the whole-cell mode before and after addition of Na^+ to the bath medium, which represents the extracellular medium. In addition, we investigated whether these transport characteristics change after plant exposure to 15 mM NaCl in the nutrient medium.

2. Experimental procedures

2.1. Plant growth conditions

Seeds of *Capsicum annuum* L., cv Mazurka, were sown in perlite. After 9 days at 30 °C in the dark, the emerged seedlings were transferred to a hydroponics system with a nutrient solution containing 3.73 mM $Ca(NO_3)_2$, 4.40 mM KNO_3, 0.97 mM KH_2PO_4, 1.92 mM $MgSO_4$, 0.89 mM K_2SO_4, Fe–EDTA and trace elements [20]. The plants were grown in a growth chamber at 20 °C with a photoperiod of 12 h (150 μmol m^{-2} s^{-1}) at a relative humidity of 70%.

Sweet pepper shows dichotomic branching [21]. Following the procedure used in commercial practice, the largest of each dichotomic branch was retained, while the other was pruned just above its first leaf. Some of the 9-week-old plants were transferred to pots containing a nutrient solution supplemented with 15 mM NaCl for 1 week for later comparison with control plants.

2.2. Protoplast isolation

Protoplasts were isolated from root tissue and stem tissue from which cortex and cambium layer were peeled off. This isolation procedure was performed without an osmotic shock and centrifugation step [22]. Briefly: up to 0.5 g plant material was placed in the enzyme medium (1% cellulase (R10 Onozuka), 0.2% macerozyme (R10 Onozuka), 10 mM MES–KOH (pH 5.5), 10 mM sucrose, 500 mM mannitol, 2 mM $MgCl_2$, 2 mM $CaCl_2$) for 16 h at 12 °C. For adult plants, a 16-h period of enzymic digestion was essential to release the protoplasts. After the incubation period, pieces of slimy tissue were picked up with a pair of tweezers and transferred to a 2-ml petri dish with wash medium (10 mM MES–KOH (pH 5.5), 10 mM sucrose, 500 mM mannitol, 2 mM $MgCl_2$, 2 mM $CaCl_2$). Protoplasts were released from the tissue by movement through the solution. Small spots were visible on the bottom of the petri dish. These spots contained 5–100 protoplasts and could easily be identified and transferred to the patch-clamp cuvette using a Pasteur pipette. The root material still contained protoplasts after this treatment and was subsequently transferred to the patch-clamp cuvette. Mechanical pressure was applied to this tissue with a pair of tweezers to release root protoplasts. The protoplasts took 2 min to adhere to the bottom of the cuvette. For experiments, protoplasts were selected with a diameter of ~ 25 m for root cells and a diameter of ~ 40 m for pith cells.

2.3. Electrophysiological measurements

Protoplasts were used in patch-clamp experiments in the whole-cell (WC) configuration. Outward-rectified cation currents (ORCs) were measured as their conductance for K^+ and Na^+. Electrodes were prepared from borosilicate glass (GC150-15, Clark Electromedical Instruments, Reading, UK). Pipettes were made with a two-stage puller (List-medical 3P-A, Darmstadt, Germany), fire-polished (Zeiss ID03 and List-medical CP-Z101) and coated with Sylgard (Dow-Corning, Midland, MI).

Dishes with protoplasts were mounted on an inverted microscope (Nikon-TMD). A piezo manipulator (Luigs and Neumann GmbH, Ratingen, Germany) was used to move

pipettes in the micrometer range. Conventional patch-clamp techniques were applied according to Ref. [23] in whole-cell configurations, performed by suction and monitored by an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA). The sealing process was monitored on a digital storage oscilloscope (Hewlett Packard 54501A 100 MHz). Pipette resistance ranged from 5 to 10 M, depending on the geometry and experimental solutions. Voltage and current data were transferred via a CED1401 A/D-converter, which was controlled by CED patch-clamp software (Cambridge, UK). Data were sampled at 2–10 kHz and analysed using (Turbo-Pascal) CED compatible software (ECOPATCH [24]).

2.4. Media used

2.4.1. Pipette solution

One millimolar HEPES–BTP (pH 7.2), 150 mM KCl (concentration depended on the desired equilibrium potential), 2 mM $MgCl_2$, 5 mM MgATP, 2 mM K_4BAPTA, 0.1 mM $CaCl_2$ (free Ca concentration: 0.009 μM) replenished with mannitol to an osmolarity of 485 mOsM kg^{-1}. In experiments in which the effect of addition of NaCl to the medium was studied, 1 mM NaCl was present in the pipette solution from the start of the experiment.

2.4.2. Bath solution

Ten millimolars MES–Tris (pH 5.5), 10 mM KCl (concentration depended on the desired equilibrium potential), 2 mM $MgCl_2$, 2 mM $CaCl_2$, replenished with mannitol to an osmolarity of 510 mOsM kg^{-1}. Where mentioned in the text, 12.5 mM (final concentration) NaCl was added in accordance with the apoplastic Na^+ concentrations found in sweet pepper in an earlier study [1].

2.4.3. Addition of blockers

Where indicated in the text, 10 M verapamil (a Ca^{2+} channel blocker that has been shown to block K^+ channels in plant cells also [25–28] while it conducts Na^+ [28]) and 100 M amiloride (a Na^+ channel blocker [29]) were added to the bath solution.

2.5. Tail current analyses

2.5.1. Determination of reversal potentials (V_r)

Experiments for determination of V_r were performed in the different protoplast species at different times before and after addition of NaCl to the bath medium (2 and 45 min, real time). In these experiments, an activating depolarizing pre-pulse (about 0 mV for 4 s) was followed by a series of depolarizing potentials which increased stepwise with 10-mV increments. V_r is calculated by fitting the deactivation currents (i.e. tail currents) beyond the capacity peak until 0 mV and by measuring the slope (Δ) of these tail currents over a period of about 200 ms and relating it to the corresponding potentials. Thus, V_r can be obtained from the ΔV relationship and is defined as the potential at which the polarity of the tail currents reverses (i.e. $\Delta = 0$).

2.5.2. Calculation of the equilibrium potentials for K^+ (EK), Cl^- (ECl) and Na^+ (ENa)

The ionic concentrations before and after addition of NaCl to the medium are expressed in ionic strengths in conformance with the Debye–Hückel theory and used in the Nernst equation at $T = 22$ °C [29].

2.5.3. Calculation of the permeability ratios

To ascertain an optimal mixing of NaCl in the solution, the values of V_r were determined 45 min after addition of

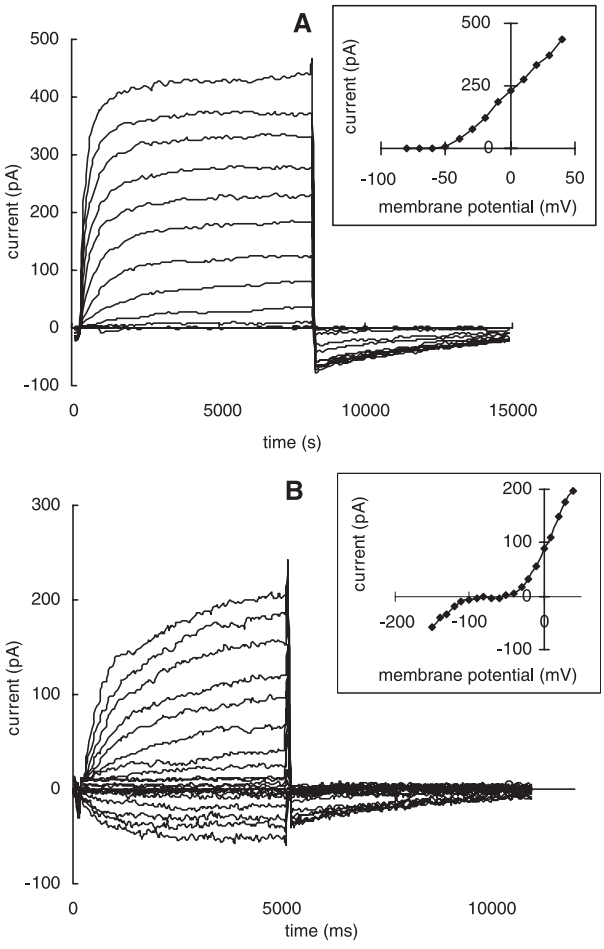

Fig. 1. Whole-cell recordings of a sweet pepper plant, grown in the absence of NaCl. (A) Outward rectifying currents of a pith protoplast in asymmetrical solutions were activated upon depolarising pipette potentials. Pulses were applied ranging from −80 to 40 mV with 10-mV steps. (B) Inward and outward rectifying of a root protoplast in asymmetrical solution currents were activated upon polarising pipette potentials. Pulses for inward activation were applied ranging from −60 to −150 mV with 10-mV steps and for outward activation ranging from −80 to 40 mV. *Insets*: *I–V* relationship corresponding to the whole-cell recordings measured at the end of each applied pulse (for A: 7.5 s and for B: 5.0 s) when the currents had reached steady state.

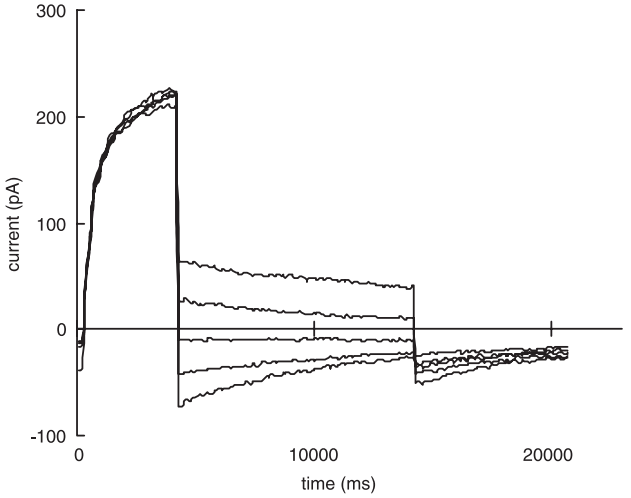

Fig. 2. Deactivation tail currents in a pith protoplast of sweet pepper grown in the absence of NaCl, appearing when after a pulse of 0 mV the voltage was returned to values ranging from −80 to −40 mV. The holding potential was −80 mV.

NaCl to the medium. These values were compared with the calculated equilibrium potentials for K^+, Cl^- and Na^+ according to the Hodgkin–Huxley model, using the equally weighed mean of the Nernst potentials of three ions [29].

2.6. Statistical analyses

Data were submitted to analysis of variance (ANOVA) by means of the statistical package GENSTAT 5. For each whole-cell patch clamp experiment, fresh protoplasts were isolated from a new, unused plant. Analysis of variance was based on completely randomized design. Data were considered to show a normal distribution. The significance of differences ($P<0.05$) was determined by Student's *t*-test.

3. Results

3.1. Whole-cell patch-clamp studies on pith and root protoplasts from sweet pepper plants grown in the absence of NaCl in the nutrient solution

The protoplasts isolated from root and stem tissue varied in size between 35 and 45 μm for pith protoplasts and between 23 and 30 μm for root protoplasts. In general, pith protoplasts contained much larger vacuoles (15–20 μm) than root protoplasts (7–12 μm).

Approximately 50 % of the successfully sealed pith and root protoplasts ($n=58$) showed no channel activity under our conditions (data not shown). Irrespective of cell type, all protoplasts with channel activity showed recordings typical of ORCs at depolarising pulse potentials ranging from −80 to +40 mV (Fig. 1A). In 8% of these protoplasts, inward rectifying currents (IRCs) were observed as well (Fig. 1B).

Activation of ORCs occurred at ca. −50 mV and showed sigmoidal time courses. Half-time values of these currents were voltage dependent and decreased from 4 to 0.4 s when pulse potentials increased from −80 to +40 mV (Fig. 1A). When the currents had reached a steady-state value (i.e. no more change of net flow of charge with time), steady-state currents were measured. Steady-state currents were clearly voltage dependent, while we had no indications for the presence of instantaneous currents. So, currents were calculated from steady-state currents after leak subtraction [30] for both ORCs and IRCs and plotted as functions of the pulse potentials (insets in Fig. 1A and B).

Deactivation of the currents could be seen in the tail currents (Fig. 2) upon stepping back to the holding potential (−80 mV). The tail currents reversed between −60 and −50 mV for both pith and root protoplasts (V_r values before addition NaCl, see Table 1). Differences between both cell types were not significant. The single exponential deactivation currents were also voltage dependent but slower than

Table 1
Whole-cell channel characteristics, standard errors (s.e.) and statistical analyses (ANOVA) of the plasma membrane of pith and root cells of sweet pepper plants grown without or with NaCl in the nutrient solution

	Cell type	Percentage decrease I (s.e.)				Reversal potential (s.e.)		
		Addition NaCl		Amiloride	Verapamil	No NaCl	Addition NaCl	
		2 min	45 min				2 min	45 min
Plants grown in the absence of NaCl	pith cells	0 (5.3)	34.2 (10.6)	16.4 (4.8)	63.0 (25.9)	−52 (2)	−48 (3)	−44 (3)
	root cells	20.4 (6.8)	58.4 (13.0)	5.1 (5.5)	18.5 (31.8)	−50 (2)	−48 (3)	−48 (4)
Plants grown in the presence of NaCl	pith cells	16.9 (9.6)	65.5 (18.4)	82.4 (9.6)	66.6 (34.0)	−55 (3)	−38 (6)	−32 (4)
	root cells	14.5 (9.6)	44.3 (18.4)	0 (6.8)	76.2 (31.8)	−58 (4)	−51 (5)	−52 (4)
Amount of cells tested in ANOVA (n)		22	21	9	7	24	21	15
Significance	plant treatment	n.s.	n.s.	0.05	n.s.	n.s.	n.s.	n.s.
	cell type	0.05	n.s.	<0.01	n.s.	n.s.	n.s.	0.013
	treatment × cell type	n.s.	n.s.	<0.01	n.s.	n.s.	n.s.	0.045

Current decrease determined at +50 mV (I_{p50}), and expressed as percentage decrease compared to I_{p50} before addition of NaCl. n.s. = not significant.

the activation currents. Their half-time values varied from 2 to 4 s for pulse potentials from −80 to −20 mV.

Both the steady-state *I*/*V* relationships (insets Fig. 1) and reversal potential near to the equilibrium potential of K^+ (EK; i.e. −65 mV; Fig. 2) show that the ORCs appeared to resemble the K^+-selective channels, although several other OR cation channels might also contribute to the ORCs found in this study.

3.1.1. Effect of Na^+ in pipette and bath solution

To evaluate the selectivity of the ORCs for Na^+, NaCl was added to the bath medium (final concentration 12.5 mM; 1 mM NaCl was already present in the pipette solution from the start of the experiment). The effect of Na^+ on membrane characteristics differed between pith and root protoplasts. Addition of NaCl resulted in an effect within 2 min (real time) on the current for root protoplasts, which manifested itself as a decrease of the current at +50 mV (I_{p50}, Table 1), whereas it did not change the I_{p50} of the pith protoplasts shortly after addition. The difference in response between cell types was significant. Approximately 45 min after addition of NaCl to the bath medium (i.e. complete bath exchange), I_{p50} was decreased for both pith and root protoplasts (Table 1) but did no longer significantly differ between both cell types.

Values of the reversal potential (V_r, Table 1) of the ORCs shifted slightly upon addition of NaCl to the bath medium, for pith cells more than for root cells, especially after 45 min. This shift indicates that the ORCs may be permeable to Na^+ to some extent, especially for the pith cells.

Addition of amiloride (Table 1), assumed to block Na^+ channels [29] and proven to have no effect on ORCs under control conditions (i.e. without Na^+ in the bath medium, data not shown), hardly decreased I_{p50} further for root cells, while it had a small effect in pith cells. Addition of verapamil, a Ca^{2+} channel blocker that has also been shown to block Na^+-permeable K^+ channels also [25–28] while it conducts Na^+ [28], blocked the channel substantially in the pith protoplasts. This is shown in Fig. 3, in which channel activities are shown before (A) and after addition of NaCl (B) and after addition of verapamil (C).

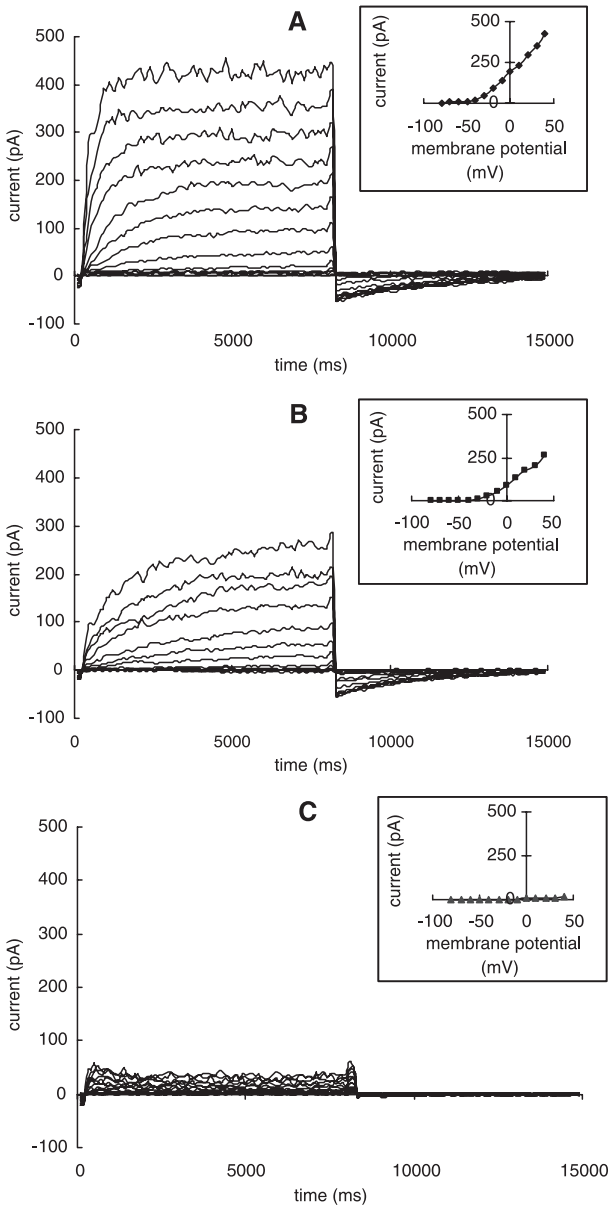

Fig. 3. Whole-cell recordings in a pith cell protoplast of sweet pepper, grown in the absence of NaCl before addition of NaCl (A), 38 min after addition of 12.5 mM NaCl (B) and after addition of 12.5 mM NaCl (90 min), 100 M amiloride (40 min) and 10 M verapamil (2 min) (C). *Insets*: *I*–*V* relationship corresponding to the whole-cell recordings measured at the end of each applied pulse (7.5 s) when the currents had reached steady state.

3.2. Pith and root protoplasts from plants grown in the presence of NaCl in the nutrient solution

Patch-clamp measurements in the whole-cell configuration showed that recordings of activation of ORCs for plants grown in the presence of NaCl in the nutrient solution for 1 week were similar to those of the control plants (see Fig. 1A for control plants). For pith cells, addition of NaCl to the bath medium decreased I_{p50} more than in the control plants (Table 1). However, differences in plant treatment (grown in the absence or presence of NaCl) were not significant.

Addition of NaCl to the bath medium caused a substantial shift of V_r in the pith protoplasts (Table 1). This again indicates permeability to Na^+ in the pith cells. V_r, however, was closer to EK and only slightly shifted upon NaCl addition in the root protoplasts (Table 1). In these cells, NaCl treatment of the plants had no effect on Na^+ conductivity Statistical analyses showed a clear, significant ($P<0.05$) difference between root and pith cells in V_r response upon addition of NaCl.

Addition of verapamil (Table 1) changed I_{p50} further for both root and pith cells. Addition of amiloride blocked the channel activity of pith protoplasts almost completely, while it had no effect in root cells. Statistical analyses showed clear significant differences in the effects of amiloride

between the two cell types and also between the two different growing conditions. The blockage pattern in pith cells is presented in Fig. 4, in which channel activities are shown before (A) and after addition of NaCl (B) and after addition of amiloride (C). Blockage of the ORC activity in pith cells by amiloride indicates that Na^+ transfer may also be involved in the ORC activity.

3.2.1. Change of conductance

The substantial shift of V_r upon addition of NaCl to pith protoplasts of plants grown in the presence of NaCl in the nutrient solution, indicates that growing plants on NaCl may change the characteristics of the ORCs. To study this phenomenon, voltage dependence of pith cells from plants grown with NaCl was compared with those of plants grown without NaCl, for which the conductance's G (calculated as $G = I/V - EK$ and normalised to $G_{max,\ 40\ mV}$) were related to the membrane potentials. Fig. 5 shows that growing plants in the presence of NaCl in the nutrient solution has a distinct effect on the channel characteristics: whereas addition of NaCl to the bath medium does not substantially change the relative conductance's of the ORC in Fig. 5A, it does significantly change the characteristics in Fig. 5B. Apparently, growing plants with NaCl in the nutrient solution induces an adaptation of the Na^+ transport characteristics in pith cells. Root cells did not show these striking changes in conductance (data not shown).

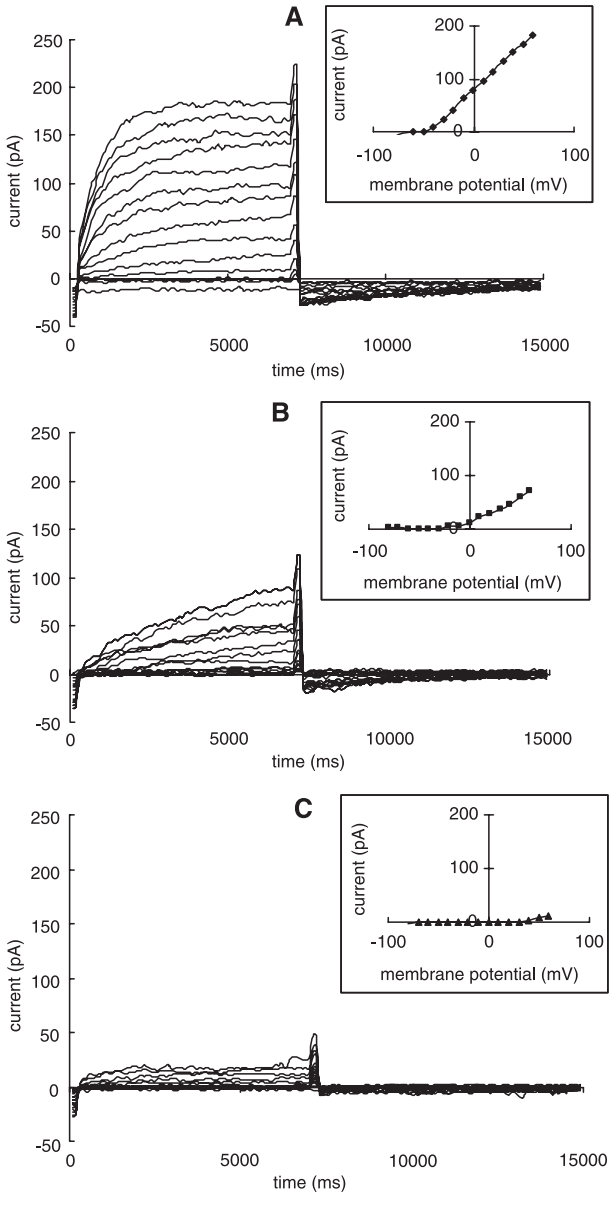

Fig. 4. Whole-cell recordings in a pith cell protoplast of sweet pepper grown in the presence of NaCl before addition of NaCl (A), 24 min after addition of 12.5 mM NaCl (B) and 40 min after addition of 12.5 mM NaCl and 2 min after addition of 100 μM amiloride (C). *Insets*: $I-V$ relationship corresponding to the whole-cell recordings measured at the end of each applied pulse (6.5 s) when the currents had reached steady state.

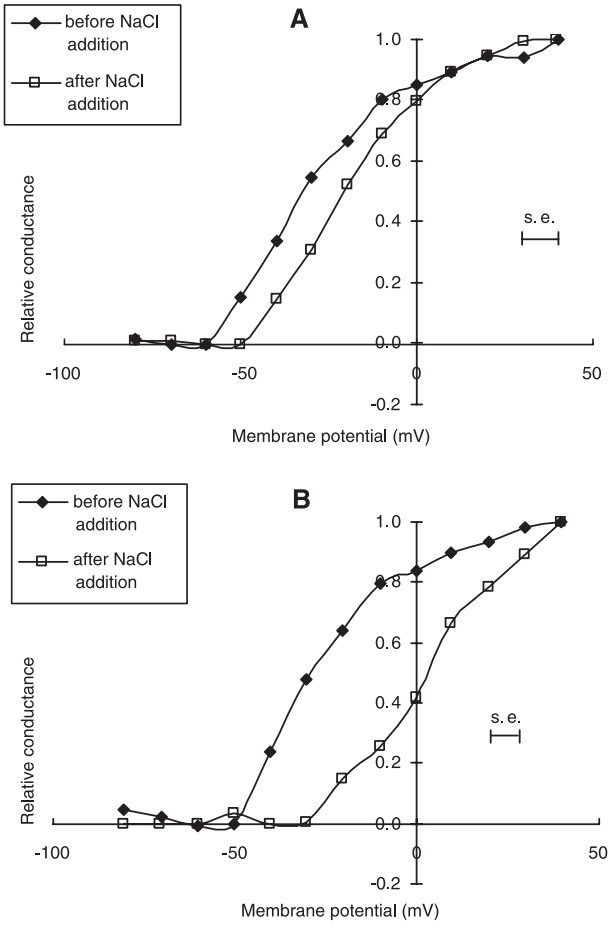

Fig. 5. Conductance ratios (G/G_{max}) of pith protoplasts of sweet pepper plants grown in the absence (A) or in the presence (B) of NaCl in the nutrient solution, measured before (♦) and after (□) addition of NaCl to the bath medium at different membrane potentials. G_{max} established at a membrane potential of 40 mV when currents had reached steady state. s.e. = standard error.

3.2.2. Permeability ratios

The permeability ratios for K^+, Cl^- and Na^+ after addition of NaCl to the bath medium are shown in Table

Table 2
Permeability ratios for K^+, Cl^- and Na^+ in the plasma membrane of pith and root cells of sweet pepper grown without (control) or with NaCl in the nutrient solution

	Control	NaCl in nutrient solution
	$p_K:p_{Na}:p_{Cl}$	$p_K:p_{Na}:p_{Cl}$
Pith cells	1:1.12:0.11	1:2.30:0.23
Root cells	1:0.84:0.08	1:0.60:0.06

Calculations are based on shift of the reversal potential 45 min after addition of NaCl to the bath medium.

2. In control plants, both pith and root protoplasts showed a permeability to Na^+ of the same magnitude as to K^+, whereas the permeability to Cl^- was low. The permeability to both Na^+ and Cl^- in pith cells was doubled when plants were exposed to NaCl for 1 week, whereas the permeability in the root protoplasts showed a decrease.

4. Discussion

The objective of this study was to evaluate whether, and if so which, channel activity is involved in Na^+ transport in root and pith protoplasts of sweet pepper. It can be concluded that:

1. Sweet pepper plants show ORC activity in both root and pith protoplasts.
2. The ORCs show conductivity for Na^+ especially in pith protoplasts.
3. Exposure of plants to NaCl in the nutrient solution for 1 week increases the conductivity for Na^+ in pith protoplasts, whereas it decreases in root protoplasts.
4. Exposure of plants to NaCl in the nutrient solution probably induces expression and formation of Na^+-permeable transporters in pith cells.

The channel activity found in this study mainly refers to voltage-dependent outward fluxes, whereas hardly any inward rectifiers are identified in the plasma membrane. In media with low Na^+, the reversal potential (V_r) was close to the equilibrium potential of the K^+ channel (EK), which indicates involvement of K^+-permeable channel activity. The shift of V_r upon NaCl addition in root and pith protoplasts suggests that for sweet pepper, the ORC is also permeable to Na^+ as already reported for cortical cells of plant roots of wheat [31]. In xylem parenchyma of barley roots, ORCs are also shown to be responsible for the release of cations, like Na^+, into the xylem vessels of the roots [32]. These authors showed a Na^+-permeability similar to that for K^+ which is in agreement with the observations in our study (Table 2).

The results show that the ORC characteristics of plants grown in the absence of NaCl differ between pith and root cells. For both cell types, addition of NaCl to the bath medium caused a decrease of I_{p50}, which may result from an inward current of Na^+ or a partial inactivation of the channel by Na^+. However, (1) the effect on I_{p50} was immediate in root protoplasts, whereas pith protoplasts responded much slower; (2) the shift of V_r in root protoplasts was smaller than in pith protoplasts; and (3) verapamil (a Ca^{2+} channel blocker that has been shown to block K^+ channels in plant cells as well [25–28]) had no effect on ORC activity of root protoplasts, whereas it completely blocked channel activity in pith protoplasts (Fig. 3). These results suggest that a voltage-dependent ORC is responsible for Na^+ transport in pith cells, whereas in root cells, the decrease of I_{p50} may rather be caused by blockage of the K^+ current than by an inward Na^+ current. Na^+ is mainly transported into root cells via non-selective cation channels [15,18,33]. These channels are voltage independent and instantaneous and not sensitive to amiloride. However, the ORC in pith cells, which may be responsible for Na^+ transport, is voltage dependent and sensitive to amiloride and verapamil. This indicates that the channel described in this study, especially for pith cells, clearly differs from the above-mentioned non-selective cation channels. For root cells, however, influx of Na^+ by non-selective cation channels as main pathway for transport cannot be ruled out, but the results of this study give no indications for this.

It is unlikely that the decrease of I_{p50} and shift of V_r upon addition of NaCl to the bath medium resulted from an affect of Cl^-, as this ion was already present in the bath and pipette solutions before NaCl addition. The ORC did show some conductivity for Cl^- which even increased for pith protoplasts when plants were grown on NaCl in nutrient solution (Table 2). The fact that the selectivity was much lower than for Na^+, however, means that the ORC will play a minor role for Cl^- transfer, especially in root protoplasts.

It is striking that only half of the successfully sealed protoplasts showed channel activity. This may be explained by several reasons; the most plausible are: damage during isolation and heterogeneity of protoplasts. However, the isolation procedure used shows a high success rate (>75%) in patch-clamp studies on channel activity [22]. Therefore, the absence of channel activity in putatively intact protoplasts is most likely caused by heterogeneity, in the form of sub-populations with different permeability characteristics.

4.1. Possibilities for adaptation

The results show that pith cells in sweet pepper plants change in reaction to the presence of NaCl in the nutrient solution. After plant exposure for 1 week, outward rectifying currents in these protoplasts showed a greater shift of V_r upon addition of NaCl to the bath medium. This suggests an increase of the permeability to Na^+. Moreover, the Na^+ channel blocker amiloride [29,34] blocked this channel activity significantly (Table 1). The appearance of channel activity that can be blocked by amiloride may result from de novo synthesis of Na^+-permeable channels. Moreover, the

changes of conductance upon addition of NaCl to the medium (Fig. 5B) are striking and also indicate a fundamental change of the plasma membrane characteristics. It has been shown very clearly that cells may respond to exposure to saline conditions by expression of a very large number of genes, regulating many cellular aspects [8]. However, the results from this study do not provide enough information to substantiate the assumption of de novo synthesis of voltage-dependent Na^+ permeable channels. This requires more research and alternative approaches, for example, microarray DNA studies.

Contrary to pith protoplasts, root cells do not change their channel characteristics following exposure to NaCl. As the shift of V_r is comparable to or smaller than the shift in the control plants, it may be concluded that the permeability of the voltage-dependent ORC for Na^+ does not change in these cells by exogenous NaCl exposure.

4.2. Selectivity under physiological circumstances

The question arises how Na^+ is preferentially accumulated in pith cells under physiological conditions and how it can suddenly be released from plants when growers renew their nutrient solutions as demonstrated with labelled Na^+ in one of our former studies [1]. As hardly any inward rectifiers are identified in the plasma membrane, it is unclear whether this channel type will play an important role in Na^+ uptake by cells. On the other hand, the ORCs show permeability to Na^+ and they may be suitable for inward-directed Na^+ currents [12]. As xylem vessels in intact sweet pepper plants contain ca. 10–20 mM Na^+ [1] and the Na^+ concentration in the cytoplasm of pith cells is considered to be much lower (i.e. 1 mM), the equilibrium potential for Na^+ (ENa) will have a value of ca. +70 mV. This will result in an inward Na^+ transport at a membrane potential $<+70$ mV. The K^+ concentrations within the cytoplasm for the in planta situation may vary between the different cell types. For leaf cells, concentrations up to 125 mM (mesophyll) or 250 mM (epidermis) have been described [35]. It seems plausible to consider a cytoplasmic K^+ concentration in the order of magnitude of the concentration used in the pipette solution in our patch-clamp experiments (i.e. 158 mM) for the pith cells of the in planta situation as well. Assuming a K^+ concentration in the xylem of 10 mM (in order of magnitude of the K^+ concentration in the nutrient solution), EK for the plasma membrane of the pith cells will then approximate to −55 mV. Then, the ORC will activate at membrane potentials >-55 mV. At physiological steady-state values of the membrane potential $\Delta\Psi$ (inside negative, i.e. −120 to −200 mV [7]), outward rectifiers will be closed and no inward Na^+ flux into the pith cells will occur. Then, accumulation of Na^+ in and release from the pith cells can only occur when either the membrane potential will depolarise upon a change of the environmental conditions as shown for xylem-parenchyma cells of barley roots [36] or when Na^+ is transported by voltage-independent channels as described for roots of maize [15], wheat [18], rye [37] and *Arabidopsis* [33]. Modelling of the Na^+ permeability of these voltage-independent channels [38,39] even showed that these channels contributed most to the Na^+ influx into rye root cells.

When growers renew their nutrient solutions, the exogenous Na^+ concentration in the root zone becomes lower than in the cytoplasm and ENa will shift to negative values. Consequently, Na^+ fluxes may become outward, supporting our findings that sweet pepper plants loaded with Na^+ in their pith cells will release all Na^+ upon transfer to a NaCl-free nutrient solution [1]. Again, an immediate depolarisation of the membrane potential upon renewal of the nutrient solution is a prerequisite for release of Na^+ from the pith cells. The results from this study are not sufficient to elucidate this aspect for in planta situations and needs further research.

It can be concluded that due to the electrophysiological characteristics of the plasma membranes, pith cells in sweet pepper show appropriate features of the voltage-dependent ORCs to enable accumulation or release of Na^+ by pith cells when the plants grow under saline conditions. The capacity to adapt to saline conditions may play an important regulatory role in Na^+ recirculation and thus in prevention of damage to the photosynthetic and other systems.

Acknowledgements

We are very grateful to Dr. J.P.F.G. Helsper, Dr. B. van Duijn and Dr. P.W.J. van den Wijngaard for critically reading the manuscript and giving helpful comments.

References

[1] M. Blom-Zandstra, S.A. Vogelzang, B.W. Veen, Sodium fluxes in sweet pepper exposed to varying sodium concentrations, J. Exp. Bot. 49 (1998) 1863–1868.
[2] B. Jacoby, Sodium recirculation and loss from *Phaseolus vulgaris* L., Ann. Bot. 43 (1979) 741–744.
[3] A.J.E. Van Bel, Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons? J. Exp. Bot. 47 (1996) 1129–1140.
[4] B.J. Barkla, O. Pantoja, Physiology of ion transport across the tonoplast of higher plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 159–184.
[5] T. Rausch, M. Kirsch, R. Löw, A. Lehr, R. Viereck, A. Zhigang, Salt stress responses of higher plants: the role of proton pumps and Na^+/H^+-antiporters, J. Plant Physiol. 148 (1996) 425–433.
[6] S.D. Tyerman, M. Skerrett, Root channels and salinity, Sci. Hortic. 78 (1999) 175–235.
[7] H. Sze, H^+-translocating ATPase. Advances using membrane vesicles, Annu. Rev. Plant Physiol. 36 (1985) 175–208.
[8] X.M. Niu, R.A. Bressan, P.M. Hasegawa, J.M. Pardo, Ion homeostasis in NaCl stress environments, Plant Physiol. 109 (1995) 735–742.
[9] A.A. Watad, M. Reuveni, R.A. Bressan, P.M. Hasegawa, Enhanced net K^+ uptake capacity of NaCl-adapted cells, Plant Physiol. 95 (1991) 1265–1269.

[10] J.I. Schroeder, J.M. Ward, W. Gassmann, Perspectives on the physiology and structure of inward-rectifying K^+ channels in higher plants: biophysical implications for K^+ uptake, Annu. Rev. Biophys. Biomol. Struct. 23 (1994) 441–471.
[11] D.P. Schachtman, J.I. Schroeder, Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants, Nature 370 (1994) 655–658.
[12] A. Amtmann, D. Sanders, Mechanisms of Na^+ uptake by plant cells, Adv. Bot. Res. 29 (1999) 75–112.
[13] E. Blumwald, G.S. Aharon, M.P. Apse, Sodium transport in plant cells, Biochim. Biophys. Acta 1465 (2000) 140–151.
[14] P.J. White, F. Lemtiri-Chlieh, Potassium currents across the plasma membrane derived from rye roots: a patch clamp study, J. Exp. Bot. 46 (1995) 497–511.
[15] S. Roberts, M. Tester, A patch clamp study of Na^+ transport in maize roots, J. Exp. Bot. 48 (1997) 431–440.
[16] S.D. Tyerman, M. Skerrett, A. Garrill, G.P. Findlay, R.A. Leigh, Pathways for the permeation of Na^+ and Cl^- into protoplasts derived from the cortex of wheat roots, J. Exp. Bot. 48 (1997) 459–480.
[17] P.H. Buschmann, R. Vaidynathan, W. Gassmann, J.I. Schroeder, Enhancement of Na^+ uptake currents, time-dependent inward-rectifying K^+ channel currents, and K^+ channel transcripts by K^+ starvation in wheat root cells, Plant Physiol. 122 (2000) 1387–1397.
[18] R.J. Davenport, M. Tester, A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat, Plant Physiol. 122 (2000) 823–834.
[19] Y. Murata, I. Obi, M. Yoshihashi, T. Ikeda, T. Kakutani, Salt adaptation of K^+ channels in the plasma membrane of tobacco cells in suspension culture, Plant Cell Physiol. 35 (1994) 637–644.
[20] A.A. Steiner, Soilless culture, Proceedings of the 6th Colloquium of the International Potash Institute, Florence, Italy, Bern, Switzerland, International Potash Institute, Bern, Switzerland, 1968, pp. 324–341.
[21] I. Rylski, *Capsicum*, in: A.H. Halevy (Ed.), Handbook of Flowering, vol. II, CRC, Boca Raton, 1985, pp. 140–146.
[22] M. Blom-Zandstra, H.T.M. Koot, J. van Hattum, S.A. Vogelzang, Isolation of protoplasts for patch-clamp experiments: an improved method requiring minimal amounts of adult leaf or root tissue from monocotyledonous or dicotyledonous plants, Protoplasma 185 (1995) 1–6.
[23] O.P. Hamill, A. Marty, E. Neher, B. Sakmann, F.J. Sigworth, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, Pflugers Arch. Eur. J. Physiol. 391 (1981) 85–100.
[24] S.A. Vogelzang, H.B.A. Prins, Plasmalemma patch-clamp experiments in plant root cells: procedure for fast isolation of protoplasts with minimal exposure to cell wall degrading enzymes, Protoplasma 171 (1992) 104–109.
[25] B.R. Terry, G.P. Findlay, S.D. Tyerman, Direct effects of Ca^{2+}-channel blockers on plasma membrane cation channels of *Amaranthus* tricolor protoplasts, J. Exp. Bot. 43 (1992) 1457–1473.
[26] S. Thomine, S. Zimmermann, B. Van Duijn, H. Barbier-Brygoo, J. Guern, Calcium channel antagonists induce direct inhibition of the outward rectifying potassium channel in tobacco protoplasts, FEBS Lett. 340 (1994) 45–50.
[27] J.T.M. Elzenga, M. Staal, H.B.A. Prins, Calcium–calmodulin signalling is involved in light-induced acidification by epidermal leaf cells of pea, *Pisum sativum* L., J. Exp. Bot. 48 (1997) 2055–2060.
[28] P.J. White, Calcium channels in higher plants, Biochim. Biophys. Acta 1465 (2000) 171–189.
[29] B. Hille, Ionic Channels of Excitable Membranes, IIth ed., Sinauer Associates Incorporated. Publishers Sunderland, Massachusetts, 1992, 607 pp.
[30] B. Van Duijn, D.L. Ypey, K.R. Libbenga, Whole-cell K^+ currents across the plasma membrane of tobacco protoplasts from cell-suspension cultures, Plant Physiol. 101 (1993) 81–88.
[31] D.P. Schachtman, S.D. Tyerman, B.R. Terry, The K^+/Na^+ selectivity of a cation channel in the plasma membrane of root cells does not differ in salt-tolerant and salt-sensitive wheat species, Plant Physiol. 97 (1991) 598–605.
[32] L.H. Wegner, K. Raschke, Ion channels in the xylem parenchyma of barley roots. A procedure to isolate protoplasts from this tissue and a patch-clamp exploration of salt passageways into xylem vessels, Plant Physiol. 105 (1994) 799–813.
[33] V. Demidchik, M. Tester, Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from *Arabidopsis* roots, Plant Physiol. 128 (2002) 379–387.
[34] O. Pantoja, J. Dainty, E. Blumwald, Tonoplast ion channels from sugar beet cell suspensions: inhibition by amiloride and its analogs, Plant Physiol. 94 (1990) 1788–1794.
[35] A.J. Karley, R.A. Leigh, D. Sanders, Differential ion accumulation and ion fluxes in the mesophyll and epidermis of barley, Plant Physiol. 122 (2000) 835–844.
[36] B. Kohler, L.H. Wegner, V. Osipov, K. Raschke, Loading of nitrate into the xylem: apoplastic nitrate controls the voltage dependence of X-QUAC, the main anion conductance in xylem-parenchyma cells of barley roots, Plant J. 30 (2002) 133–142.
[37] P.J. White, M. Tester, Potassium channels from the plasma membrane of rye roots characterized following incorporation into planar lipid bilayers, Planta 186 (1992) 188–202.
[38] P.J. White, M. Ridout, The K^+ channel in the plasma membrane of rye roots has a multiple ion residency pore, J. Membr. Biol. 143 (1995) 37–49.
[39] P.J. White, The molecular mechanism of sodium influx to root cells, Trends Plant Sci. 4 (1999) 245–246.