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Patch Clamp Analysis of the Dominant Plasma Membrane K^+ Channel in Root Cell Protoplasts of *Plantago media* L. Its Significance for the P and K State

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Abstract. Ion channels in the plasma membrane of root cell protoplasts of *Plantago media* L. were studied with the patch clamp technique in the cell-attached patch and outside-out patch configuration. An outward rectifying potassium channel was dominantly present in the plasma membrane. It appears responsible for the diffusional part, dominated by the K^+ diffusion potential, of the cell membrane potential, in vivo. This channel is activated at potentials near to and more positive than the K^+ diffusion potential. The dependence of this ion channel on K^+ activity and voltage has been characterized. The current-voltage relationships of the open channel at various K^+ concentrations are described by a four-state model. The membrane potential of intact protoplasts appears either dominated by the K^+ diffusion potential, the protoplast is then said to be in the K state, or by the pump potential generated by the plasma membrane-bound proton pump/H^+ ATPase, the P state. An experimental procedure is described to determine in cell-attached patch mode the state of the protoplast, either K or P state.

Key words: Potassium channel — K state — P state — Patch clamp — Membrane potential — Root cell

Introduction

The free-running membrane potential (E_m) of plant cells is generally dominated by two parallel potential differences, the potassium diffusion potential (E_K) and the pump potential (E_p). The latter is generated by the plasma membrane (PM)-bound H^+ ATPase (PM-H^+ ATPase or proton pump).

Prins and coworkers (De Boer, 1985; Maathuis & Prins, 1990) measured the E_m of root cells of *Plantago media* in excised roots. The root cells appeared to exist in two states. E_m was either close to the K^+ diffusion potential or much more hyperpolarized. Maathuis and Prins (1990) measured E_m at KCl concentrations between 1 and 100 mM and concluded that E_m of *P. media* roots under the given experimental conditions, was close to E_K. We will refer to this state of the cell as the K state (Bisson & Walker, 1982; Beilby, 1986). On the other hand, De Boer (1985), also using *P. media* root cells, showed that E_m could hyperpolarize to values 50 to 80 mV more negative than E_K in long duration experiments.

In these experiments a wound effect, due to isolation of root and the succeeding microelectrode impalement, probably caused cells to be in the K state at the start of an experiment. Within two hours, E_m slowly recovered and became more negative than E_K and could then remain hyperpolarized for 30 hr. Apparently under these conditions E_p dominated E_m. This hyperpolarized state is often called the pump or P state (Bisson & Walker, 1982; Beilby, 1986; Cruz-Mireles & Ortega-Blake, 1991).

The existence of a K state indicates the presence of a dominant K^+ conductance in the plasma membrane. Earlier whole-cell patch clamp experiments on *P. media* root cell protoplasts showed that a depolarization of E_m, to values near and more positive than E_K, activated outward rectifying currents (Vogelzang & Prins, 1992). These currents were highly selective for potassium.

In the present study, we applied the patch clamp technique to examine the state, P or K, of the root cell protoplasts of *P. media* and to establish the contribution of ion channels to the K state. To this end, ion channels activated in cell-attached patch (CAP) and outside-out patch (OOP) were compared. A dominant K^+ chan-

nel was identified and characterized with respect to its (i) ion selectivity, (ii) E_K-dependent channel activity, (iii) K^+ affinity and (iv) the K^+ activity and voltage dependence of its conductance. A four-state translocator model (Gradmann, Klieber & Hansen, 1987; Bertl, 1989) has been used to describe the single channel current voltage (*I-V*) relationship for a range of K^+ gradients. It will be discussed how in CAP this K^+ channel can be used to determine the P or K state of isolated protoplasts. The procedure used for this is based on the fact that when the protoplast is in the K state and a dominant K^+-specific conductance is present in the patch, the reversal potential can be calculated from the K^+ concentration in bath and pipette alone without knowing the cytoplasmic ion concentrations. Comparison of this calculated reversal potential with the one actually measured then indicates whether the protoplast is indeed in the K state or in the P state.

Materials and Methods

Isolation of Protoplasts

Root cortex protoplasts of *P. media* L. were isolated using the method of Vogelzang and Prins (1992). This method enables a rapid isolation of protoplasts with minimal exposure of the plasma membrane to the cell wall degrading enzymes. The cortex cell protoplasts were collected on the bottom of a "Leiden culture dish" (Ince, Van Dissel & Diesselhoff, 1985). Before the start of an experiment, the measuring chamber was perfused with the bath solution for a period of 15 to 30 min. The perfusion removed only the debris, while the intact protoplasts remained attached to the bottom of the dish.

Solutions

Unless noted otherwise, the solutions had the following compositions. Bath medium (mM): 350 mannitol, 10 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 5 MES, adjusted to pH 6.0 with 0.5 M BTP. Pipette medium (mM): 230 mannitol, 100 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1 K_4BAPTA (Sigma), 5 HEPES, adjusted to pH 7.2 with 0.5 M BTP. When potassium concentrations other than those mentioned above were used, the osmolarity was kept constant with mannitol to 387 and 446 mOsm for bath and pipette solutions, respectively.

Patch Clamp Experiments

Conventional patch clamp techniques were applied (Hamill et al., 1981) in the CAP and OOP configurations with an EPC-7™ patch clamp amplifier (List Electronics, Darmstadt, FRG) as previously published (Vogelzang & Prins, 1992). Transfer of voltage and current data, via a CED™ 1401 A/D-converter, was controlled by patch clamp software of CED (Cambridge, UK). Data were sampled at 1 to 12 kHz and stored on a personal computer.

Throughout the records, the flow of positive charges directed from the cytoplasmic side to the extracellular side (or negative charges in the opposite direction) is a positive current and defined as going outward.

Calculations

For analysis of single channel recordings, CED-compatible software was developed (obtainable upon request). This software allows a fast determination of single channel *I-V* curves. The parameters of the K^+ translocating four-state model (Gradmann et al., 1987; Bertl, 1989), *see below,* were determined by coupling the simplex algorithm (Caceci & Cacheris, 1984) to Turbo Pascal matrix-handling graphical routines, written for this procedure.

Permeability ratios for K^+ over Cl^-, P_K/P_{Cl} ratio, were calculated according to the Goldmann-Hodgkin-Katz voltage equation after a correction for ionic activities as described in the Debye-Hückel theory (1923). The P_K/P_{Cl} ratio quantifies the ability of a K^+ channel to discriminate K^+ going in from Cl^- going out (inward current) or K^+ going out from Cl^- going in (outward current). Measured values are expressed as means ± standard deviation (SD).

Identification of Ion Channels

Ion channels were identified based upon differences in conductance, rectification or kinetic behavior. Inward and outward rectifying channels were called, respectively, IRC and ORC. The outward rectifying conductance of a cation channel mainly depends on intracellular cation concentrations, the inward rectifying conductance on extracellular cation concentrations. In CAP, only the extracellular ion concentrations can be controlled. Therefore, in this configuration only inward rectifying conductances were used for comparison and identification of ion channels. The conductances were calculated from the slope of the inward currents of the *I-V* curves. As a rule, a distinction was made between conductances, measured in CAP, (from low to high conductances) if they differed by a factor of two or more.

Results

Ion Channels in the Cell-attached Patch Configuration

Using the above-described method, we identified eleven groups of channel conductances in CAP, five ORCs and six IRCs (Table 1). These data suggest that eleven different channels are present in the plasmalemma; however, these groups have not been observed together in one patch. Therefore, overlap cannot be excluded, specially since in CAP the whole-cell resistance and intracellular fluid composition are unknown.

One dominantly present ORC exhibited characteristic flickering kinetics. Hyperpolarization of the membrane potential always caused flickering behavior of the inward current (Fig. 1) with mean open and closed times of 1 to 3 msec. This channel was designated ORC-f, for outward-rectifying channel flickering. Extracellular Ca^{2+} in the range of 0.1 to 10 mM did not influence the mean open and closed times (*result not shown*).

Figure 2 shows a current trace of a patch in which three depolarization-activated ion channels were present. The ORC-f was present in more than 80% of the CAP measurements. Usually one to five ORC-f ion

Table 1. Ion channel conductances in the CAP configuration

Type	Range of K^+ activity (mM)		Conductance ± SD (pS)	No. of observations
ORC1			7 ± 3	2
ORC2			15 ±	1
ORC3			75 ± 7	3
ORC4			130 ±	1
ORC-f	4	17	22 ± 6	7
ORC-f	35	45	40 ± 7	12
ORC-f	58	78	49 ± 5	10
IRC1	50	100	6 ± 2	7
IRC2	50	100	14 ± 1	5
IRC3		100	28 ± 5	5
IRC4		50	53 ± 4	3
IRC5		100	73 ± 1	3
IRC6	50	100	127 ± 16	4

The conductance of ion channels in CAP activated either upon a hyperpolarizing potential, the IRCs, or upon a depolarizing potential, the ORCs. Also given are the number of observations and the pipette K^+ activity range. The conductance of the ORC-f has been calculated from their flickering inward current which depends on the pipette K^+ activity. The ORC1,2,3,4 did not conduct inward current; their conductance was calculated from outward currents which depend on the unknown cytosolic K^+ activity. A distinction was made between conductances (from low to high conductances) if they differed by a factor of two or more. An exception was made for IRC5 which was observed in the same patch as IRC4. The channels clearly differed in conductance.

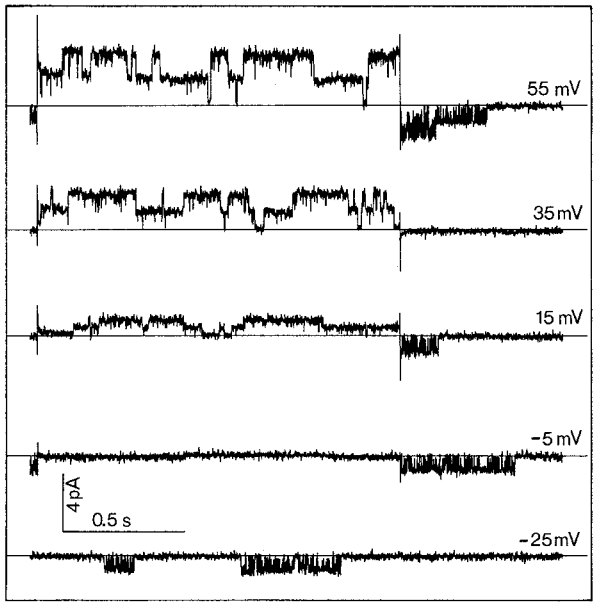

Fig. 1. Recordings of single channel events of ORC-f channels in CAP from cortex root cells of *P. media*. Five traces are displayed with their corresponding pulse potential on the right-hand side of each trace. Depolarizing voltage-clamp steps were applied (20 to 80 mV at 20 mV intervals) from a holding potential of −25 mV. The pulse potentials represent the difference between the reversal potential and the pulse pipette potential ($V_{r(CAP)} - V_{pulse(pip)}$). This correction results in a display of inward (negative) and outward (positive) K^+ current. Notice the flickering behavior of the inward currents. The traces are shifted along the Y-axis so that the current is zero with all channels closed. This amounts to a leak subtraction of 40 GΩ.

channels were active in one patch, the other channels were far less frequently observed (Table 1).

Six hyperpolarization-activated inward rectifying channels were found. None of these ion channels was dominantly present in CAP. Usually the activity of these channels decayed within 5 min after obtaining a gigaseal.

Ion Channels in the Outside-Out Patch Configuration

The conductances measured in OOP were arranged according to the classification of channel conductances measured in CAP (Table 1). One ORC and five IRCs were identified in OOP (Table 2). Comparison of the conductances indicated that all channels found in OOP were also found in CAP. A number of channels observed in CAP, namely ORC1,2,3,4 and IRC2 (Table 1), were not observed in OOP. As in CAP, the dominant ORC showed flickering and was accordingly identified as ORC-f.

Apart from the unitary conductances (one ORC and five IRCs), a significant current was observed in OOP in which no unitary changes could be detected. One possibility is that this current flows through a number of very small unitary conductances making its contribution to the total outward current very significant, while the individual currents through the single conductance could not be distinguished (Fig. 3). Although the exact nature of this conductance is still not clear, for convenience we will refer to these conductances as sub-pico channels or sub-pico conductances. Preliminary analysis of whole-cell data indicates that these sub-pico channels can conduct 30% of the total outward current and can be blocked with tetraethylammonium (TEA). Apparently, the total sub-pico conductance is proportional to the surface of the voltage-clamped membrane. We conclude that it is not an artifact caused by a voltage-dependent seal. Sub-pico currents were not observed in CAP.

"Normal" leakage currents flow through relatively voltage-independent nonselective background conductances. In contrast, here the sub-pico channels conduct a depolarization-activated outward going current, as does the ORC-f (Fig. 3). A depolarizing voltage jump from −120 to +40 mV caused an exponential activation of the outward current which reached a level of 4.7 pA after three seconds. Deactivation by a hyperpolarizing voltage jump from +40 to −120 mV caused a large inward current (−6.7 pA) which decayed expo-

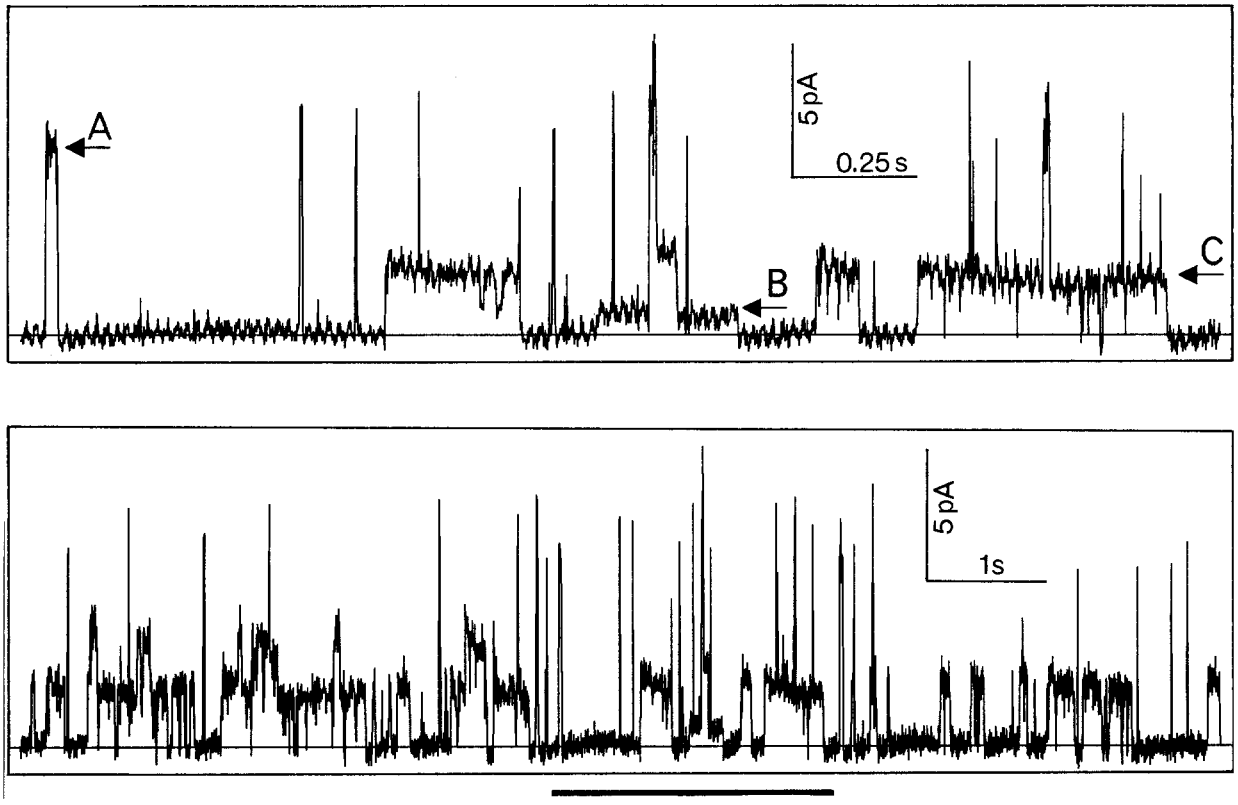

Fig. 2. Recordings of single channel events in the cell-attached patch configuration displaying the presence of three different depolarization-activated ion channels in one patch. The lower trace has a timescale of 10 sec, the upper trace corresponds with the part of the lower trace marked with a bar. In the upper trace, three different ion channels marked *A*, *B* and *C*, are shown. The pipette potential was −120 mV.

Table 2. Ion channel conductances in the OOP configuration

Type	Range of K^+ activity (mM)		Conductance ± SD (pS)	No. of observations	P_K/P_{Cl} ratio ± SD
ORC-f	6	18	20 ± 3	3	>550 ± 45
ORC-f	40	75	47 ± 2	9	>550 ± 45
IRC1		75	8 ±	1	2 ±
IRC2				0	
IRC3	50	100	30 ± 4	2	5 ± 1
IRC4	75	100	51 ± 3	2	2 ± 1
IRC5		100	73 ±	1	3 ±
IRC6	75	100	133 ± 6	2	6 ± 4

The conductance of ion channels in OOP activated either upon a hyperpolarizing potential, the IRCs, or upon a depolarizing potential, the ORCs. Also given are the number of observations, the pipette K^+ activity range and the P_K/P_{Cl} ratio. The conductances measured in OOP were arranged according to the classification of channel conductances measured in CAP (Table 1).

nentially. The preceding hyperpolarization step from a holding potential of −40 to −120 mV caused a much smaller inward current (−2.7 pA) demonstrating that fewer sub-pico conductances were activated at −40 than at +40 mV. The sub-pico conductance thus behaves in a voltage-dependent manner.

The ORC-f was highly selective for K^+ in contrast to the IRCs (Table 2). The ORC-f had a P_K/P_{Cl} ratio higher than 550. The reversal potential ($V_{r(OOP)}$) plotted against E_K (Fig. 4) yielded a straight line with a slope of nearly one (correlation coefficient 0.99) and an intersection close to zero millivolts. $V_{r(OOP)}$ of the ORC-f thus equals E_K. Addition of 100 mM Na^+Cl^- to the extracellular side of the plasmalemma (without

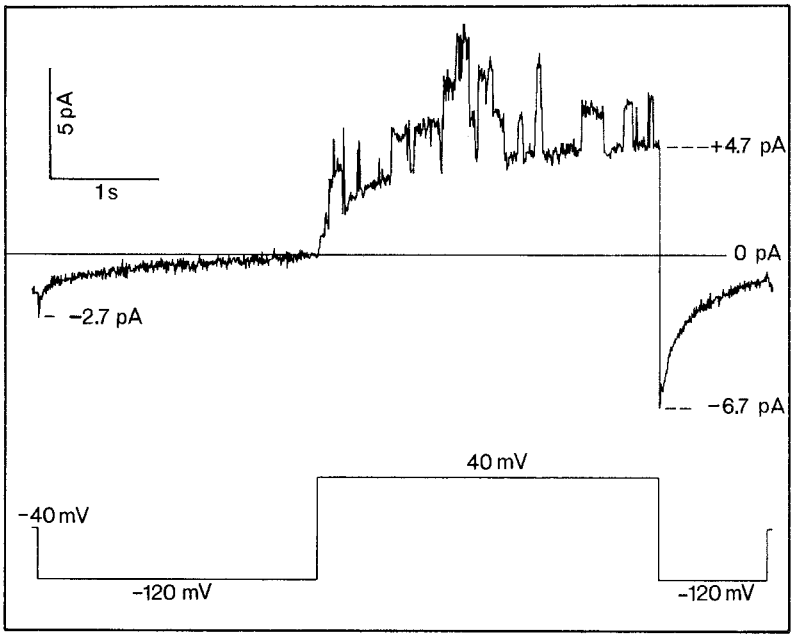

Fig. 3. Recording of single channel events in the OOP mode displaying a hyperpolarizing pulse of −120 mV, interrupted by a depolarizing potential of +40 mV. Depolarization caused the activation of the ORC-f and the sub-pico conductances. After 3 sec, the outward current carried by the sub-pico conductances reached a value of +4.7 pA. The return to the hyperpolarizing potential (−120 mV) caused an inward peak current of −6.7 pA and deactivation of the sub-pico conductances. The traces are shifted along the Y-axis so that the current is zero with all channels closed. This amounts to a leak subtraction of 11 GΩ.

Na^+Cl^- on the cytoplasmic side) did not significantly change $V_{r(OOP)}$ of the ORC-f. Perfusion of the extracellular solution with 50 mM TEA resulted in a complete block of the ORC-f (*data not shown*).

$V_{r(OOP)}$ of the sub-pico conductances could be estimated from the difference between the current at the end of a +40 mV pulse and the current at the start of a successive −120 mV pulse. From this, the P_K/P_{Cl}-ratio was calculated to be larger than three, assuming a linear *I-V* curve. Single channel *I-V* curves of sub-pico channels of course could not be produced as the unitary current steps could not be detected. Preliminary data of whole-cell experiments, not shown here, indicate a higher selectivity for K^+. According to these data, the P_K/P_{Cl} ratio would be in the order of 300.

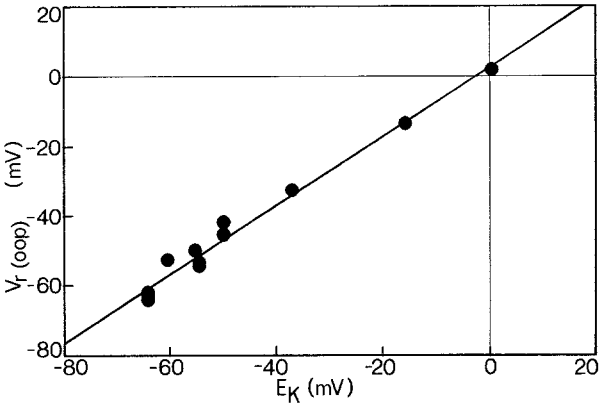

Fig. 4. $V_{r(OOP)}$ plotted *vs.* E_K. $V_{r(OOP)}$ and E_K were determined for 12 different depolarization-activated outward rectifying ion channels (ORC-f). The correlation coefficient was 0.99.

E_K-dependent Channel Activity

Voltage-dependent activation of the ORC-f shifted with E_K and was always observed at potentials near and more positive than E_K. A change from potentials more positive than E_K to potentials more negative than E_K only resulted in flickering of ORC-f if the channel was in an open state at the end of the depolarizing potential (Fig. 1). Flicker activity always decayed within several seconds. Sometimes "spontaneous" flickering occurred at constant potentials slightly more negative than E_K. The lowest trace of Fig. 1 gives an example of such "spontaneous" flickering. Here the holding potential was 25 mV more negative than E_K. Typically, such spontaneous flicker activity was completely absent if the holding potential was 50 mV more negative than E_K. Apparently, the ORC-f can conduct currents at holding potentials slightly more negative than E_K. Its threshold potential for activation lies between 25 and 50 mV more negative than E_K.

Dependence on K^+ Concentrations

In CAP, the conductance of the ORC-f was tested for dependence on the external K^+ activity. The inward rectifying conductance saturated above 58 mM reaching an average maximum of 49 ± 5 pS (Table 1). As expected, the outward rectifying conductance did not depend on external K^+ activity but remained relatively constant (23 ± 5 pS). In CAP, the outward rectifying conductance depends only on the cytoplasmic K^+ activity. Maathuis and Prins (1990) estimated the cyto-

plasmic K^+ concentration of *P. media* root cells to be 107 mM corresponding to an internal K^+ activity of 80 mM. As mentioned above, the outward rectifying conductance measured under these conditions was 23 ± 5 pS. In contrast, here the inward rectifying conductance at a comparable external K^+ activity of 80 mM was about twice as large, 49 ± 5 pS. This asymmetry is also reflected in the *I-V* curves made in CAP (Fig. 1) and OOP (Fig. 6).

The inward rectifying conductance of the ORC-f saturates at high K^+ activity at the extracellular side (Fig. 5). Fitted with the Michaelis-Menten equation, the data from CAP and OOP could be compared, on the assumption that the inward rectifying conductance mainly depends on the K^+ activity on the extracellular side of the plasmalemma. Best fits were obtained with a maximum conductance (G_{max}) of 54 pS for CAP and 62 pS for OOP. The corresponding K_m values were 10 and 17 mM, respectively. Both the G_{max} and the K_m values were lower in CAP than in OOP although not significantly. In CAP, a low conductance of the cell plasma membrane in contact with the bath solution and in series with the patch membrane may have caused an underestimation of the conductance in CAP.

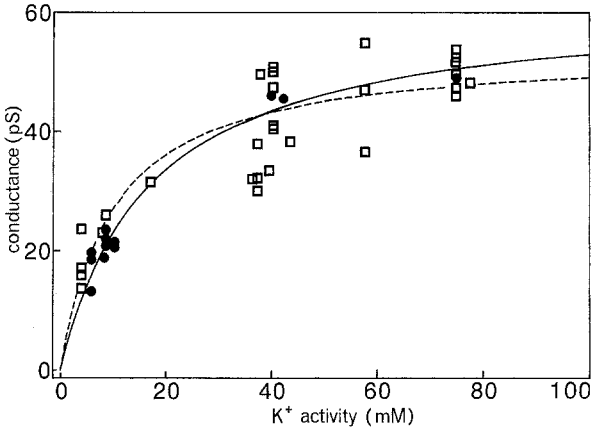

Fig. 5. The conductance, calculated from the slope of the *I-V* curves of inward currents of ORCs-f in the CAP (□) and OOP mode (●), plotted against the K^+ activity of pipette (CAP) and bath (OOP) solution. Two Michaelis-Menten curves are drawn through the data points; the dotted line represents a fit of the CAP data with a V_{max} of 54 pS and a K_m of 10 mM, the uninterrupted line gives the fit of the OOP data with a V_{max} of 62 pS and a K_m of 17 mM.

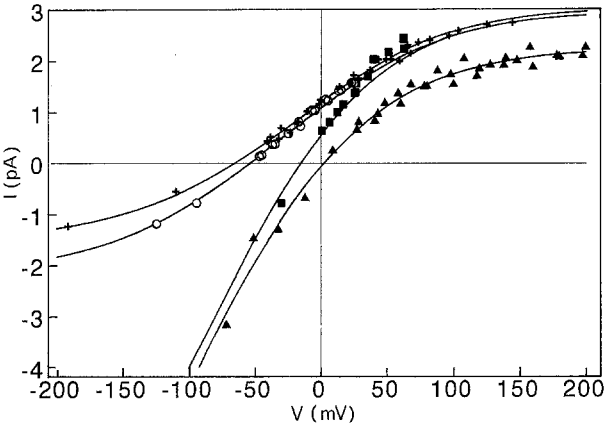

Fig. 6. The *I-V* curves of four ORC-f channels, measured in OOP at different ionic conditions. $[K^+]_{in}$ = 80.3 mM and $[K^+]_{out}$ = 5.9 mM (+); $[K^+]_{in}$ = 74.8 mM and $[K^+]_{out}$ = 8.6 mM (○); $[K^+]_{in}$ = 76.0 mM and $[K^+]_{out}$ = 40.0 mM (■); $[K^+]_{in}$ = 40.0 mM and $[K^+]_{out}$ = 42.0 mM (△). The lines through the data points result from a single set of parameters of the four-state model, which predict the *I-V* curves for the different ionic conditions.

Modeling Single Channel Current-Voltage Curves

Ion channels may be characterized by modeling the behavior of single channel *I-V* curves under different ionic conditions as described by Gradmann et al. (1987). The procedure allows a prediction of the shape of steady-state *I-V* curves at various substrate concentrations. For the present experimental results, we used a four-state model described by Bertl (1989) for a K^+ channel present in the tonoplast of *Chara corallina*. This four-state cyclic model (Fig. 7) has one single charge translocating step (Class I transporter, Hansen et al., 1981). The observed currents result from N1 and N2, which designate the amount of transporters being in state 1 (charged binding site, facing inside) and state 2 (charged binding site, facing outside). The transition rates, k_{12} and k_{21}, between these two states are voltage sensitive and may be described as:

$$k_{12} = k_{12}^o \exp\left(\frac{+zeV}{2kT}\right) \tag{1a}$$

and

$$k_{21} = k_{21}^o \exp\left(\frac{-zeV}{2kT}\right) \tag{1b}$$

where k_{12}^o and k_{21}^o are the voltage-sensitive rate constants k_{12} and k_{21} at $V = 0$ mV, z is the valence of the charged K^+ ion crossing the membrane, e the elementary charge, k the Boltzmann constant and T the absolute temperature on the Kelvin scale. The factor two showing up in Eq. (1) is based on the assumption of a symmetric Eyring barrier (Läuger & Stark, 1970). In this four-state model, the effective rate constants k_{32} and k_{41} will depend on the respective K^+_{out} and K^+_{in} concentrations:

$$k_{32} = k_{32}^* [K^+]_{out} \tag{2a}$$

and

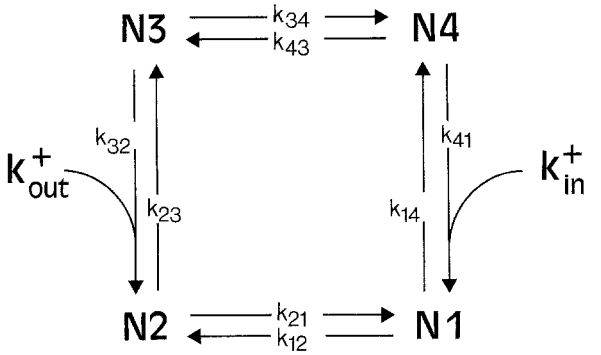

Fig. 7. Scheme of a four-state reaction for uniport of potassium ions with probabilities N_1 to N_4 and eight rate constants; k_{12} and k_{21} are voltage sensitive.

$$k_{41} = k^*_{41}\,[K^+]_{in} \tag{2b}$$

For steady-state conditions, a linear equation system can be solved (Bertl, 1989) leading to the following *I-V* equation for the four state model:

$$I(V) = ze\frac{k_{12}X1 - k_{21}X2}{X1 + X2 + X3 + X4} \tag{3}$$

with

$$X1 = k_{32}k_{21}\,(k_{41} + k_{43}) + k_{34}k_{41}\,(k_{21} + k_{23}) \tag{4a}$$
$$X2 = k_{41}k_{12}\,(k_{32} + k_{34}) + k_{43}k_{32}\,(k_{12} + k_{14}) \tag{4b}$$
$$X3 = k_{23}k_{12}\,(k_{41} + k_{43}) + k_{43}k_{14}\,(k_{21} + k_{23}) \tag{4c}$$
$$X4 = k_{14}k_{21}\,(k_{32} + k_{34}) + k_{34}k_{23}\,(k_{12} + k_{14}) \tag{4d}$$

To obtain the parameters for the model shown in Fig. 7, single channel *I-V* curves were made at different ionic conditions. Equations (3) and (4) were used to estimate the parameters of the four-state model for the data plotted in Fig. 6. The results of these calculations are presented in Table 3 and displayed as lines in Fig. 6. This figure shows that the four-state model can describe accurately the single channel *I-V* curves of the ORC-f under different ionic conditions.

P or K State of E_m of Intact Protoplasts

The free-running membrane potential, E_m, of protoplasts depends on the pump potential E_p and the parallel diffusion potential, generally dominated by E_K. In the whole-cell configuration E_m can be determined in current clamp. However, there is generally a rapid equilibration between the content of the pipette and the cytoplasm. Therefore, the whole-cell configuration cannot be used to measure E_m as it is in the intact protoplast, nor can it be decided if the intact protoplast is in the K or P state. On the other hand, impalement of the protoplast with a small tip/high resistance microelectrode is often problematic, due to the lack of turgor and uncertainty of the tip position (in the cytosol or in the vacuole). In CAP, the cytoplasmic concentrations are not affected by the pipette content. Although it is not possible in CAP to measure E_m directly, it is possible to determine how much E_m differs from $E_{K(cell)}$ and thus to decide whether the protoplast is in the P or K state. It should be emphasized here that only the deviation from $E_{K(cell)}$ is estimated and not the true value of E_m. To do this, the presence of a highly selective K^+ channel in the patch is needed. It is then possible in CAP to predict the reversal potential when a protoplast is in the K state $V_{r(CAP,K)}$.

E_K of the cell interior with respect to the pipette ($E_{K(patch)}$) and with respect to the bath ($E_{K(cell)}$) both depend on the cytosolic K^+ activity. As will be explained below, they thereby eliminate the need to know the cytoplasmic K^+ activity to calculate $V_{r(CAP,K)}$.

In CAP, pulses are applied across both patch and cell membrane in series, and the voltage drop will be distributed between the two in a proportion determined by their relative conductances. When current is flowing, the voltage drop across each will be set by the condition of equal flow, but across their respective conductances. At each, the following will apply:

$$I_m = I_K + I_p \tag{5a}$$

where I_m is the total membrane current, I_K the K current and I_p the pump current (if the pump is active). Therefore:

$$I_m = g_K(V_m - E_K) + g_p(V_m - E_p) \tag{5b}$$

where g_K and g_p are the conductances of the K channels and pump, respectively, E_k the K-equilibrium potential for that membrane, and E_p the pump potential.

$$I_m = (g_K + g_p)V_m - g_K E_K - g_p E_p \tag{5c}$$

Thus, at the patch (where with 100 mM K^+ outside, and a comparable concentration inside, the K channels are dominant and the pump will be off or can be ignored):

$$I_{patch} = g_{K(patch)}(V_{patch} - E_{K(patch)}) \tag{6}$$

V_{patch} is the membrane potential across the patch, $g_{K(patch)}$ and $E_{K(patch)}$ likewise refer to the patch. Over the rest of the cell:

$$I_{cell} = g_{K(cell)}(V_{(cell)} - E_{K(cell)}) + g_{p(cell)}(V_{cell} - E_{p(cell)}) \tag{7}$$

or

$$I_{cell} = (g_{K(cell)} + g_{p(cell)})V_{cell} - g_{K(cell)}E_{K(cell)} - g_{p(cell)}E_{p(cell)}$$

$I_{cell} = I_{patch}$ in magnitude, but one is inward and the other outward.

$$V_{cell} = \frac{I + g_{K(cell)}E_{K(cell)} + g_{p(cell)}E_{p(cell)}}{g_{p(cell)} + g_{K(cell)}} \quad (8)$$

If $I = 0$ at the reversal potential ($V_{r(cell)}$):

$$V_{r(cell)} = \frac{g_{K(cell)}E_{K(cell)} + g_{p(cell)}E_{p(cell)}}{g_{p(cell)} + g_{K(cell)}} \quad (9)$$

At the patch $I = 0$ gives $V_{patch} = E_{K(patch)}$. The pulse is the sum of the two voltages:

$$V_{pulse} = \frac{g_{K(cell)}E_{K(cell)} + g_{p(cell)}E_{p(cell)}}{g_{p(cell)} + g_{K(cell)}} + E_{K(patch)} \quad (10)$$

If there is no pump activity then:

$$V_{r(CAP)} = E_{K(cell)} + E_{K(patch)} \quad (11)$$

where $V_{r(CAP)}$ is the reversal potential observed when the cell is in the K state. The Nernst potential for K^+ across the patch ($E_{K(patch)}$) is given by:

$$E_{K(patch)} = -58 \text{ mV} \log \left(\frac{[K^+]_{pipette}}{[K^+]_{cytosol}} \right) \quad (12)$$

and $E_{K(cell)}$ by:

$$E_{K(cell)} = -58 \text{ mV} \log \left(\frac{[K^+]_{cytosol}}{[K^+]_{bath}} \right) \quad (13)$$

Inserting Eqs. (12) and (13) into Eq. (11) yields the reversal potential of a K^+ channel in CAP for a protoplast in the K state:

$$V_{r(CAP,K)} = -58 \text{ mV} \log \left(\frac{[K^+]_{pipette}}{[K^+]_{bath}} \right) \quad (14)$$

We defined the right term of this equation as $V_{r(CAP,K)}$. Notice that $V_{r(CAP,K)}$ is independent of $[K^+]_{cytosol}$. If the pump is active there will be a discrepancy which is coined the residual potential (V_{res}, Eq. 15):

$$V_{res} = \frac{g_{p(cell)}(E_{p(cell)} - E_{K(cell)})}{g_{p(cell)} + g_{K(cell)}} \quad (15)$$

or

$$V_{res} = V_{cell} - E_{K(cell)} \quad (16)$$

V_{res} is equal to the deviation of the actually measured $V_{r(CAP)}$ from the calculated $V_{r(CAP,K)}$:

$$V_{res} = V_{r(CAP)} - V_{r(CAP,K)} \quad (17)$$

V_{res} has been determined for 25 protoplasts at different K^+ activities in the bath solution (Fig. 8*A*). Over the entire K^+ range, two groups could be identified (Fig. 8*B*). First, the 18 protoplasts with K state potentials ($V_{r(CAP)} \approx V_{r(CAP,K)}$). Second, the five cells that were in a P state ($V_{r(CAP)} < V_{r(CAP,K)}$ and $V_{res} \approx -90$ mV). Notice that the V_{res} value of these latter cells behaves independently of the K^+ activity of the bath.

Discussion

The ORC-f and the Sub-Pico Conductance

The ORC-f shift in activity with E_K indicates a K^+-sensing gating mechanism for this channel. K^+ sensing by inward rectifying channels has been reported in guard cells of stomata by Schroeder and Fang (1991) and in barley aleurone protoplasts (Bush et al., 1988). Such a gating mechanism creates a K^+ transport system which, because of its kinetic control, can adapt to changing external K^+ concentrations. Schroeder and Fang (1991) reported the presence of an inward rectifying K^+ channel in guard cells with a K_m of 3.5 mM. It was concluded that this conductance allows for a low affinity uptake of K^+. Apparently, the ORC-f observed here with a K_m between 12 and 17 mM is an example of a transport system with a lower affinity.

The single channel *I-V* curves obtained in CAP and OOP were asymmetrical. Such asymmetrical *I-V* curves of ion channels have been observed in membranes of several plant species (Schroeder, Hedrich & Fernandez, 1984; Bertl & Gradmann, 1987; Bertl, 1989). The eight rate constants of the four-state model (Table 3) were compared with those found for a *Chara* tonoplast K^+ channel (Bertl, 1989; A. Bertl, *personal communication, see* footnote [a] in legend of Table 3) and those from a *Vicia faba* K^+ channel (Gradmann et al., 1987). For all channels, the slowest rate constants were the empty site reorientation constants (k_{34} and k_{43}). In all cases, the inward reorientation of the empty side was slower than the outward reorientation, resulting in a smaller outward than inward rectifying conductance. The outside K^+ debinding constant (k_{23}) is the only constant that is the same for all three channels. The outside K^+ binding constant (k^*_{32}) and the inward-going empty site reorientation (k_{34}) constants are equal for *P. media* and *Vicia faba*. Two of the constants, the outward-going empty site reorientation (k_{43}) and the inside K^+ binding (k_{41}), found for the ORC-f were very sim-

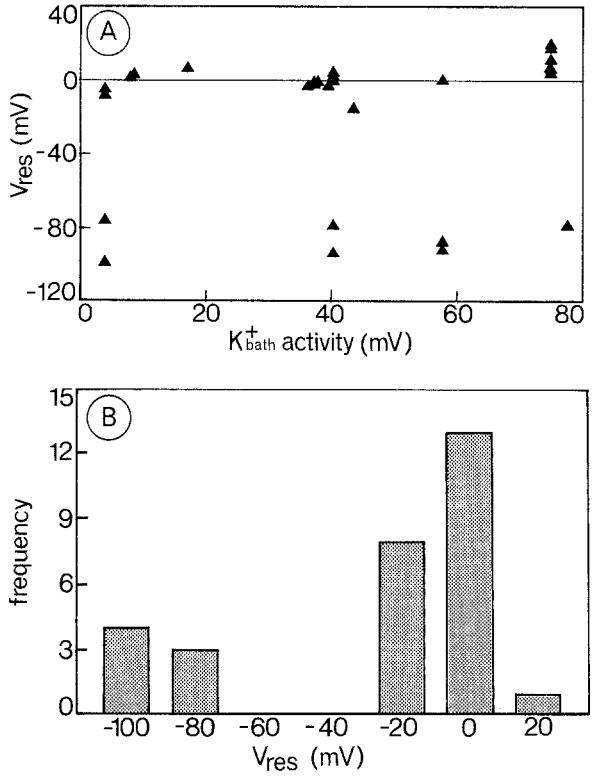

Fig. 8. (*A*) The residual potential (V_{res}) plotted *vs.* the K^+ activity of the bath solution. V_{res} is the difference between the measured reversal potential ($V_{r(CAP)}$) of an ORC-f channel in CAP and the calculated reversal potential of a cell in a K state ($V_{r(CAP,K)}$). (*B*) Frequency histogram for V_{res} from the corresponding CAP measurements.

ilar to those of *Chara*. The inside K^+ debinding constant (k_{14}) of ORC-f was very low compared to those of the other channels. Most rate constants were of the same order of magnitude, except for the K^+ translocation constants (k^o_{12} and k^o_{21}) which were much higher in *V. faba*.

The kinetics of the sub-pico conductance(s) were comparable with those of "normal" unitary conductances. The single exponential activation and deactivation curves indicate kinetic behavior similar to that of an ion channel that has one open and one closed state. However, unitary changes of this conductance were not observed in OOP measurements, implying that its conductance was below the detection level. The conductance of a single channel must have been in the range of femtosiemens rather than picosiemens. Therefore, we propose the name sub-pico conductance, although the true nature of this conductance awaits further clarification. It seems less likely that the sub-pico conductance is an artifact comparable to the normal leak, as it is voltage dependent and selective for K^+. Preliminary whole-cell data, not shown here, indicate an even higher selectivity for K^+, with respect to Cl^-, than indicated by the present data.

K and P State in Protoplasts

The majority of the protoplasts, 18 out of 25, were in the K state with a V_{res} close to zero, or, otherwise stated, with E_m close to E_K. The other protoplasts all had a V_{res} of -90 mV, which indicates that these protoplasts were in the P state. Protoplasts in the K state and in the P state were observed over the entire bath K^+ activity range tested, from 5 to 80 mM (Fig. 8*A*). Thus, in protoplasts at least, the K or P state is not determined by the external K^+ activity.

By definition, E_m in the P state is dominated by E_p, the electrogenic potential of the proton pump. From this, one should expect that in the P state, E_m is rather insensitive to the external K^+ activity, as often observed in intact root cells. This does not seem to be the case in the protoplasts. E_m being always 90 mV more negative than E_K implies that the slope of the relation between E_m and the external K^+ activity is always around 58 mV per decade in the P as well as in the K state, assuming that the intracellular K^+ concentration remains constant. Evidently, the protoplasts in the P state must have some regulatory mechanism of E_m that uses E_K as a reference and has a K^+-sensing mechanism as was concluded for the ORC-f, suggesting some kind of coupling between the two. The transition from K to P state, and vice versa, observed in intact root cells (De Boer, 1985) and here in protoplasts, shows that the proton pump and K^+ conductances (G_p and G_K, respectively) may change dramatically, indicating kinetic control (Gradmann & Bertl, 1989). The dominant presence of ORC-f in CAP as well as in OOP, its high selectivity for K^+, and its activity at potentials near E_K makes this channel a very likely candidate for maintaining the stable K state in *P. media* root cell protoplasts. At potentials more positive than E_K, outward rectifying currents carried by this channel will tend to drive E_m towards E_K. Its inward rectifying currents would do the same in the opposite direction. The selectivity, P_K/P_{Cl} ratio, of the IRCs (Table 2) is such that they will tend to drive E_m to values more positive than E_K. In the CAP experiments with protoplasts, this would mean a positive V_{res}. This was never observed; V_{res} was either close to zero or -90 mV. On the other hand, the outward current of the sub-pico conductance might contribute to the K state given its selectivity for K^+.

That ORC-f is activated at potentials more positive than E_K and deactivated at more negative potentials forms also a kind of "flip-flop" mechanism for regulation of E_m at two stable states. When the cell is in the P state, $E_m << E_K$, the ORC-f channels will be mainly in the closed state and E_m will be dominated by E_p, where E_p is determined by the energy charge of the cell/protoplast. If by some unknown mechanism E_m becomes depolarized, the ORC-f will be activated. This will bring the cell in the K state. In this state, small vari-

Table 3. Parameters of the four-state reaction cycle model

Meaning	Constants[b]	*P.media*	*C.corallina*	*V.faba*		
		Value	Value	Value	Ratio	Ratio
		A	B	C	A/B	A/C
K^+ translocation $i \rightarrow o$	k_{12}^o	5.3E3	1.3E4	1.5E2	0.4	35.3
K^+ translocation $o \rightarrow i$	k_{21}^o	9.0E3	2.7E3	2.0E2	3.3	45.0
K^+ debinding o	k_{23}	6.7E4	5.3E4	9.5E4	1.3	0.7
K^+ binding o	k_{32}^*	1.7E3	6.7E3	1.7E3	0.3	1.0
Empty site reorient $o \rightarrow i$	k_{34}	2.9E1	1.1E2	2.8E1	0.3	1.0
Empty site reorient $i \rightarrow o$	k_{43}	3.1E2	2.5E2	3.2E1	1.2	9.7
K^+ binding i	k_{41}^*	7.6E3	5.0E3	2.6E4	1.5	0.3
K^+ debinding i	k_{14}	1.7E4	7.9E4	8.3E4	0.2	0.2

Parameters of the four-state model (Fig. 7) for the *I-V* relationships of the *P.media* ORC-f channel. Data were compared with similar values of K^+ channels from *C.corallina* tonoplast (Bertl, 1987, *personal communication*[a]) and *V.faba* plasmalemma (Gradmann et al., 1987). i = inside. o = outside.

[a] The first eight parameters in the third table of Bertl (1989) should be multiplied by a factor of 6.3.

[b] Rate constants expressed as 10^6 sec^{-1} except for k_{32}^* and k_{41}^*: 10^6 $sec^{-1}M^{-1}$.

ations in proton pump activity will not affect E_m. However, if by some mechanism a large hyperpolarization with respect to E_K is induced, the K^+ conductances of ORC-f will be deactivated and E_m will be dominated by E_p and the cell is again in the P state.

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References

Beilby, M.J. 1986. Factors controlling the K^+ conductance in *Chara*. *J. Membrane Biol.* **93:**187–193

Bertl, A. 1989. Current-voltage relationships of a sodium-sensitive potassium channel in the tonoplast of *Chara corallina*. *J. Membrane Biol.* **109:**9–19

Bertl, A., Gradmann, D. 1987. Current voltage relationships of potassium channels in the plasmalemma of *Acetabularia*. *J. Membrane Biol.* **99:**41–49

Bisson, M.A., Walker, N.A. 1982. Transitions between modes of behavior (states) of the charophyte plasmalemma. *In:* Plasmalemma and Tonoplast: Their Functions in the Plant Cell. D. Marmé, E. Marrè and R. Hertel, editors. pp. 35–40. Elsevier Biomedical Press B.V., Amsterdam

Bush, D.S., Hedrich, R., Schroeder, J.I., Jones, R.L. 1988. Channel-mediated potassium flux in barley aleurone protoplasts. *Planta* **176:**368–377

Caceci, M.S., Cacheris, W.P. 1984. Fitting curves to data: The simplex algorithm is the answer. *Byte* **5:**340–362

Cruz-Mireles, R.M., Ortega-Blake, I. 1991. Effect of Na_3VO_4 on the P-state of *Nitella* translucens. *Plant Physiol.* **96:**91–97

De Boer, A.H. 1985. The energy metabolism of excised *Plantago media* roots and its relation to the plasma membrane electrical potential difference. *In:* Xylem/Symplast Ion Exchange: Mechanism and Function in Salt-Tolerance and Growth. Ph.D. thesis. Rijks Universiteit Groningen, Groningen, The Netherlands

Debye, P., Hückel, E. 1923. Zur Theorie der Elektrolyte. II Das Grenz-gesetz für die elektrische Leitfähigkeit. *Phys. Z.* **24:**305–325

Gradmann, D., Klieber, H.G., Hansen, U.P. 1987. Reaction kinetic parameters for ion transport from steady-state current-voltage curves. *Biophys. J.* **51:**569–585

Gradmann, D., Bertl, A. 1989. Physiological control of membrane currents in plants. *Plant Physiol. Biochem.* **27:**587–593

Hamill, O., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell free membrane patches. *Pfluegers Arch.* **391:**85–100

Hansen, U.P., Gradmann, D., Sanders, D., Slayman, C.L. 1981. Interpretation of current voltage relationships for "active" ion transport systems: I. Steady-state reaction-kinetic analysis of Class-I mechanisms. *J. Membrane Biol.* **63:**165–190

Ince, C., Van Dissel, J.T., Diesselhoff, M.M.C. 1985. A teflon culture dish for high magnification microscopy and measurements in single cells. *Pfluegers Arch.* **403:**240–244

Läuger, P., Stark, K. 1970. Kinetics of carrier mediated ion transport across lipid bilayer membranes. *Biochim. Biophys. Acta* **211:**548–566

Maathuis, F.J.M., Prins, H.B.A. 1990. Electrophysiological membrane characteristics of the salt tolerant *Plantago maritima* and the salt sensitive *Plantago media*. *Plant and Soil* **123:**233–238

Schroeder, J.I., Hedrich, R., Fernandez, J.M. 1984. Potassium-selective single channels in guard cell protoplasts of *Vicia faba*. *Nature* **312:**361–362

Schroeder, J.I., Fang, H.H. 1991. Inward-rectifying potassium channels in guard cells provide a mechanism for low-affinity potassium uptake. *Proc. Natl. Acad. Sci. USA* **88:**11583–11587

Vogelzang, S.A., Prins, H.B.A. 1992. Plasmalemma patch clamp experiments in plant root cells: procedure for fast isolation of protoplasts with minimal exposure to cell wall degrading enzymes. *Protoplasma* **171:**104–109