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Partial Purification of a Potassium Channel with Low Permeability for Sodium from Tonoplast Membranes of *Hordeum vulgare* cv. Gerbel

Barbara Klughammer, Marion Betz, Roland Benz†, and Karl-Josef Dietz
Lehrstuhl für Botanik 1 and †Lehrstuhl für Biotechnologie der Universität, 8700 Wüzburg, Germany

Summary. A potassium-specific tonoplast channel was identified by reconstitution of tonoplast polypeptides into planar lipid bilayer membranes. Highly purified tonoplast membranes were solubilized in Triton X-100-containing buffer and fractionated by size-exclusion chromatography. The protein fractions were assayed for ion channel activity in a planar bilayer system, and the potassium channel was routinely recovered in specific fractions corresponding to an apparent molecular mass of 80 kDa. In symmetrical electrolyte solutions of 100 mM potassium chloride, the potassium channel had a single-channel conductance of 72 pS. Substates of the channel with conductances of 17, 33 and 52 pS were frequently observed. After identification of the channel in low or high KCl, addition of sodium acetate or sodium chloride caused only insignificant conductance changes. This result suggested that the channel was not or little permeable for sodium or chloride, whereas it had similar single-channel conductance for rubidium and caesium ions as compared with potassium ions. The channel is presumably responsible for the equilibration of potassium between the vacuole and the cytosol. The role of the channel in the physiology of the barley cell under salt stress is discussed.

Key Words ion channel · partial purification · planar bilayer · potassium · sodium · tonoplast

Introduction

In fully expanded plant cells, the tonoplast membrane separates the large central vacuole, which occupies up to 95% of the cell volume, from the surrounding cytosol. Large concentration gradients are maintained between the two compartments. Whereas vacuolar sodium and free calcium concentrations exceed by far cytosolic concentrations, potassium and magnesium are frequently accumulated in the cytosol. Efficient deposition of sodium inside the vacuole is the main mechanism by which salt-tolerant plants handle salt-stress situations (Flowers & Yeo, 1988).

Transport proteins, carriers and channels, mediate solute transport across the tonoplast. Some of them have been characterized. By use of the patch-clamp method, a "slow"- and a "fast"-type ion channel have been identified (Hedrich & Neher, 1987; *see also* Bentrup, 1989). However, these ion channels lack the specificity needed for maintenance of known *trans*-tonoplast ion gradients. Both channels were described to conduct K^+ and Na^+ with similar efficiency. Thus, the reported tonoplast channels have either lost their specificity in these studies or are closed under physiological conditions of plant life. The only report about a tonoplast ion channel with a larger conductance towards potassium than sodium was published by Kolb, Köhler and Martinoia (1987).

In a recent publication, we reported successful incorporation of solubilized tonoplast material into planar lipid bilayers (Klughammer et al., 1992). A number of different channels have been detected in protein extracts of tonoplast membrane. These channels have highly different single-channel conductances and varying ion specificities. These results suggest a more complex composition of the tonoplast with respect to ion channels than has been discussed in previous reports (Hedrich et al., 1988).

In this communication, we report the reconstitution of tonoplast polypeptides into lipid bilayer membranes. These polypeptides were solubilized and fractionated by size-exclusion chromatography. The results suggest that a defined fraction contained a tonoplast channel with a high selectivity for potassium over sodium ions.

Materials and Methods

Barley (*Hordeum vulgare*, cv. Gerbel) was grown in soil culture in a growth chamber. The conditions were: 14 hr light, 22°C/10 hr dark, 18°C. Primary leaves were harvested from 10-day-old plants and used for vacuole isolation. Vacuoles were isolated according to the method of Martinoia, Heck and Wiemken (1981).

Tonoplast membranes corresponding to 1-ml vacuolar volume were sedimented (120,000 × *g*; 4°C; 30 min). The pellet was resuspended in 200 μl of buffer containing 20 mM Tris, pH 7.2, 50 mM NaCl and 2% (vol/vol) Triton X-100 and solubilized by incubation on ice for 30 min, followed by a freeze/thaw cycle using liquid nitrogen. The sample was then fractionated by size-exclusion chromatography (Superose 6 HR, Pharmacia, Uppsala, Sveden) (Betz & Dietz, 1991). Forty fractions were collected. For the initial screening, three consecutive fractions were pooled and tested for channel-forming activity using the bilayer technique (Klughammer et al., 1992). The composition of the electrolyte solutions in the bilayer chamber is given in the text and legends to the figures. The two chambers of the test cuvette were filled with electrolyte solution, the bilayer was painted across the central hole, and sample was added under continuous stirring. After reconstitution of channel activity *I-V* curves were performed by applying different voltages in the range from −120 to +120 mV. Evaluation of the experiments was usually done by hand.

One-dimensional SDS-PAGE of polypeptides was performed in principle as described by Laemmli (1970). The gels were stained with silver nitrate according to Blum, Baier and Gross (1987). If necessary, protein samples were concentrated by freeze drying at −40°C for 5 to 7 days.

Results

Molecular Characterization of the Potassium Channel

Isolated tonoplast polypeptides were solubilized in buffer containing Triton X-100 and separated by size-exclusion chromatography. From fraction 37 to 40, ion channel activity could be reconstituted into planar bilayer membranes. In contrast to these and a few specific fractions, no current fluctuations corresponding to opening and closing events of single channels were observed in most other fractions (*cf.* Klughammer et al., 1992). When we compared the elution of the channel-forming activity with the elution of standard polypeptides of known relative molecular masses, the apparent molecular mass of the potassium channel may be on the order of about 80 kDa (Fig. 1). However, although the polypeptide composition of the active fractions was not very complex and contained only a few dominant bands upon silver staining (*see* Fig. 1), it was not possible to attribute the channel-forming activity to a distinct band.

Identification of a Tonoplast Ion Channel of Maximum Single-Channel Conductance of 72 pS in 100 mM KCl

In 100 mM KCl, the ion channel revealed a single-channel conductance of 69–74 pS (mean 71.9 ± 2.9 pS). Figure 2*A* shows typical current traces of the

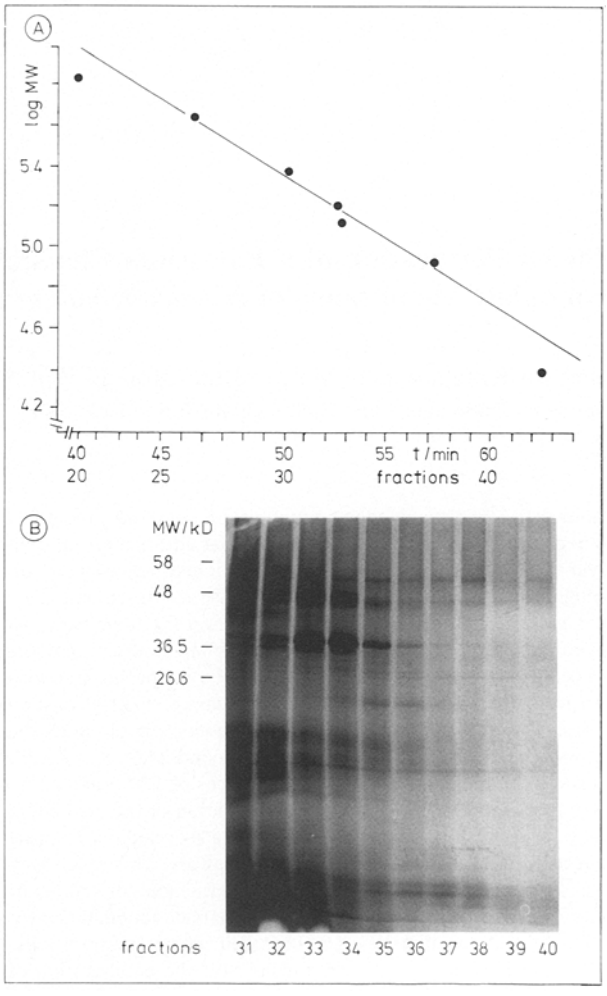

Fig. 1. Estimation of the relative molecular mass of the channel protein from calibration of the Superose 6 HR column with standard proteins of known molecular mass (*A*). The fractions with maximum channel activity (fraction 37) reveals only a small number of distinct polypeptides after separation on a 12.5% SDS-PAGE followed by silver staining (*B*).

channel in 100 mM KCl and membrane potentials between +70 and −70 mV. The analysis of the current recordings (i.e., the current-voltage relationship) demonstrated the existence of several substates (Fig. 2*B*). In this experiment, the substates had single-channel conductances of 70, 51, 33 and 17 pS. As an explanation for this complex pattern of conductances one could argue for a channel complex consisting of four subunits, each of which has a conductance of approximately 17 pS. In some experiments, a substate of even lower conductance (8–9 pS) was also seen. Figure 3 shows an example of these substates of the channel. It was obvious from the single-channel recordings that the substates did not represent other channels since we observed tran-

sitions between the 72-pS state and the different substates. These observations provided strong evidence that the distinct conductance levels represented various substates of one distinct channel. A statistical analysis on the transients which occurred between the various conductance levels was performed in an experiment where one channel had been incorporated into the bilayer. A change in conductance of 50 pS occurred in 36% of all current jumps. The probabilities to observe the other transients were: 24% for 17.5 pS; 20% for 70 pS; 18% for 35 pS and 2% for 9 pS. This analysis was based on 247 events. Only conductance jumps were considered which led to a stable new conductance level of at least 0.2-sec duration. This was necessary due to the relatively slow time resolution of our setup in the continuous mode of registration. This analysis further demonstrates that the different current jumps are caused by one channel with distinct substates.

The Channel is Selective for Potassium over Chloride

To test the specificity of the tonoplast channel we performed the following experiment. The membrane was formed in 100 mM KCl, and one channel was reconstituted. Then the KCl concentration was raised on one side to 240 mM by the addition of concentrated salt solution and the current was recorded for different voltages. Current-voltage curves were performed from these recordings (Fig. 2*C*). The reversal potential, $U_{I=0}$ (i.e., the potential, at which the current through the channel was zero), was approximately -15 mV for the different states of the channel. This result suggested a permeability ratio for K^+ over Cl^- of 6:1 for the channel according to the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949; Benz, Janko & Läuger, 1979). This means that the channel could also be permeable for chloride, since we would expect a reversal potential of -22 mV for a 2.4-fold gradient in the case of potassium permeability alone. On the other hand, we could not exclude the possibility that Gouy-Chapman effects of charges located at the channel mouth decrease the reversal potential (*see below*).

The Single-Channel Conductance Saturated for Increasing Ion Concentrations

The single-channel conductance was under the conditions of the asymmetric potassium chloride concentrations (100 *versus* 240 mM) about 100 pS. The conductance of the 52- and 17-pS substrate levels increased under the same conditions to a single-channel conductance of 69 and 25 pS, respectively. The dependence of the channel activity on the substrate concentration was investigated in detail. Membranes were formed in solutions of different KCl concentrations, c, and the channel was reconstituted into these membranes. Table 1 summarizes the single-channel conductances, $G(c)$, of the open channel and substates given as a function of the KCl concentration. They were analyzed with the following equation by assuming single occupancy of the binding site inside the channel (Läuger, 1973; Benz & Hancock, 1987)

$$G(c) = G_{\max} \frac{c}{K_s + c}.$$

$G_{\max}$ is the maximum single-channel conductance at very high salt concentrations and K_s is the Michaelis-Menten constant. The Equation means that $G(c)$ could be analyzed by Lineweaver-Burk plots (*see* Fig. 4). The maximum single-channel conductances of the 72-, 52-, 34- and 17-pS substates were 100, 70, 48 and 25 pS, respectively. It has to be noted that in these experiments the 17- and 34-pS substates were less frequently observed, which means that the experimental data were somewhat weak for these substates. However, the data points obtained for them suggested that the binding site had in these cases the same affinity for potassium as in the open state. The stability constant, K, for the binding of potassium to the binding site was approximately 25 liter/mol, which corresponded to a half-saturation constant, K_s, of 40 mM. It should be mentioned that substitution of concentrations by activities led to the same result. Also on the basis of activities, the half-saturation constant was close to 40 mM (*results not shown*).

Interaction of the Channel with Other Cations

To study the permeability of other ions through the channel we performed similar experiments as reported above in the presence of other salts. Using LiCl we were not able to detect any channel activity although we would have expected to observe channels according to our experience with KCl. In NaCl we measured channels with a single-channel conductance of 13.6 pS for the open state and rarely substates (Fig. 5). This means that the channel was clearly selective for potassium over sodium. It is interesting to note that the tonoplast channel had in 100 mM CsCl slightly higher single-channel conduc-

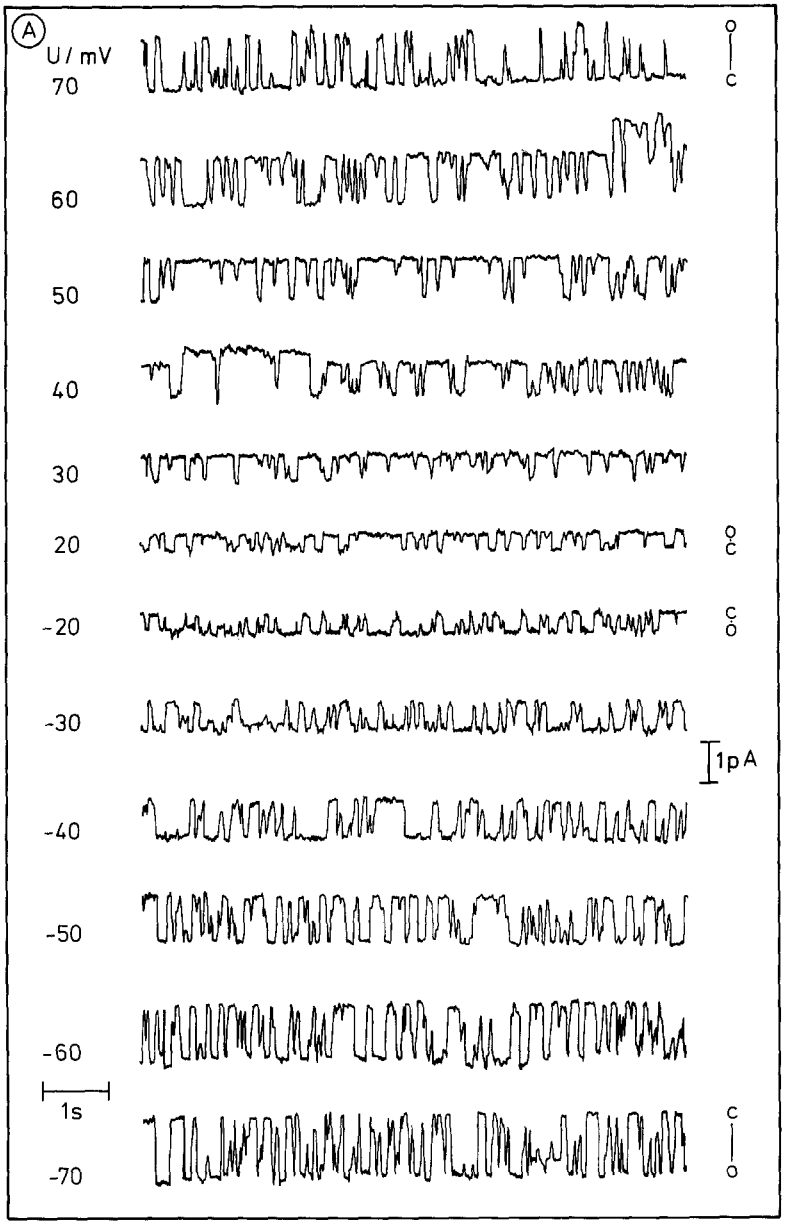

Fig. 2. (*A*) Single-channel current recordings after reconstitution of the 72-pS tonoplast channel into planar bilayer membranes. The voltage was adjusted from +70 to −70 mV. The electrolyte solution contained 100 mM KCl and 5 mM $MgCl_2$.

tance than in KCl. These results suggested that the channel is exclusively permeable for cations. In another set of experimental conditions the channel was first incorporated and identified in symmetrical potassium chloride solution. Then other salts were added to both sides of the membrane and their influence was studied on the single-channel conductance of the tonoplast channel. Figure 6 shows a single-channel recording of this type. The channel was first recorded in a symmetric 30-mM KCl solution (upper trace), then 30 mM sodium acetate was added to both sides of the membrane (lower trace). The addition of sodium acetate had only a small influence if any on the conductance of the channel. Table 2 shows that the conductance of the 52-pS substate of the channel did not increase but decreased a little after addition of 70 mM Na acetate, 70 mM NaCl or 70 mM LiCl. These results supported the assumption that Li^+ and Na^+ are not or only a little permeable through the channel. On the other hand, the decrease of the single-channel conductance indicated that Gouy-Chapman (i.e., ionic strength) effects may be involved in cation transport through the channel.

The addition of potassium acetate, KCl or RbCl

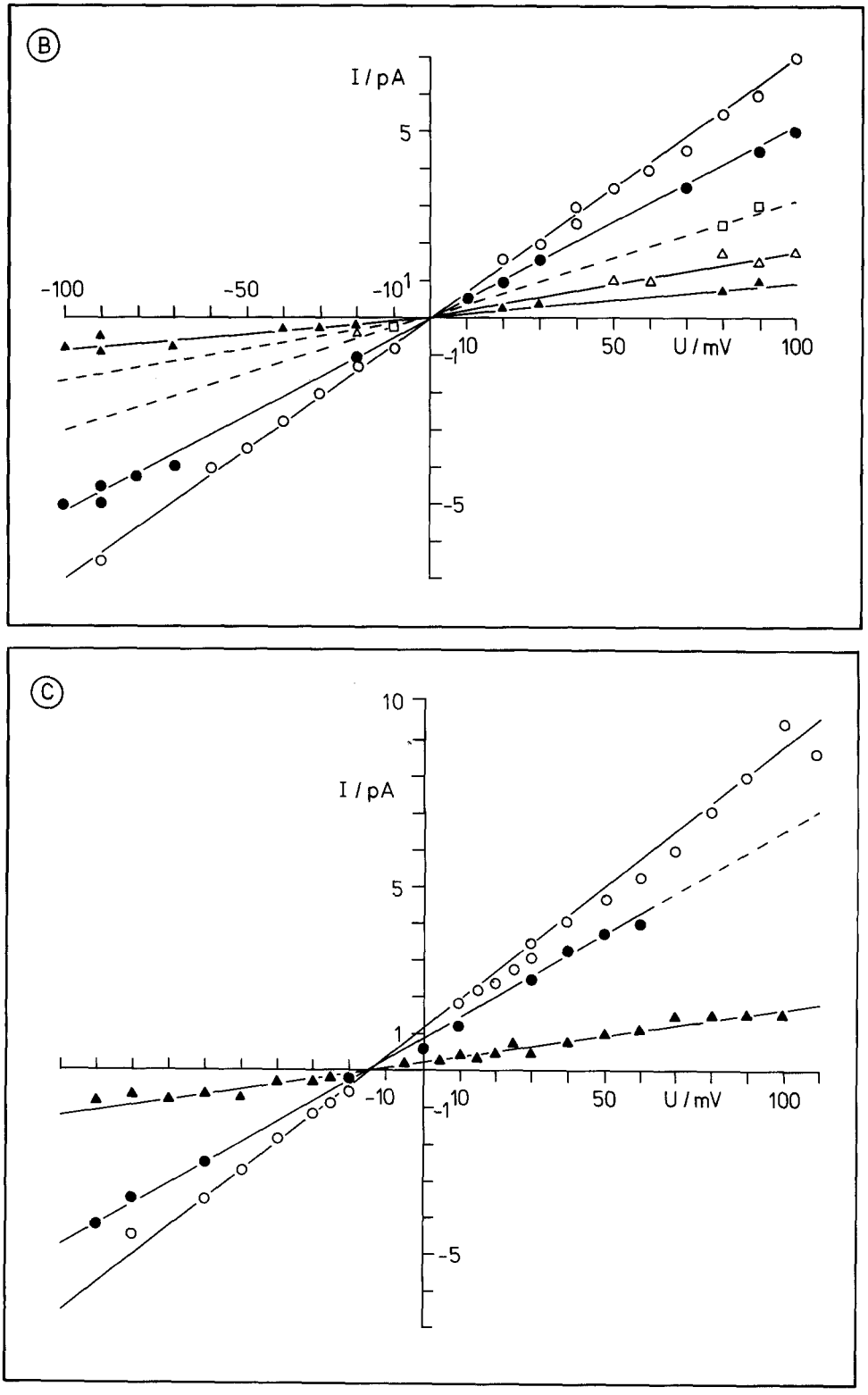

Fig. 2. (*B*) The current/voltage plot demonstrates the existence of sublevels of conductance. Symbols: (○): 70 pS; (●): 51 pS; (□): 32 pS; (△): 17.6 pS; and (▲): 9 pS. (*C*) After increasing the KCl concentration in the *cis*-chamber to 170 mM, the current/voltage curves indicate cation selectivity. The increased electrolyte concentration led to only a minor change in single-channel conductance. Symbols: (○): 76 pS, (●): 56 pS: and (▲): 14.2 pS.

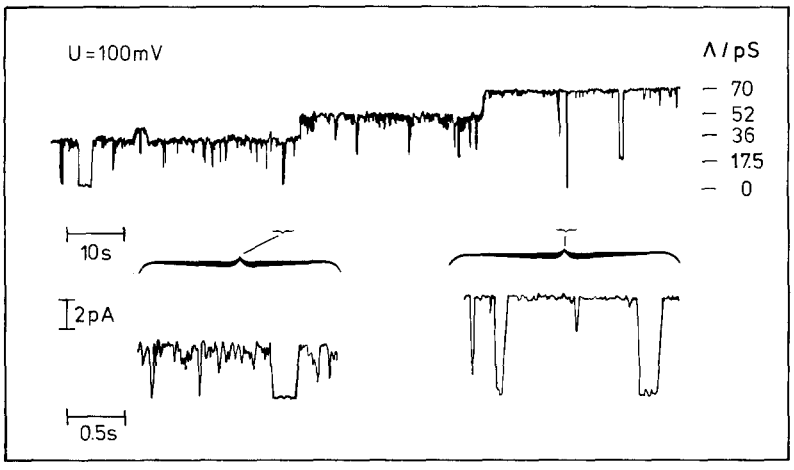

Fig. 3. Transients between the substates of the potassium channel. A current trace was selected to demonstrate that the different conductance levels must be attributed to one type of channel and not to a population of different channels. The chamber contained 100 mM KCl and 5 mM $MgCl_2$. The upper trace was recorded at low time resolution; therefore, two periods were extended in the lower traces to show that the 36-pS sublevel (left extension) and the 72-pS sublevel (right extension) closed to the basal conductivity of the membrane. It should be noted that the noise level was lowest in the fully closed state and also low after complete opening to the 72-pS state.

Table 1. Single-channel conductances of the substates of the tonoplast potassium channel at different valtages[a]

Concentration (c/mM)	Activity (a/mM)		Conductance $G(c)$/pS		
10	9	20	13	—	—
20	18	33.4	21	—	—
30	26	43.8	34	20	10.5
50	42	52.6	37	25	—
60	49	56	40	30	—
100	77	72	50	34	17
200	144	76	55	—	—
240	169	100	59	—	25
300	206	100	66	—	—

[a] The chamber solution contained KCl, as indicated in the table, suplemented with 5 mM $MgCl_2$. For comparison, activities of KCl are also given. They were interpolated from the data of Pytkowicz and Johnson (1979) and neglect the effect of the added $MgCl_2$.

led to an increase of the conductivity of the 52-pS substate (*see* Table 2). The increase caused by potassium acetate was the same as caused by KCl. This is another support for the hypothesis that the channel was impermeable for chloride. The addition of 70 mM CsCl caused no effect on single-channel conductance. Measurements were also performed in the presence of other potassium-containing salts, such as 50 mM K_2SO_4. In this case the single-channel conductance of the open state was only a little smaller than in 100 mM KCl, probably caused by the larger ionic strength of the sulfate-containing salt solution.

The channel was also affected by $BaCl_2$. In the presence of 10 mM $BaCl_2$, a 30% decrease of conductance was observed which may be caused by competition between barium and potassium ions for binding to the binding site, by blockage of the channel by Ba^{2+} or by Gouy-Chapman effects. The opening and closing probabilities were not affected by Ba^{2+}.

In previous patch-clamp studies (Hedrich, Flügge & Fernandez, 1986), a rectifying characteristic was described for the channel current. As incorporation of membrane proteins occurs in the bilayer system in a random manner, our results may not directly be compared with results obtained in the whole vacuole configuration by patch-clamp analysis. However, it should be mentioned that the incorporated channel switched more often in certain experiments at high positive potentials and in others at high negative voltages, i.e., the opening and closing events occurred more frequently at high voltage of one direction as compared to high voltages of the other direction.

Discussion

Mesophyll vacuoles from barley plants have been used for patch-clamp studies by Hedrich et al. (1986) who reported on an ion channel with low selectivity ($K^+ \leftrightarrow$ malate^{2-}). Its conductivity was 60–80 pS in symmetrical 50 mM K_2malate (i.e., 100 mM K^+) solutions. The channel revealed inwardly rectifying functions. Kolb et al. (1987) observed a potassium channel with a single-channel conductance of 105–121 pS in symmetrical KCl solutions of 250 mM concentrations. This channel also carried charges preferentially into the vacuole. The channels described in these previous reports and our reconstituted 72 ± 2-pS channel may be identical. Ion conductivity is similar in all cases if our observation of substrate saturation and the KCl concentrations are taken into account which were used in the various experimental approaches. Further evidence for identity of the channels is the substate conductance

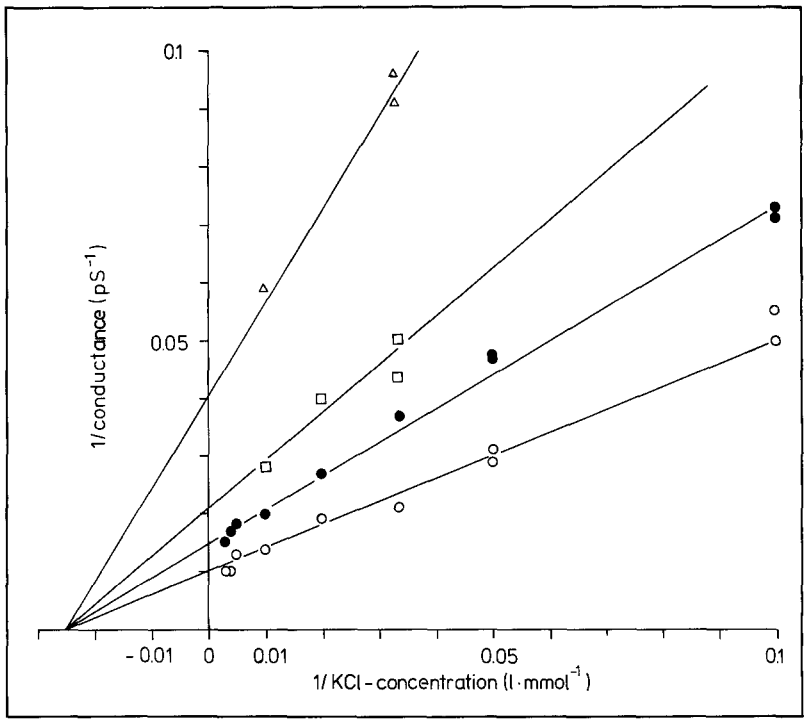

Fig. 4. Change in conductance of the tonoplast channel as a function of the KCl concentration in the chamber solution. The inverse relationship between substrate concentration and conductance gives identical K_M values as intersection of the regression lines with the x-axes of 40 mM KCl for all substrates. Maximal conductivities of 100 pS (○: 71.9 ± 2.9 pS in 100 mM KCl, n = 7), 69 pS (●: 51.7 ± 3.1 pS in 100 mM KCl, n = 7), 48 pS (□: 32.7 ± 4.4-pS level in 100 mM KCl, n = 6) and 25 pS (△: 17.1 ± 1.1-pS level in 100 mM KCl, n = 5) can be taken from the graph.

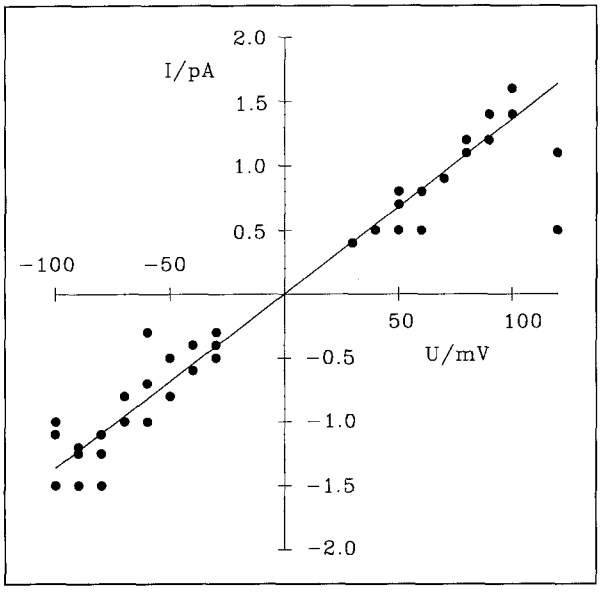

Fig. 5. The tonoplast K^+ channel has a single-channel conductance of 13.6 pS in 100 mM NaCl solution. A regression curve from three independent experiments is shown.

level described by Kolb et al. (1987). Evaluation of an amplitude histogram derived from single-channel recordings by patch-clamp analysis showed a conductance level of 18 pS (in 250 mM KCl solution) which is in good accord with our 17-pS conductance state. These similarities provide evidence that we have reconstituted and identified the main cation channel of the tonoplast membrane.

However there also exist differences between both channels which require explanation: The selectivity of potassium over sodium was more pronounced in our experiments than estimated by Kolb et al. (1987). These authors replaced the initial electrolyte solution containing 250 mM KCl by a solution of 125 mM KCl and 125 mM NaCl. The single-channel current dropped from about 121 to 79 pS. Assuming a linear relationship between single-channel conductance and ion concentration (which may not be given according to our data) up to 250 mM, a selectivity of K^+ over Na^+ of 6 has been calculated by Kolb et al., 1987.

Our results provide some evidence that the channel is permeable to cesium and rubidium besides potassium and impermeable for sodium, lithium and chloride. The addition of divalent cations and anions lead to a decrease of the single-channel conductance, which may be caused by Gouy-Chapman effects and/or competition between different ions for the binding site.

SDS-PAGE of the active fractions obtained by size-exclusion chromatography on Superose 6 HR reveals only a few bands even when polypeptides of 5×10^7 vacuoles had been loaded on the SDS-polyacrylamide gel followed by silver staining. The question arises if we could expect to see the band of the channel protein in a gel. This is a critical point and difficult to answer. Assuming 1000 channels per

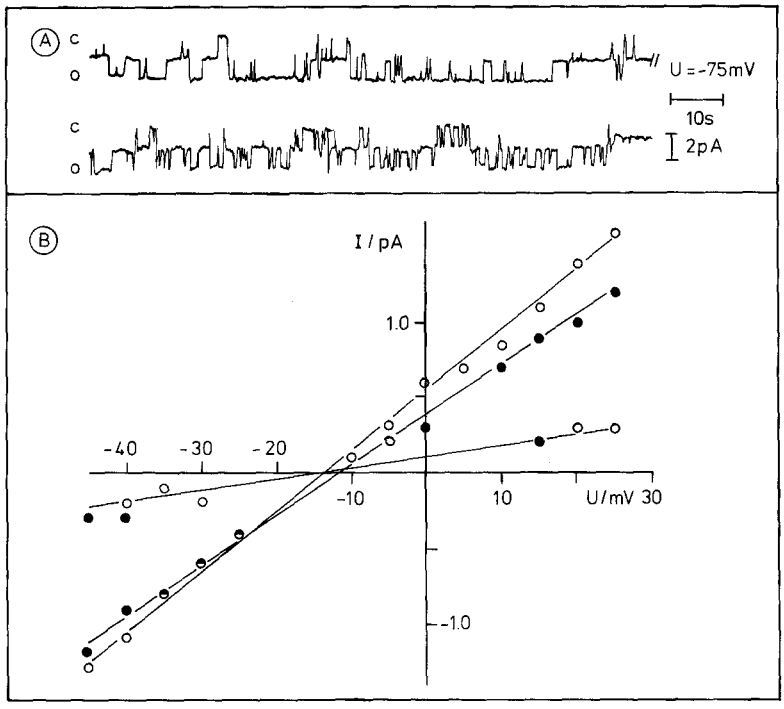

Fig. 6. Different permeability of the reconstituted channel for potassium and sodium. The current amplitude of the channel did not increase after addition of sodium acetate. The electrolyte solution initially contained 30 mM KCl and 1.5 mM $MgCl_2$. At the time point indicated, 30 mM Na acetate was added to both chambers (lower trace) (*A*). Symmetrical 100 mM Na acetate were added to a KCl gradient of 100 mM (*cis*)/50 mM KCl (*trans*-chamber). The current/voltage plot even shows a small decrease of conductance after addition of the Na acetate (filled circles) (*B*).

Table 2. Conductance of the reconstituted potassium channel in the presence of different cations and anions[a]

(A) Additions	Basic electrolyte	
	30 mM KCl	100 mM KCl
	Conductance, pS	
None	31	52
LiCl	n.m.	47 (70 mM)
NaCl	n.m.	50 (70 mM)
Na acetate	27 (30 mM)	46 (70 mM)
KCl	50 (70 mM)	56 (70 mM)
K acetate	45 (30 mM)	56 (100 mM)
RbCl	n.m.	56 (100 mM)
CsCl	n.m.	52 (70 mM)
(B)	Conductance, pS	
KCl (100 mM)	72 ± 3	52 ± 3 (n = 10)
K_2SO_4 (50 mM)	67	45
Cs_2SO_4 (50 mM)	77	55
NaCl (100 mM)	12	n.d.
LiCl (100 mM)	n.d.	n.d.

[a] (A) The "basic electrolyte solution" contained KCl at concentrations of either 30 or 100 mM suplemented with 5 mM $MgCl_2$. Additions of other electrolyte solutions were made as indicated. Their concentrations are given in brackets. The relatively small changes in single-channel conductances after addition of other salts observed in the case of 100 mM KCl as basic electrolyte solution can be explained with the saturation characteristic of the channel.
(B) Comparison of potassium, caesium, sodium and lithium permeability.
Abbreviations: n.m. = not measured, n.d. = not detected.

vacuole with a relative molecular mass of 80 kDa, the gel would contain 0.5 ng of channel protein. That amount of protein is close to the detection limit of silver staining (if the protein was stained by silver), and the question has to be left open.

An important characteristic of this channel is its strong selectivity of K^+ over Na^+. Na^+ is accumulated inside the vacuole under saline conditions via a Na^+/H^+ exchanger (Blumwald, Cragoe & Poole, 1987), whereas K^+ may be distributed between the vacuole and the cytoplasm according to a Nernst equilibrium (K.-J. Dietz, *unpublished*). This makes it difficult to transport Na^+ and K^+ through the same channel. Activation of the channel would dissipate the differential ion gradients at the tonoplast including the proton gradient. Potassium selectivity of the channel prevents a futile cycle which involves energized sodium uptake and passive release of sodium and allows maintenance of salt gradients which are necessary for survival of plants under conditions of salt stress. The ionic selectivity was $Cs^+ \approx Rb^+ > K^+ > Na^+ \gg Li^+$ which means that the selectivity follows the mobility sequence of the ions in the aqueous phase. This selectivity sequence could also mean that the tonoplast channel is a wide channel which has only a small field strength in the selectivity filter (i.e., the binding site). The permeability for rubidium, cesium and potassium is high, whereas the permeability for anions, sodium and lithium is low. The hydration energy decreases in the order $Li > Na > K > Rb > Cs$. This suggests that the translocation involves a partial dehydration of the

cations, which may only be possible for large cations with smaller hydration shells (Hille, 1984; Moczydlowski, 1986).

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