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Single Potassium Channels in Membranes of Isolated Mesophyll Barley Vacuoles

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Summary. Voltage-dependent K channels could be identified in on-cell and excised patch-clamp records on membranes of isolated plant cell vacuoles. The current through a membrane patch is dominated by a channel population with a conductance of about 121 pS in symmetrical 250 mM KCl solution. The single channel adopts at least two conducting levels, the 121-pS state being most frequently observed. The channel shows outward rectification, representing a cation flux into the vacuoles. The rectification appears to be caused by a vanishing open probability and a short channel lifetime at hyperpolarizing voltages. A selectivity ratio of potassium over sodium of about 6 was derived as an estimate. Occasionally, an additional population of K channels with a single-channel conductance of approximately 18 pS is observed. This channel type exhibits outward rectification as well.

Key Words K channels · rectification · open probability · plant vacuoles · patch clamp

Introduction

Ions play an important role in the preservation of the plant water potential. Uptake of salts enables the plant to maintain turgor without expending energy for the synthesis of organic osmotica. Most of these ions are stored in the large central vacuole that accounts for about 80% of the plant cell volume. The vacuole functions as a storage unit for sugars and organic and inorganic ions (Boller & Wiemken, 1986). In spite of the importance of ion transport across tonoplast membranes, only a few transport studies using isolated vacuoles have been conducted. Uptake of malate and chloride in vacuoles of barley mesophyll leaves is energy dependent (Martinoia et al., 1985, 1986), for which participation of the tonoplast-bound H^+-ATPase (Sze, 1985) is suggested.

Homeostatic mechanisms are thought to maintain an ion distribution which facilitates metabolism (Kaiser, Weber & Sauer, 1983). Nitrate, which is predominately located in the vacuole (Martinoia, Heck & Wiemken, 1981), disappears during senescence (Christensen, Below & Hageman, 1981), whereas chloride or sodium, which are not metabolized, seems not to be released (Jeschke, 1979). Therefore, mechanisms controlling the ion transport and rectification at the tonoplast membrane have to be postulated.

In the present paper we used the patch-clamp technique to investigate ion transport into isolated vacuoles (Kolb, Köhler & Martinoia, 1986*a*). Plant electrophysiological investigations using the patch-clamp technique have been few so far. The plasmalemma of wheat mesophyll protoplasts contains different channels (Moran et al., 1984). They have been distinguished by their conductance, but the corresponding ion selectivity is unknown. A potassium channel of 37 pS has been observed in the plasmalemma of *Vicia faba* guard cells (Schroeder, Hedrich & Fernandez, 1984). This channel is ten times more selective for potassium than for sodium. In a recent study, Bentrup et al. (1985) investigated the tonoplast of *Chenopodium rubrum* vacuoles. From the analysis of current noise, a K channel of 0.5 pS was postulated in symmetrical 46 mM KCl. For the cytoplasmic drop of *Chara australis*, Lühring (1986) could show a K-channel conductance of up to 165 pS, depending on the accessability of both sides of the membrane for potassium ions.

The major result of this work is the identification of a K channel of large unit conductance. The percentage of channel open time was measured as a function of membrane potential. At hyperpolarizing voltages the channel could not be detected, suggesting outward rectification. It was found that the potential-dependent gating properties are similar to the results obtained for maxi-K channels in membranes of muscle cells (*cf.* Lattorre & Miller, 1983) and epithelial cells (Kolb, Brown & Murer, 1986*b*). A kinetic analysis of open and closed times is presented. We carefully analyzed the problem of the

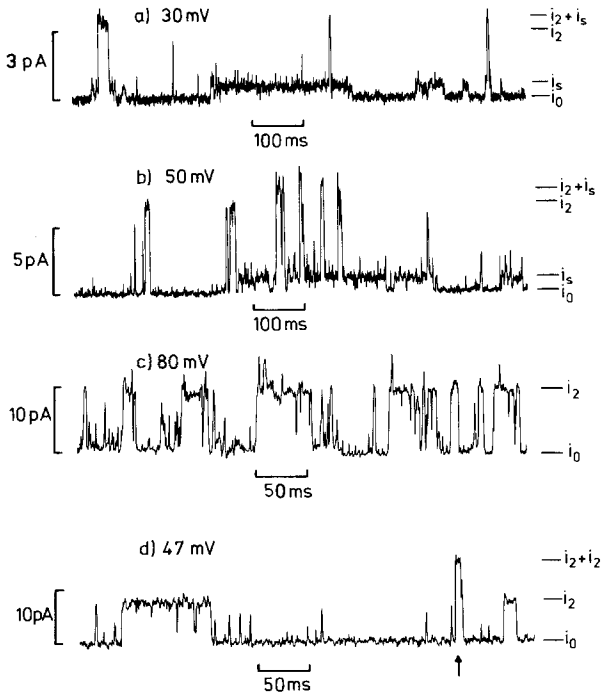

Fig. 1. Current records at different depolarizing membrane (≡ pipette) potentials as indicated. Tonoplast-attached pipette configuration with high-K solution in the bath and pipette. Positive currents from the pipette into the vacuole are presented as upward currents (*see* Materials and Methods). (*a–c*) Current traces of one experiment. At 30 mV (*a*) and 50 mV (*b*) two channel populations are visible, which show different current amplitudes and kinetical patterns. For clearer presentation, at 30 mV a record section was selected where the current pulses of larger amplitude showed longer open times. On the right-hand side the corresponding mean current levels are marked: i_o denotes the commonly closed state, i_s the smaller current amplitude, and i_2 the larger current amplitude. The sum of $i_s + i_2$ is given as well. (*d*) Current fluctuations of a further experiment where only the channel of larger amplitude was observed. The superposition of two simultaneously open channels is marked

number of conducting states and concluded that the channel adopts at least two.

Materials and Methods

Preparation of Protoplasts and Vacuoles

Protoplasts from young barley leaves were prepared as described previously (Kaiser, Martinoia & Weimken, 1982). Vacuoles were isolated according to Martinoia et al., 1981. For patch-clamp studies 50–100 μl of the vacuole preparation was added to 1–2 ml of 250 mM KCl, containing 50 μg/ml neutral red. The electrolyte concentration of the vacuole is 400–450 mM (nitrate 40–60 mM, chloride 50–70 mM, phosphate 50–70 mM, sulfate 10–20 mM, potassium 130–180 mM, sodium 20–50 mM). Vacuoles were allowed to settle on the bottom of a petri dish, which was treated with 1 mg/ml L-polylysine for about 5 min. L-polylysine treatment enhanced the adherence of vacuoles considerably and therefore facilitated the formation of gigaseals. The vacuoles were spherical with a diameter of 20–30 μm. Purity was as referred elsewhere (Kaiser et al., 1982).

Patch-Clamp Experiments

Patch-clamp experiments were carried out according to the method of Neher and Sakmann (*cf.* Hamill et al., 1981). The experiments were performed in the vacuole-attached configuration of the micropipette, or also on inside-out patches of the tonoplast. Throughout the records potentials are denoted as membrane potentials. Since the electrolyte of the micropipette can be considered as a substitute for the cytoplasmic solution, the membrane potential is equivalent to the applied pipette potential, referred to a vacuole potential of 0 mV. Positive currents from the pipette into the vacuole are presented as upward currents and defined as outward currents. The current records were stored on an FM tape recorder (Racal Store 4) with a frequency response of DC to 20 kHz. The records were actively low-pass filtered (Krohn-Hite Model 3342) at a cuf-off frequency (−3 db frequency) of 1.5 kHz.

Electrolyte Solutions

For all current records, the bath contained a solution of 250 mM KCl, 20 mM HEPES, 10 mM glucose with a pH of 7.4 (high-K medium) to mimick the normal cytoplasmic ionic composition. The patch electrode contained, unless stated otherwise, the high-K medium with no $CaCl_2$ added. The ionic selectivity was investigated by varying the pipette electrolyte. In this case, KCl was replaced by equal amounts of NaCl. Experiments were performed at room temperature (20–30°C).

Data Analysis

For data analysis, the recorded patch current was played back at 1/32 of the recording speed into a strip-chart recorder (Gould Brush 2200S). Open-close events and the corresponding current amplitudes were digitized using a Hewlett Packard 9874A digitizer. For derivation of frequency histograms of open and closed times, the half-amplitude threshold analysis (Colquhoun & Sigworth, 1983) was applied. Sojourns within single-channel events to lower current levels lasting less than 500 μsec were considered as open times. The derived frequency distributions were fitted by exponential functions using a least squares fitting routine.

To estimate the percentage of time during which a channel stays open (open probability), the sum of consecutively measured open times was divided by the observation time.

Results

Channel Populations and States

Figure 1 shows current records of an active tonoplast patch in the on-cell mode at different depolarizing membrane potentials. As will be demonstrated

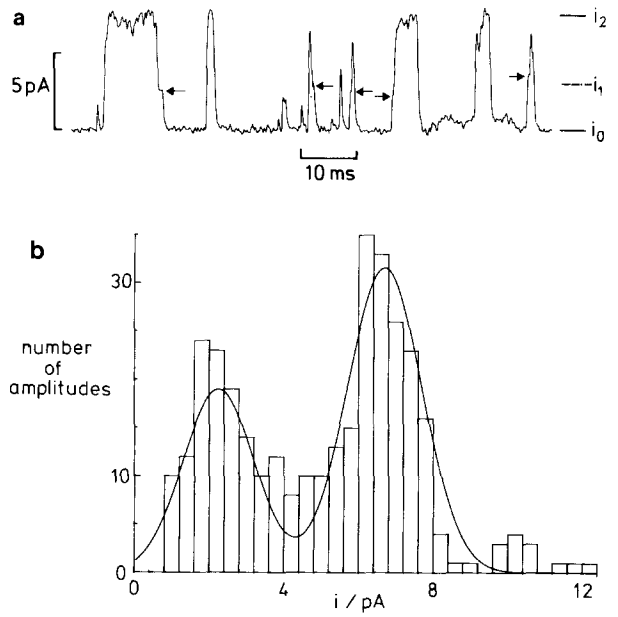

Fig. 2. (*a*) Current pulses of single-channel events at 58 mV. Pronounced steps in the rising and falling phase of the current pulses are marked by arrows. On the right-hand scale, the different mean channel states are denoted as i_o (closed state), i_1 (substate) and i_2 (fully open state). The mean values of i_1 and of i_2 were taken from the corresponding amplitude histogram of *b*. For further explanations *see* text. The experimental conditions were as used for Fig. 1. (*b*) Amplitude histogram of different current states (i_1, i_2) within single current pulses (*see a*) at 58 mV. The amplitude histogram was fitted by a superposition of two Gaussian functions. The following values were derived: $i_1 = 2.25 \pm 0.95$ pA, $i_2 = 6.7 \pm 1$ pA. The total number of amplitude values is 321. The experimental conditions were as used for Fig. 1

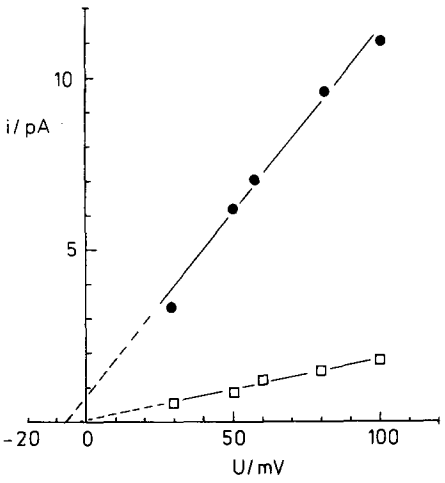

Fig. 3. Current amplitudes (*i*) *vs.* applied membrane potential (*U*) of single-channel events. The current values were obtained from evaluation of the corresponding amplitude histograms. The drawn line represents a fit of the ohmic relation to $i_2(U)$ (● points) of the fully open channel state (i_2, *see* Fig. 2*a*) which yields $g_2 = 105$ pS. For the second type of current amplitudes (□ points, *see also* marked events in Fig. 1) $g_s = 18$ pS is derived. By linear extrapolation of both lines a common zero current potential of about −7 mV is found. The data were as used for Fig. 1

below, the record shows a cationic current directed into the vacuole. Opening and closing events of this type could be observed in a total of 12 experiments, but giga-seal formation was only successful about every 10 to 20th try. Figure 1*a–c* shows that besides a channel of large current amplitude, a second population appears with a significantly smaller amplitude and an obviously distinguishable current pattern. Both channel populations open and close independently, as may be seen from the current traces of Fig. 1*a* and *b*. For the channel of smaller amplitude, a conductance of $g = 18 \pm 4$ pS ($n = 4$) is derived (*see also* Fig. 3). But in the following, we will concentrate on the analysis of the current pulses of large amplitude which dominate the records. For this purpose, those current records were selected in which only this channel population could be identified (Fig. 1*d*). Dependent on the open probability of one channel (*see below*), we could observe two simultaneously open channels of equal amplitude (*see* marked event in Fig. 1*d*).

The current fluctuations ending on larger current amplitudes show a characteristic stepwise increase and decrease as may be seen from the time-expanded current traces of Fig. 2*a*. To find out whether these steps are related to defined channel states, frequency histograms of the corresponding amplitude levels were evaluated (Fig. 2*b*). At least two conducting current levels can be identified, denoted by i_1 and i_2, in the order of increasing amplitude. As Fig. 2*a* demonstrates, the current increases and decreases stepwise, and state i_2 is the one most frequently observed (Fig. 2*b*). This results from the fact that most of the current pulses ending on i_2 appear to virtually never enter the intermediate current level during the "on-" and "off-set," which may be caused by the limited frequency resolution of the setup. In Fig. 3 the current amplitude, i_2, is plotted as a function of the membrane potential U. The figure indicates that a linear relationship exists within the potential range of approximately 30 to 100 mV. For the corresponding single-channel conductance, a value $g_2 = 121 \pm 14$ pS ($n = 9$) is derived. From a linear extrapolation of $i_2(U)$ to zero current, a potential intercept of -9.2 ± 5.2 mV ($n = 9$) is derived. A change to the excised patch configuration shifts the zero current potential to -0.2 ± 2.4 mV ($n = 4$) in symmetrical high-K electrolyte, and the single-channel conductance stays unchanged within the experimental error. This potential shift is caused by a change of the K^+ concentration at the

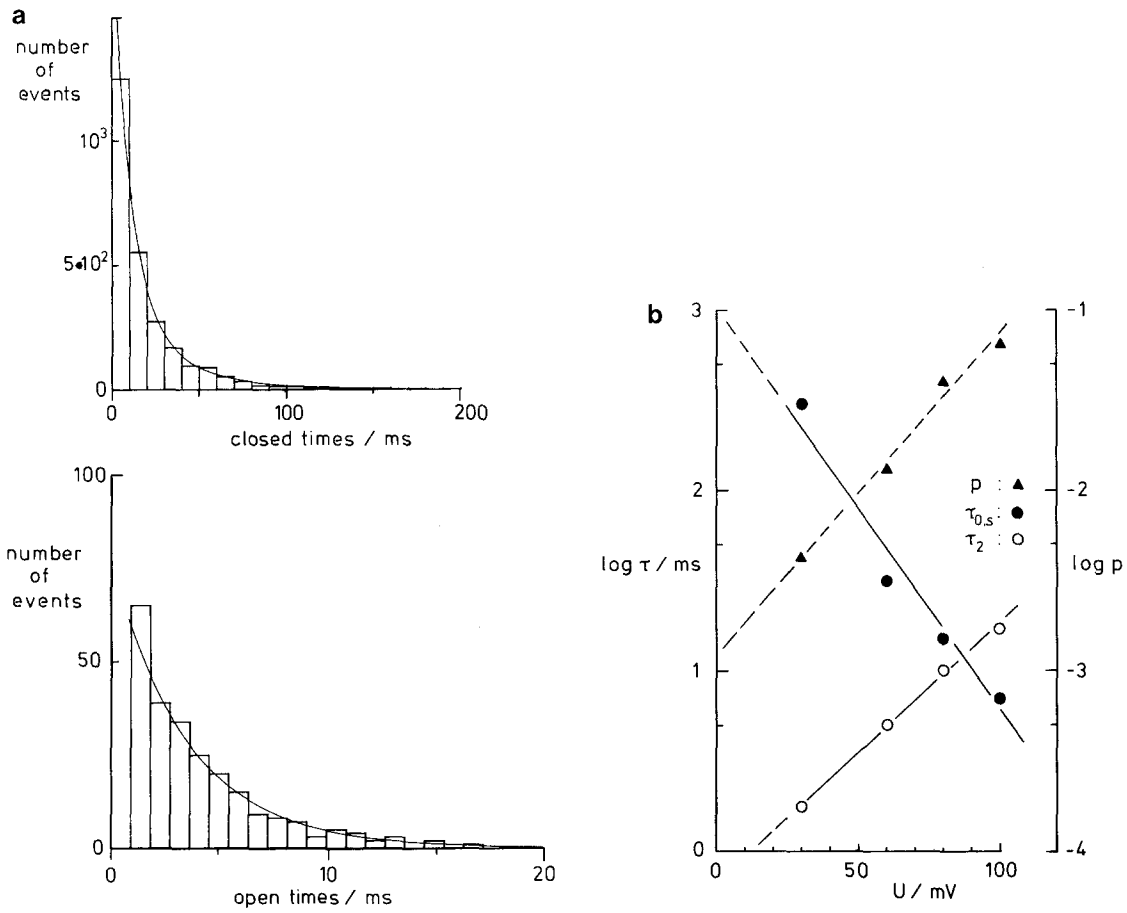

Fig. 4. (*a*) Frequency histograms of lifetimes of current pulses in state i_2 (fully open state) and state i_o (closed state) at 58 mV (for notation *see* Fig. 2*a*). For this plot all transitions shorter than 0.5 msec were discarded. The curves represent fits of either one or a sum of two exponential functions to the corresponding frequency histogram, respectively. A mean open time of τ_2 = 4.4 msec (250 events, eight events are outside the shown time scale) and two mean closed times of 9.4 and 30.5 msec (2560 events, 21 events are outside the shown time scale) were obtained. All events were considered for the fits, respectively. The experimental conditions were as used for Fig. 1. (*b*) Mean lifetime of current fluctuations in state i_o ($\tau_{0,s}$, (●), closed state) and in the fully open state i_2 (τ_2 (○)) as function of membrane potential. Regression lines were fitted to the semilogarithmic plot of these values. Using Eq. (1) the following parameters were derived: $\tau_{0,s}(0)$ = 1.1 msec, $\delta = -0.13\ mV^{-1}$; $\tau_2(0)$ = 0.7 msec, $\alpha = 0.041\ mV^{-1}$. In addition, the channel open probability of an individual channel is plotted (▲). The corresponding scale is given on the right ordinate. $p(U)$ was estimated as outlined in Materials and Methods. By extrapolation $p(0) = 0.0012$ is derived and for the corresponding voltage sensitivity, $3.5\ mV^{-1}$. The experimental conditions were as used for Fig. 1

inside of the tonoplast, from about 150 mM in the intact vacuole to 250 mM for the excised patch configuration. As a further test whether the observed current is an outward potassium current, the KCl concentration of the pipette solution was reduced from 250 to 125 mM and replaced by an equal amount of NaCl. This reduction was followed by a decrease of the single-channel conductance to 79 ± 9 pS (n = 3) and a zero current potential of +8 ± 3 mV (n = 3), derived by extrapolation. Application of the Goldman equation for K and Na as the only transported ions yields an estimate for the selectivity ratio of K over Na of about 6. With the electrolyte compositions of the bath and patch-pipette used, an inward current was not observed. Chloride-selective channels could not be identified.

Open-Close Kinetics and Open Probability

As an approximation of the channel kinetics, we considered the frequency distribution of the most often adopted state, i_2, by neglecting the appearance of state i_1. For pulse durations longer than about 1 msec, the corresponding frequency distribution could suitably be described by a single exponential function. At 58 mV, a mean lifetime of τ_2 = 4.4 msec is derived (Fig. 4*a*). A correlation between

the appearance of state i_1, in either the rising or falling phase of a current pulse, and the lifetime of state i_2 was not observed. The voltage dependence of τ_2 could be described by the Eyring equation

$$\tau_2(U) = \tau_2(0) \cdot \exp(\alpha \cdot U) \quad (1)$$

where $\tau_2(0)$ is the extrapolated value of $\tau_2(U)$ at 0 mV and α the voltage sensitivity in mV^{-1}. The derived parameter values are $\alpha = 0.041\ mV^{-1}$ and $\tau_2(0) = 700\ \mu sec$ (Fig. 4*b*), whereby $\tau_2(0)$ could vary by a factor of two between different experiments.

The frequency distribution of channel shut times is presented in Fig. 4*a* as well. As a first approximation, the frequency distribution could be fitted by a superposition of two exponential functions. By analogy to Eq. (1) we obtained a voltage dependency with a voltage sensitivity of $-0.13\ mV^{-1}$ for the slower characteristic time constant $\tau_{0,s}$ (Fig. 4*b*). By extrapolation, $\tau_{0,s}(0) = 1.1$ msec was calculated. Despite the fact that we took the visible number of simultaneously active channels in the patch into account (Kolb et al., 1986*b*), values three times larger could be found in the estimate of $\tau_{0,s}(0)$ in different experiments. The deviation of the kinetic parameters might be caused by the variability of the electrolyte composition within the vacuole (*see also* Materials and Methods) as a consequence of growth conditions. The voltage dependency of the open probability could be described by a single exponential as well. A voltage sensitivity of $3.5\ mV^{-1}$, and an extrapolated value of 0 mV of $p(0) = 1.3 \times 10^{-3}$ was estimated as an upper limit.

Discussion

In the present paper, single-channel currents in a tonoplast are presented for the first time. Two populations of K channels could be identified in cell-attached and excised patch-clamp experiments. The records are dominated by an outward-rectifying K-channel of 121 pS in symmetrical 250 mM KCl. Occasionally, a K channel of 18 pS with an obviously different kinetical current pattern (Fig. 1*a*,*b*) was also identified. The outward rectification was observed in the cell-attached as well as in the excised patch configuration of the tonoplast. The corresponding shift of the zero current potential of about 10 mV in depolarizing direction indicates that the vacuole is intact with a lower K concentration ($\approx$150 mM) than in the bath (250 mM). Further evidence for an outward K current is obtained from a significant decrease of the single-channel conductance to 78 pS by replacement of 125 mM KCl out of 250 mM by NaCl in the pipette. A characteristic feature of the large K channel is the appearance of at least one well-distinguishable sublevel, which is preferentially observed in the "on-" and "off-set" of a single current pulse (Fig. 2*a*). Sublevels are a widespread phenomenon for channels in membranes of animal origin. They have been observed in the acetylcholine-activated cation channel (Hamill & Sakmann, 1981), the proton-activated Cl channel (Hanke & Miller, 1983), the Ca-dependent maxi-K channel (Barrett, Magleby & Pallotta, 1982), the serotonin-dependent K channel in *Aplysia* (Siegelbaum, Camarelo & Kandel, 1982), and a large anionic channel in myotubes and macrophages (Schwarze & Kolb, 1984). But only in the case of the proton-activated Cl channel could the kinetics of the sublevel be analyzed. For the other channel types, as for the presented large K channel, the substate was visible at random and could not be analyzed in terms of Poisson distributed open-close kinetics. This might be caused by the limitation in the frequency resolution of the patch-clamp technique, which virtually supresses the short-lived substates of a channel (Colquhoun & Sakmann, 1985).

The kinetic analysis concentrated on the fully open channel state and on open-close events lasting longer than 500 μsec. Within this limit, one-channel open state and two-channel closed states could be discriminated. In the latter case, the slower characteristic shut time exhibits a steep dependency on the applied voltage, like the mean open time (Fig. 4*b*).

It is interesting to note that the corresponding voltage sensitivities are in the same order of magnitude as observed for the voltage-dependent time constants of the Ca-dependent maxi-K channel in animal cell membranes (Magleby & Pallotta, 1983; Kolb et al., 1986*b*). The voltage-dependent kinetics result in a voltage-dependent open probability, which increases roughly exponentially with depolarization. Despite the large single-channel conductance, the low open probability reduces the contribution of the large K channel to the mean macroscopic current to less than 1 fA at zero membrane potential. By depolarization of 100 mV, the current contribution increases to about 0.7 pA. At cell-attached configuration and zero membrane potential, an open probability of less than 1.3×10^{-3} is estimated (Fig. 4*b*). Assuming that the isolated vacuoles retain their normal membrane potential in the chosen bath solution, the channel would be active with this low probability under normal conditions. An outward rectification can be explained by the small open probability and short channel lifetime ($\leq$700 μsec, as extrapolated from Fig. 4*b*) at hyperpolarizing voltages.

The described channel cannot be compared

with the K channel postulated by Bentrup et al. (1985) in tonoplasts from *Chenopodium rubrum*. A single K channel of 0.5 pS was estimated at symmetrical K of 46 mM from the analysis of current noise from whole cell records. This value is more than two orders of magnitude smaller than that for the large K channel described in this paper. In protoplast membranes of *Vicia faba*, K channels were also observed (Schroeder et al., 1984). These channels have a smaller conductance of 37 pS at comparable symmetrical KCl concentration (225 mM), and neither a significant voltage-dependent gating mechanism nor a rectifying behavior was observed.

Lühring (1986) could demonstrate a potassium channel in the cytoplasmic drop of *Chara* with a maximal conductance of 165 pS at symmetrical potassium concentrations of 150 mM. This value would well agree with our findings. For these K channels subconductance levels were also reported. But in comparison to our results, no rectification of the K channel at hyperpolarizing potentials was observed.

Finally, we want to discuss the possible contribution of this large K channel to the overall ion transport across the tonoplast. It is known that the tonoplast contains H^+-ATPase driven anionic transport systems (Martinoia et al., 1986). In C3 plants, accumulation of anions is not linked to an acidification of the vacuole (Winter et al., 1982, G. Schröppel-Meier, *personal communication*). Therefore, high amounts of potassium and/or sodium have to be transported into the vacuole. Na^+/H^+ and K^+/H^+ antiport systems (Blumwald & Poole, 1985; Scherer & Martiny-Baron, 1985) have been postulated for the tonoplast. The observation of a K channel suggests that potassium is not only transported into the vacuole by an energy-consuming process, but also by facilitated diffusion through a channel.

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