Biochimica et Biophysica Acta, 1104 (1992) 308–316

BBAMEM 75521

Reconstitution of vacuolar ion channels into planar lipid bilayers

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(Received 4 June 1991)
(Revised manuscript received 27 September 1991)

Key words: Anion transport; Ion channel; Lipid bilayer; Reconstitution; Tonoplast; Vacuole

Vacuolar ion channels were characterized after reconstitution into planar lipid bilayers. (1) Channel activity was observed after incorporation of tonoplast-enriched microsomal membranes, purified tonoplast membranes or of solubilized tonoplast proteins. (2) Channels of varying single-channel conductances were detected after reconstitution. In symmetrical 100 mmol l^{-1} KCl, conductances between 1 and 110 pS were frequently measured; the largest number of independent reconstitution events was seen for single-channel conductances of 16–25 pS (28 experiments), 30–42 pS (26), 49–56 pS (15) and 64–81 pS (15). Channel current usually increased linearly with voltage. (3) In asymmetrical solutions, cation-, non-selective and, for the first time for the tonoplast, anion-selective channels were detected. Ca^{2+}-dependent regulation of channel opening was not observed in our reconstitution system. (4) Permeability was also observed for Cl^-, NO_3^-, SO_4^{2-} and phosphate. (5) After fractionation of tonoplast proteins by size exclusion chromatography, ion channel activity was recovered in specific fractions. (6) Some of these fractions catalyzed sulfate transport after reconstitution into liposomes. The results suggest that different channels are active at the tonoplast membrane at a larger number than has been concluded from previous work.

Introduction

The large central vacuole of plant cells occupies upto 95% of the volume of plant cells. It is an effective storage compartment for various solutes whose concentration in the cytosol must be controlled in order to maintain cellular function. The concentrations of cations, such as sodium, and of anions, such as nitrate and chloride, are low in the cytosol and may be high in the vacuole, depending on growth conditions and the extent of salt stress [1,2]. Concentration gradients across the tonoplast of malate, potassium and phosphate are adjusted in response to metabolic demands [2,3]. This requires regulated transport across the tonoplast membrane. Using intact vacuoles, various transporters have been characterized kinetically. H^+-ATPase- or H^+-pyrophosphatase-dependent transport was observed for anions such as chloride, malate and phosphate [3–5]. A Na^+-H^+ exchanger has been described for oat tonoplast vesicles [6]. Two types of rather unspecific ion channels have been identified at the tonoplast of sugar beet by the patch clamp technique [7,8]. These channels transport cations such as Na^+ and K^+, but are also permeable for anions (Ref. 9, for reviews, see Refs. 10 and 11). It is not yet easy to see how they can facilitate specific transport of different ions *in vivo*.

In this communication we reconstitute tonoplast membrane proteins into planar bilayer membranes. In numerous studies with bacterial and animal membrane fractions, planar bilayer membranes have been successfully used to characterize pore or channel forming activities and to identify channel proteins [12–14].

Several questions can be addressed with this technique: (1) Are tonoplast channels reconstituted into planar bilayer membranes in an active state? (2) Is activity maintained after solubilization of the membranes? (3) How many different channels can be attributed to the tonoplast and what is their characteristics? (4) Can the method be used to identify individual tonoplast channel proteins by screening fractions from chromatographic separations of tonoplast membrane proteins for ion channel activity?

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Materials and Methods

Preparation of intact vacuoles, tonoplast membranes and microsomal tonoplast fractions. Intact vacuoles were isolated from primary leaves of 10–12 day old barley seedlings (*Hordeum vulgare* cv. Gerbel) according to the method of Martinoia et al. [15]. Tonoplasts were sedimented at 120000 × *g* and 4°C for 45 min and resuspended in a buffer containing 10 mmol l^{-1} Tricine-imidazole (pH 7.6), 4 mmol l^{-1} $MgCl_2$ or 10 mmol l^{-1} Tricine-imidazole (pH 7.6), 4 mmol l^{-1} $MgCl_2$ and 700 mmol l^{-1} urea. Microsomal membranes were prepared as described in Thume and Dietz [16].

Bilayer technique. Samples were tested for channel activities using the lipid-bilayer method [17]. The cuvette consisted of two chambers of 4 ml volume each separated by a diaphragm with a central hole of 0.8 mm diameter. The hole was precoated by applying 5 μl of lipid (either phosphatidylethanolamine or diphytanoylphosphatidylcholine) solution in chloroform (2% w/v). After 5 min of air-drying, the chambers of the cuvette were filled with 4 ml of electrolyte solution (100 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, pH 6.5 unbuffered, if not indicated otherwise). Then, a solution (2 μl) containing 1% (w/v) lipid, 10% (v/v) butanol and 89% (v/v) *n*-decane was painted across the hole with a teflon-loop. After the membrane turned black, the sample was added under continuous stirring. Two calomel electrodes were used to apply a voltage between the two chambers. One chamber was held at zero potential (trans chamber), the other at potentials between + 150 mV and − 150 mV variable in steps of 5 mV (cis chamber). The zero potential-electrode was connected to a current to voltage converter based on a Burr Brown operational amplifier. The amplifier signal was monitored on a storage oscilloscope and protocolled on a chart recorder. The input signal was amplified by a factor of upto 10^{10}. Usually the signal was filtered with a time resolution of 33 Hz, in some cases of 100 Hz which is necessary to reduce the background noise caused by the large size of the lipid bilayer membrane.

Two different approaches were used to reconstitute channel activity into the planar bilayers:

(1) Fusion of membrane vesicles. The osmolarity of the sample was adjusted with urea to 700 mosmol, the osmolarity of the electrolyte solution of the trans-chamber was increased with urea to 450–500 mosmol, whereas the osmolarity of the cis-chamber was held at 220 mosmol. Sample was added to the trans-chamber. In these experiments, phosphatidyl ethanolamine (Avanti Polar Lipids, Birmingham, AL, USA) was used as lipid. The content of the chambers was stirred for 5–10 min and the changes in conductance upon incorporation of channels was recorded [18].

(2) Reconstitution of channel proteins after solubilization with Triton X-100. The tonoplast membranes were suspended in 10 mmol l^{-1} Tricine-imidazole (pH 7.6), 4 mmol l^{-1} $MgCl_2$ and 2% (v/v) Triton X-100 (Pierce Chemical Company, Oud Beijerland, The Netherlands) at room temperature for 5 min. 5–10 μl samples were pipetted into each chamber. The reconstitution frequency was unchanged when the solubilization mix was freed from non-solubilized proteins and membranes by centrifugation (result not shown). In these experiments, diphytanoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL, USA) was used as lipid to form the bilayers.

Chromatographic separation of solubilized polypeptides. Tonoplast membranes corresponding to about $3 \cdot 10^8$ vacuoles were solubilized in 200 μl of a buffer containing 20 mmol l^{-1} Tris (pH 7.2), 50 mmol l^{-1} NaCl and Triton X-100 at a concentration of 2% (v/v). Solubilized proteins were separated by size exclusion chromatography on a prepacked Superose 6 HR-column (Pharmacia, Uppsala, Sweden), which was equilibrated with 20 mmol l^{-1} Tris (pH 7.2), 50 mmol l^{-1} NaCl and 0.1% (v/v) Triton X-100 at a flow rate of 0.3 ml min^{-1}; 40 fractions of 0.3 ml were collected. The fractions were divided into aliquots and stored in liquid N_2 (−196°C) until further use.

Reconstitution of tonoplast polypeptides into liposomes. Tonoplast polypeptides were incorporated into phosphatidylcholine-liposomes by a freeze-thaw cycle as described in Thume and Dietz [16]. Liposomes and proteoliposomes were incubated in the presence of $^{35}SO_4^{2-}$ (50 Bq μl^{-1}). Liposomes were separated from the surrounding medium by applying the liposomal suspension onto a Sephadex G-75 column. The turbid fraction was collected and counted for radioactivity.

Reconstitution of nitrate-, sulfate-, chloride- and phosphate-conducting channels. To measure nitrate-, sulfate-, phosphate- and chloride-conductances, electrolyte solutions were prepared from the corresponding acids. The pH value of the solutions was adjusted to about pH 6.5 with the impermeable cation *N*-methylglucamine. Mg^{2+} ions were added in the form of Mg-gluconate to a final concentration of 5 mM.

Results

Reconstitution of ion channels from tonoplast-enriched microsomal fractions, tonoplast vesicles or solubilized tonoplast membranes

In symmetrical KCl-solution, channel activity was detected upon addition of tonoplast-enriched microsomal membranes or tonoplast vesicles to the chambers. Without adding any sample no opening and closing events could be detected. Fig. 1 shows examples of current recordings which were obtained from different samples. Usually, 2–10 min after adding the tonoplast

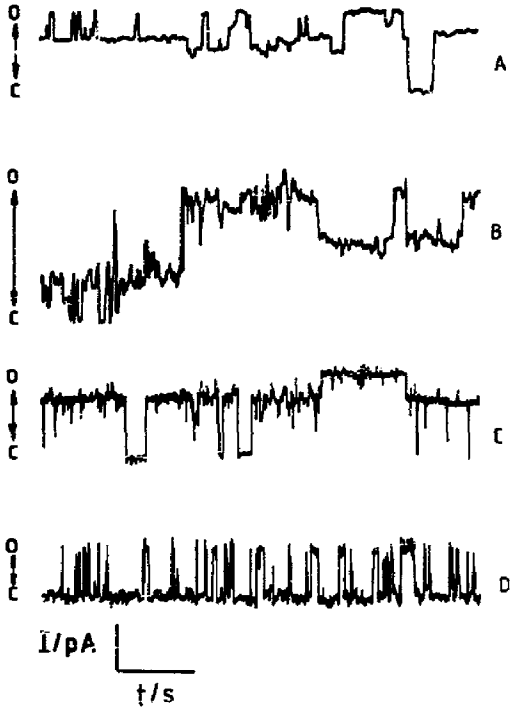

Fig. 1. Recordings of ion channel activities reconstituted into planar bilayers from a tonoplast-enriched microsomal fraction (A; the time bar corresponds to 20 s; +50 mV; the conductance was 60 pS), or from vacuolar vesicles obtained after freezing of isolated intact vacuoles (B; t = 10 s; +50 mV; 40 pS), or from vesicles of sedimented tonoplasts (C; t = 2 s; +50 mV; 24 pS), or from tonoplast membranes solubilized in 0.1% Triton X-100 (D; t = 20 s; +50 mV; 20 pS). The chambers of the cuvette contained 100 mmol/l KCl and 10 mmol/l $MgCl_2$. The electrolyte solution was unbuffered with a pH of 6.5.

preparation to the chambers, frequent opening and closing events of ion channels were observed. Channel activity was also seen when the tonoplast polypeptides were solubilized in detergent prior to the addition to the chamber. Without mechanical stress, stability of the membranes reached up to five hours. Analysis times for reconstituted channels was variable. It was in the range of 30 min to 3 h without much change in opening and closing frequency.

Selectivity and voltage-dependence of two tonoplast channels with 57 pS and 24 pS

The characteristics of two channels is shown in more detail in Fig. 2. Voltage was varied between +50 and −50 mV and channel currents were recorded. The amplitude of the currents through the channels were linearly dependent on the voltage. The single-channel conductance of the channels was 57 pS, and 24 pS, respectively. After the measurement in symmetrical KCl solution, an asymmetrical electrolyte distribution was established by increasing the electrolyte concentration in the cis-chamber from 100 mmol l^{-1} KCl to 430 mmol l^{-1} KCl. Under these conditions, the reversal potential calculated from the Nernst-equation corresponded to ±37,5 mV. A second current-voltage curve was measured. Again closing and opening events of two channels were observed. The single-channel conductances increased after the increase in ion concentration. The conductance of the 57 pS channel which revealed no ion selectivity increased to 150 pS. Assuming a linear relationship between ion concentration and single-channel conductance we would have expected a conductance 4.3-times as high as 57 pS (245 pS). This result indicates substrate saturation. The conductance of the 24 pS channel which was anion-specific (the

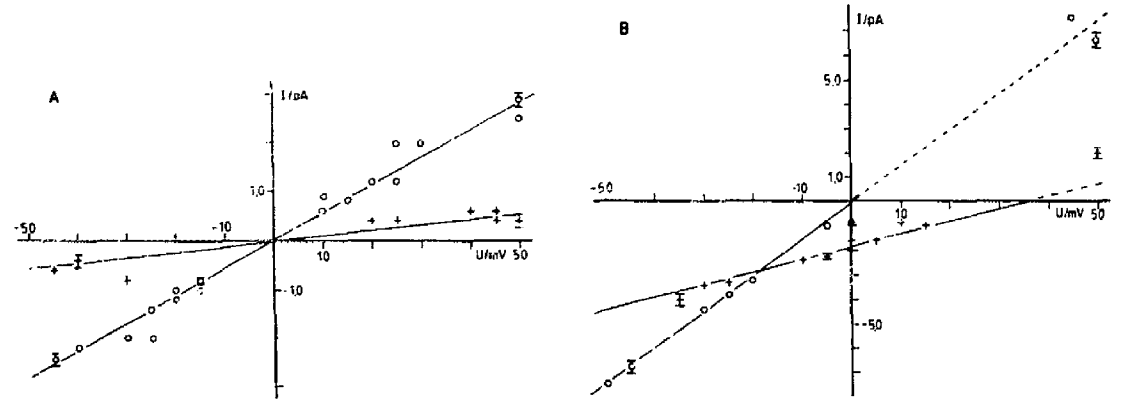

Fig. 2. Voltage-dependent current of reconstituted tonoplast channels. The current amplitudes obtained in 0.1 mol/l KCl, 0.01 mol/l $CaCl_2$ (A) or, after increasing the KCl in the cis chamber to 0.43 mol/l (B) were analyzed and plotted as I-V curves. Symbols: (+) = 24 pS; (○) = 57 pS.

current through the channel was zero at +37 mV, the Nernst potential for Cl^-) increased only to 49 pS which indicates again substrate saturation at higher concentrations.

Variability of channel activities after reconstitution of tonoplast membranes

Different ion channels showed large differences in single-channel conductance after incorporating tonoplast proteins into the bilayer membrane Figure 3 summarizes conductances which were observed in different experiments. The conductance steps were chosen in dependence of the mean error of the measurements and were based on the functional relationship that the magnitude of the error is proportional to the square root of the single-channel conductance. Only channels are included in the figure which were characterized by a current analysis of at least five different voltages. Each of these single voltage steps of every experiment reflects several to more than one hundred opening and closing events. The histogram shows that the most frequent events of reconstitution were observed for channels with a single-channel conductance of 16–25 pS (28 experiments. This corresponds to a 40% probability of detection in single experiments), 30–42 pS (26), 49–66 pS (15) and 64–81 pS (15) (in 100 mmol l^{-1} KCl). Interestingly, channel activities were observed over the whole range from < 4 pS to > 196 pS. This observation clearly needs clarification of the possible causes for the channel heterogeneity (see Discussion). A detailed analysis of the channels is not possible as long as the reconstitution is performed with complex populations of different channels. Fig. 3 shows that preparations of highly purified tonoplast membranes contain a large number of different channels. Therefore, we started a chromatographic separation of solubilized tonoplast proteins in order to enrich specific channels in distinct fractions.

Channel activity in fractions from chromatographically separated tonoplast polypeptides

To investigate the heterogeneity of ion conducting activities, we separated solubilized tonoplast polypeptides by gel filtration (Fig. 4A). The silver-stained electrophoretogram of the 40 collected fractions showed good separation of most and little separation of some polypeptides. In particular, a 24 kD polypeptide was present in all fractions. This polypeptide is reported to be developmentally regulated, heavily glycosylated and tends to form higher molecular weight aggregates. It is also responsible for the heavy protein stain in fractions 25–28 ([19]; Kloske, personal communication). Individual fractions were tested for channel forming activities. Most of them showed no or little channel activity (Fig. 4 C). Fractions 1–3 showed different channels with conductances of 20 pS, 30 pS, 70 pS and 190 pS. These first fractions contained unsolubilized membrane vesicles and therefore are likely to reflect the whole pattern of activities contained in the starting material.

In fraction 6, channels were detected with a single-channel conductance of 4 pS in 100 mmol l^{-1} KCl. Channel activites were also repeatedly found in fractions 15 and 16 (40 pS and 100 pS), in fraction 35 (42 pS; Fig. 5) and in fraction 39 (42 pS). The spectrum of channels detected after separation of tonoplast proteins by gel filtration corresponded to the spectrum of channels which can be reconstituted from whole tono-

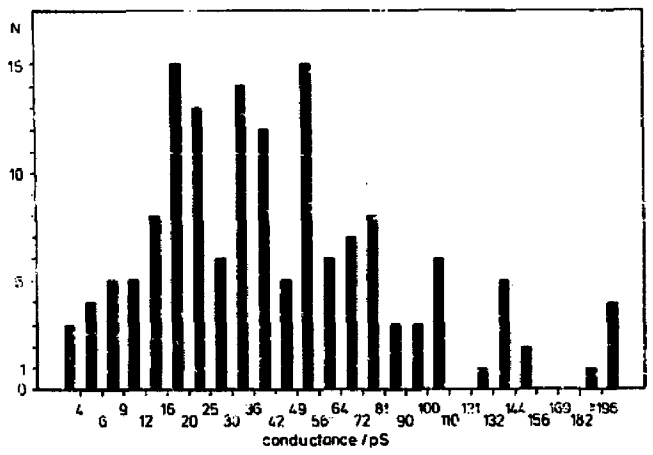

Fig. 3. Histogram of reconstitution events of ion channels with specific conductances. Tonoplast vesicles were incorporated into the planar bilayers. Channel reconstitution events from independent experiments were ordered according to their single-channel conductance. Each event corresponds to a channel which was characterized at least by five different voltages.

plasts. Single-channel conductances range from 4 pS to 190 pS.

Fig. 4 compares the $^{35}SO_4^{2-}$-transport activity of reconstituted proteoliposomes with the activity profile seen after reconstitution of tonoplast polypeptides into planar bilayer membranes. As for reconstitution into liposomes the amount of fractionated tonoplast material was limiting. SO_4^{2-} was chosen arbitrarily as an example to test for anion permeability. SO_4^{2-} is particularly suitable for liposomal reconstitution experiments, as the basic rate of SO_4^{2-} uptake by phosphatidylcholine liposomes was very low. In fraction 6, a single-channel conductance of 16 pS was observed in the bilayer system in the presence of 1 mol l^{-1} sulfuric acid neutralized with the impermeable cation *N*-methylglucamine. This result with bilayer membranes coincides with the activity of the same fraction in the liposome reconstitution system.

The 42 pS channel of fraction 35 was further characterized after application of an ion gradient. It revealed only a small increase in single-channel conductance after the KCl concentration in the cis chamber had been increased from 100 mmol l^{-1} (Figs. 5A and B) to

TABLE I

Reconstitution of anion-conducting channels into planar bilayer membranes from solubilized tonoplast membranes

The electrolyte solution consisted of the anion as indicated. The pH was adjusted to about pH 6.5 with *N*-methylglucamine. Mg gluconate was present at a concentration of 5 mmol l^{-1}. The conductance was derived from the slope of *I-V* curves obtained from several measurements.

Ion	Concentration (mol l^{-1})	Conductance (pS)		
Cl^-	0.1	42	20	10
NO_3^-	0.1	57	22	
SO_4^{2-}	0.1	9		
	1.0	16		
$H_2PO_4^-/HPO_4^{2-}$	1.0	10	5.7	1.4

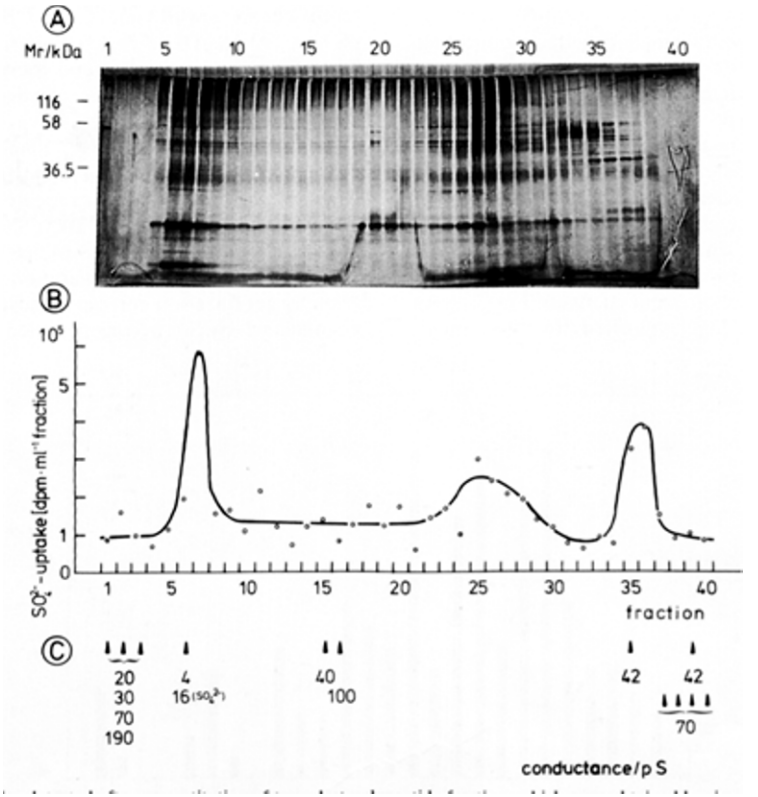

Fig. 4. Channel activities detected after reconstitution of tonoplast polypeptide fractions which were obtained by size exclusion chromatography. Tonoplast membranes of $3 \cdot 10^8$ vacuoles were sedimented, solubilized and fractionated on superose 6 HR-particles as described in Methods. Fig. 4A shows a silver stained SDS-PAGE of the obtained fractions 1–40. In 4B, 50 µl of each fraction were incorporated into phosphatidylcholine liposomes as described in Thume et al. [16] and analyzed for [^{35}S]sulfate permeability. In 4C, results from incorporating ion channels into planar bilayer membranes are summarized. Either KCl (0.1 mol/l) or SO_4^{2-} (1 mol/l)/*N*-methylglucamine-permeability was investigated after adding 10 µl of the chromatographic fractions to the chambers. The numbers give the conductance of the channels in pS.

480 mmol l^{-1} (Fig. 5C). The intersection with the abscissa shifted to −25 mV which indicates partial cation selectivity. As the conductance only increased to 66 pS, the channel shows substrate saturation. We would have expected a reversal potential of −40 mV for a 4.8-fold gradient in the case of potassium perme-

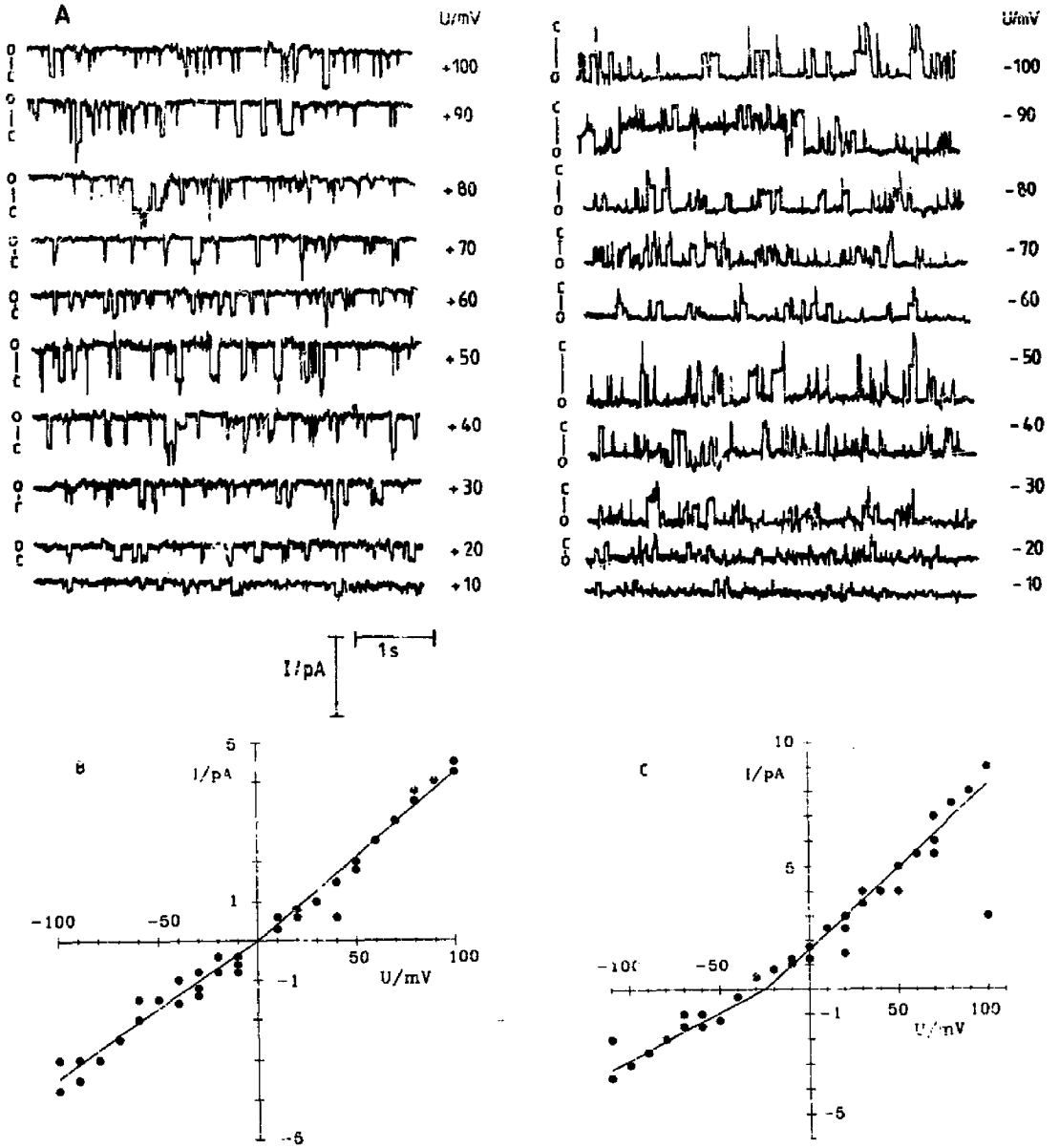

Fig. 5. Current traces and *I-V* curve of the 42-pS channel detected in fraction 35 after size exclusion chromatography of tonoplast polypeptides on a Superose 6HR-column. These data represent one example of several independent measurements. Current recordings under symmetrical conditions (0.1 mol/l KCl, 5 mmol/l $MgCl_2$) are shown in (A) at varying voltages. For $I \geq 60$ mV, the current bar corresponds to 1 pA, for $I < 60$ mV to 0.4 pA. The current amplitudes were analyzed and plotted as *I-V* curve (B). After increasing the KCl concentration of the cis chamber to 0.48 mol/l, the *I-V* curve shown in (C) was obtained.

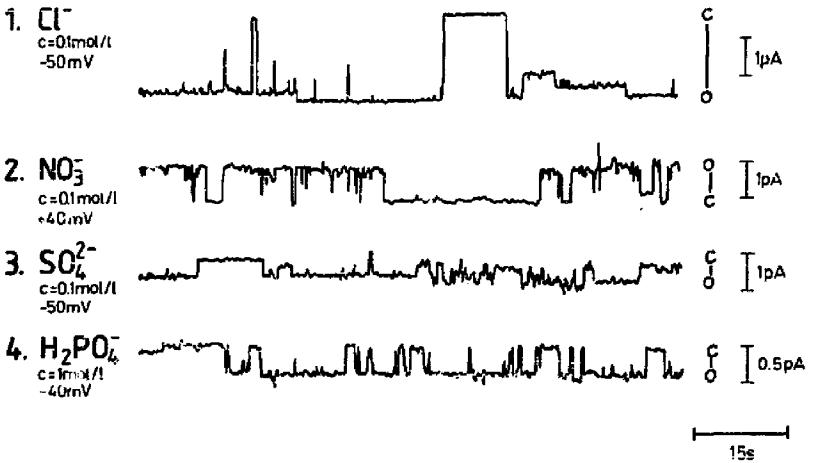

Fig. 6. Anion permeability of reconstituted tonoplast channels. The electrolyte solutions were made up from the corresponding acid at the concentration indicated and neutralized with *N*-methylglucamine. The conductivities were: 42 pS for Cl^-, 22 pS for NO_3^-, 9 pS for SO_4^{2-} and 10 pS for $H_2PO_4^-$ (the latter at 1 molar concentration; all the others at 0.1 molar concentration).

ability alone. The incomplete shift to −25 mV suggests that the channel also conducts Cl^-, however at a about 5.7-fold lower activity than K^+.

Reconstitution of nitrate-, sulfate-, chloride- and phosphate-conducting channels

In samples of Triton X-100-solubilized tonoplasts, single-channel conductances were detected for all tested anions (Table I) (Fig. 6). Channels for chloride and nitrate had higher conductances than channels for sulfate and phosphate. The comparison has to be taken with care as only the direct exchange of the cuvette content after successful reconstitution and characterization of a channel will provide clear evidence for the single-channel conductance of the different tonoplast channels for various ions.

Discussion

What can reconstitution tell us?

Ion transport across the tonoplast membrane has been studied with intact vacuoles, microsomal membrane fractions and tonoplast vesicles. The possible involvement of ion channels in transtonoplast ion transport has been discussed only recently. Experimental results from patch clamp studies demonstrated the existence of three or four different types of ion channels in the vacuolar membrane, a slowly activating and a rapidly activating channel for monovalent cations and anions, a potassium channel and a Ca^{2+}-channel [8,20,21,22]. The specificity of the cation channels is low [7]. For this reason, their function is difficult to assess. Clearly a further characterization of the ion channels is required to fully understand their role in ion transport.

Two approaches are feasable: On the one hand, electrophysiological investigations may be extended to more complex systems such as permeabilized protoplasts or cells in which possible cytoplasmic regulatory mechanisms are still likely to be at work. The whole cell configuration in the patch clamp analysis is a powerful technique bridging the data on excised membrane patches and the work with intact vacuoles. On the other hand, we are interested in the characterization and identification of ion channels for biochemical analysis of the parent proteins. Reconstitution as a method to detect channel activity after disruption of membrane integrity is the first step to address these questions. In this communication we demonstrate that tonoplast channels can be incorporated into planar bilayer membranes. The density of ion channels at the tonoplast is unlikely to be as high as in membranes of cells specialized for rapid ion conductance, for example stomata [10]. The efficiency of channel incorporation in the planar bilayer membrane is low. The observation of more than one simultaneously opening channels in the bilayer system suggests (as a function of the open probability) that more than the resolved number of channels have incorporated into the bilayer membrane [23]. Therefore, repeated reconstitution of identical

channels from different tonoplast preparations provides good evidence for the localization of the characterized channel in the tonoplast membrane.

Channel heterogeneity

The large variability of channel conductance after reconstitution of tonoplast membranes is interesting and puzzling. The function of plant vacuoles in specific compartmentation of ions requires specific transporter proteins. With the exception of the Ca^{2+} conducting channel, the tonoplast channels which have been characterized upto now show insufficient specificity to account for the *in vivo* observed specific ion movement. Therefore the identification of an anion specific channel of 24 pS conductance marks an important step towards an improved understanding of ion movement into and out of the vacuole.

Although this is promissing, the diversity of channel activities seen after incorporation of tonoplast material into planar bilayers may require some discussion. The diversity may be explained by different causes:

(i) The tonoplast contains a larger number of different ion channels than usually conceived. The standard methodology of the patch clamp technique allows only to detect spontaneously active or specifically activated, for example Ca^{2+}-regulated channels. Regulatory mechanisms must be understood before activity of specifically regulated channels can be observed. Reconstitution of proteins into planar bilayer membranes might override regulation and produce channel responses different from those observed in intact systems. We favour this first explanation.

(ii) The diversity could also be caused by changes in the molecular structure or arrangement of the channel or other proteins during reconstitution. Proteins consisting of multiple subunits may reveal altered activity after a reduction of subunit number. The model of multi-subunit (barells)-channels is often used to explane substate levels of conductances (cf. for example, Refs. 24 and 25). After incorporation into planar bilayers, such substructures may catalyze ion tranport at lower conductance. An example of such a change in electrical characteristics after reconstitution is the K^+-permeability associated with the CF_0-subunit of the ATP synthase of the thylakoid membrane after extraction of the hydrophilic CF_1-complex [26]. Following solubilization, reconstitution may also alter the subunit structure of channel proteins. This was observed for subunit III of the chloroplast ATPase. Reconstitution led to various aggregation states of the protein with distinct conductance levels [27].

(iii) Possible contaminations of the tonoplast membrane preparations with other cellular membranes or with microorganisms could lead to the incorporation of channels from other than tonoplast sources. As judged from marker enzyme actvities, vacuoles isolated from barley mesophyll protoplasts are very pure and are contaminated with other cellular material by less than 1% [28]. The media were routinely frozen to suppress growth of microorganisms. Of course, it can not be excluded that in some rare cases, channels from other than tonoplast origin incorporate into the bilayer.

Enrichment of channels by size-exclusion chromatography

Our results demonstrate that activities of tonoplast channels can be separated by chromatographic methods. This is the first step towards a molecular understanding of the tonoplast channels beyond the electrophysiological characterization by patch clamp analysis. It will be important to investigate the channel heterogeneity in partially purified fractions in order to understand specificity of channels and their role in ion compartmentation *in vivo*.

The survey of tonoplast ion channels by Hedrich et al. [7] shows that a slow-vacuolar type channel exists in all tested vacuoles. This channel has a conductance of 50–80 pS in 100 mM KCl and exhibits low ion-selectivity. However, inter-species-variability was large. Obviously, data obtained with different methods, such as patch-clamp and planar bilayer technique, can only be compared for one specific tissue and species. There only exist two reports for barley where tonoplast ion channels were analyzed with the patch-clamp technique [21,22]. However, quite different conditions were chosen by the authors for their analysis. In 250 mM KCl, a single channel conductance of 121 pS was reported by Kolb et al. [21], whereas a conductance of 60–80 pS was observed by Hedrich et al. [22] in 50 mM K_2malate. Kolb et al. [21] did not characterize the specificity towards K^+ and Cl^-. In the case of Hedrich et al. [22], K^+ was about twice as permeable than malate at pH 7.3. Although, we also detected single-channel conductances in the range of 60–80 pS in 100 mM KCl, this type of channel was less frequently observed than channels with lower conductance. Interestingly, both groups describe a voltage-dependent open probability of the tonoplast channels. Our here reported channels did not reveal such behaviour. This may be caused by the solubilization of the channel proteins, followed by reconstitution into artificial bilayers which in contrast to the polar tonoplast has symmetrical properties.

Only (partially) purified fractions of tonoplast proteins will allow to characterize specific types of tonoplast channels in detail.

Acknowledgements

We are grateful to Dr. Enrico Martinoia (ETH Zürich) for helpful discussions. We also acknowledge continuous support of the work by Prof. Ulrich Heber.

This work was performed within the Sonderforschungsbereich 176 of the Julius-Maximilians-Universität Würzburg and was supported by the Fonds der Chemischen Industrie.

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