Plant Cell Physiol. 48(12): 1747–1757 (2007)
doi:10.1093/pcp/pcm147, available online at www.pcp.oxfordjournals.org

Characteristics of Anion Channels in the Tonoplast of the Liverwort *Conocephalum conicum*

Kazimierz Trebacz [1,*], Gerald Schönknecht [2], Halina Dziubinska [1] and Agnieszka Hanaka [1]

[1] *Department of Biophysics, Institute of Biology, Maria Curie-Sklodowska University, Akademicka 19, PL-20-033 Lublin, Poland*
[2] *Department of Botany, Oklahoma State University, 104 Life Sciences East, Stillwater, OK 74078-3013, USA*

Isolated vacuoles of the liverwort *Conocephalum conicum* thallus cells were investigated using the patch-clamp technique. At high cytosolic Ca^{2+} activities, slowly activating currents were evoked by positive potentials. The currents were conducted by the SV (slow-vacuolar) channel. When isolation of vacuoles was carried out at high Mg^{2+} and low Ca^{2+} concentration and the same proportion of the cations was kept in the bath, currents were recorded at negative potentials. Once activated, these currents persisted even after replacing Mg^{2+} with K^+ in the bath. Sr^{2+} and Ba^{2+} were also effective activators of the currents. With a Cl^- gradient, 10 mM in the bath and 100 mM in the lumen, currents were significantly reduced and the current–voltage characteristics shifted towards the reversal potential of Cl^-, indicating Cl^- selectivity. Currents almost vanished after substituting Cl^- with gluconate. They were strongly reduced by anion channel inhibitors 4,4′-diisothicyanatostilbene-2,2′-disulfonic acid (DIDS; 1 mM), anthracene-9-carboxylic acid (A9C; 2 mM) and ethacrinic acid (0.5 mM). Single-channel recordings revealed a 32 pS channel activating at negative voltages. It is concluded that the currents at negative potentials are carried by anion channels suitable for conducting anions from the cytosol to the vacuole. The anion channels were weakly calcium dependent, remaining active at physiological calcium concentration. The channels were almost equally permeable to Cl^-, NO_3^- and SO_4^{2-}, and much less permeable to malate^{2-}. Anion channels did not respond to ATP addition. cAMP (10 μM) had a weak effect on anion channels. Protein kinase A (0.4 U) added to the medium caused no significant effect on anion channels.

Keywords: Anion channel — *Conocephalum conicum* — Magnesium — Patch-clamp — SV channel — Vacuole.

Abbreviations: A9C, anthracene-9-carboxylic acid; AP, action potential; CAM, crassulacean acid metabolism; CDPK, calcium-dependent protein kinase; DIDS, 4,4′-diisothicyanatostilbene-2,2′-disulfonic acid; FV channel, fast vacuolar channel; PKA, protein kinase A; SV channel, slowly activating vacuolar channel; VK channel, vacuolar potassium channel; VCL channel, vacuolar anion channel permeable to chloride; VMAL, vacuolar anion channel permeable to malate.

Introduction

Transport mechanisms across the vacuolar membrane–tonoplast are responsible, among others, for osmoregulation, signaling, regulation of cell metabolism and sequestering of toxic compounds. It is thus of great importance to understand vacuolar transport systems. The patch-clamp technique offers the possibility of direct examination of ion channels in isolated vacuoles. Most published data concern cation-permeable channels, mainly slow activating vacuolar (SV) channels. SV channels have been characterized in a large number of plant species and under different experimental conditions mimicking cytoplasmic and intravacuolar factors (Barkla and Pantoja 1996, Allen and Sanders 1997, Krol and Trebacz 2000, Pottosin and Schönknecht 2007).

Far less is known about anion channels in plant vacuoles (Barkla and Pantoja 1996, Allen and Sanders 1997, Krol and Trebacz 2000). Two major groups of anion channels have been characterized so far in higher plants: channels permeable to both malate and some other organic anions, such as fumarate and succinate (VMAL), and chloride channels (VCL), permeable to inorganic ions (Ping et al. 1992, Barkla and Pantoja 1996, Allen and Sanders 1997). Vacuolar malate channels have been investigated mainly in crassulacean acid metabolism (CAM) plants (Iwasaki et al. 1992, Cheffings et al. 1997, Pantoja and Smith 2002, Hafke et al. 2003), but are also known in C_3 plants (Pantoja et al. 1992, Plant et al. 1994, Cerana et al. 1995). There are also channels permeable to both chloride and malate (Pei et al. 1996). A common feature of most anion channels in higher plant vacuoles is their kinetics of activation consisting of a fast, instantaneous component followed by a slow component with a time constant of up to 5 s. Anion channels exhibit inward rectification, i.e. they are suited for anion transport into the vacuole in the physiological tonoplast potential range −10 to −50 mV. Most of the anion channels are weakly affected by cytosolic Ca^{2+}. The transporters of higher plant vacuoles responsible for anion re-entry into the cytosol remain to be characterized.

*Corresponding author: E-mail, trebacz@biotop.umcs.lublin.pl; Fax, +48-81-5375901.

In contrast to higher plants, anion channels conducting Cl^- preferentially from the vacuole to the cytosol have been characterized in giant *Characean* algal vacuoles (Tyerman and Findlay 1989, Berecki et al. 1999, Berecki et al. 2001). These channels are activated by an increase in cytosolic free calcium concentration, $[Ca^{2+}]_{cyt}$, up to 100 μM at pH 7.4 and <5 μM at pH 6.0 (Berecki et al. 2001).

The object of our study, the liverwort *Conocephalum conicum*, is phylogenetically located between algae and higher plants. It belongs, as other liverworts do, to the oldest terrestrial plants (Qiu et al. 1998). *Conocephalum* has a simple structure without conducting bundles and stomata. It is an excitable plant. It generates action potentials (APs) in response to different stimuli (Paszewski et al. 1982, Dziubinska et al. 1983, Trebacz and Zawadzki 1985, Trebacz et al. 1997, Krol et al. 2003). Recently, *Conocephalum* has become the subject of patch-clamp studies (Trebacz and Schönknecht 2000, Trebacz and Schönknecht 2001). It has been demonstrated that the vacuole of *C. conicum* possesses functional SV channels. SV channels are ubiquitous among terrestrial plants (*Embryophyta*) but are absent in algae (Hedrich et al. 1988). Here we present evidence that anion channels similar to VCL and VMAL channels of higher plants exist in the tonoplast of this liverwort. Different aspects of anion channel characteristics, including permeability, susceptibility to inhibitors and selected metabolic factors, are presented.

Results

Divalent cations activate currents at negative potentials

Currents recorded with isolated vacuoles differed tremendously depending on the ionic composition of the solution used for both isolation and perfusion. In solutions containing 2 mM $CaCl_2$ and 100 mM KCl in the bath (and in the pipet), currents passing through SV channels were recorded (Trebacz and Schönknecht 2000). SV currents exhibited typical slow activating kinetics at positive command potentials with nearly no current at negative potentials (Fig. 1A). Calcium in the bath is necessary to activate the SV channel (Hedrich and Neher 1987, Ward and Schroeder 1994). The SV channel is an unselective cation channel, mainly conducting K^+ but also Ca^{2+} with the bath solutions present. In contrast, under high magnesium and low calcium concentration (50 mM $MgCl_2$, no $CaCl_2$ added) in the bath, significant currents were recorded at negative potentials (Fig. 1B) and nearly no currents at positive potentials. These magnesium-activated currents displayed characteristic kinetics consisting of an instantaneous phase followed by a slow phase. None of the abundant vacuolar cation channels (SV, FV or VK) shows this voltage dependence and kinetics, and none should be active under the conditions (high Mg^{2+} no Ca^{2+}) applied (Brüggemann et al. 1999, Pei et al. 1999). Current–voltage characteristics of SV and Mg^{2+}-activated currents are compared in Fig. 1C and D.

Once currents at negative potentials had been activated by magnesium, increasing the Ca^{2+} concentration in the bath resulted in the appearance of large SV currents at positive potentials (Fig. 2A, C). As before, SV channels were activated by Ca^{2+}, but this time the current was mainly carried by Mg^{2+}. Negative currents were slightly increased by cytosolic Ca^{2+} (see below). This demonstrates that SV currents at positive potentials and magnesium-activated currents at negative potentials are regulated independently. Depending on the ionic composition of the bath, these currents can be recorded separately (Fig. 1) or together (Fig. 2). Substituting 50 mM $MgCl_2$ with 100 mM KCl in the bath (at 2 mM Ca^{2+}) caused only small changes in current amplitudes (Fig. 2B, D). Thus, the negative currents activated by magnesium persisted even after removal of Mg^{2+} from the bath.

Different Mg^{2+} concentrations were applied at the cytosolic side (bath) to test which concentrations are required to activate negative currents. The threshold of activation was between 2 and 5 mM $[Mg^{2+}]_{cyt}$; Mg^{2+} binding can be described quantitatively with a $K_m = 11.4$ mM (Fig. 3).

To test the specificity of Mg^{2+} in channel activation, Mg^{2+} was substituted by Sr^{2+} or Ba^{2+} (all 50 mM). These divalent cations were almost as effective in activating currents at negative potentials as Mg^{2+}, provided that they were applied already during vacuole isolation (Fig. 4). This indicates that the activation of currents at negative potentials was not an Mg^{2+}-specific effect, but could be brought about by other divalent cations as well. High Ca^{2+} concentrations (50 mM) resulted in bursting of isolated *Conocephalum* vacuoles. This prevented us from testing the effect of high Ca^{2+} on channel activation at negative potentials. At 2 mM Ca^{2+} which was routinely used to activate SV channels, no currents were activated at negative potentials (Fig. 1A).

Currents at negative potentials are due to anion channel activity

To characterize further the currents activated by divalent cations, experiments were performed with vacuoles that had been isolated in the presence of 50 mM $MgCl_2$. This high Mg^{2+} concentration resulted in the stable and reliable activation of currents at negative potentials.

To identify the ion channel that is responsible for whole-vacuole currents at negative potentials, single-channel recordings were performed. The recordings were carried out with small cytoplasm-out patches immersed in a bath solution known to activate the negative currents,

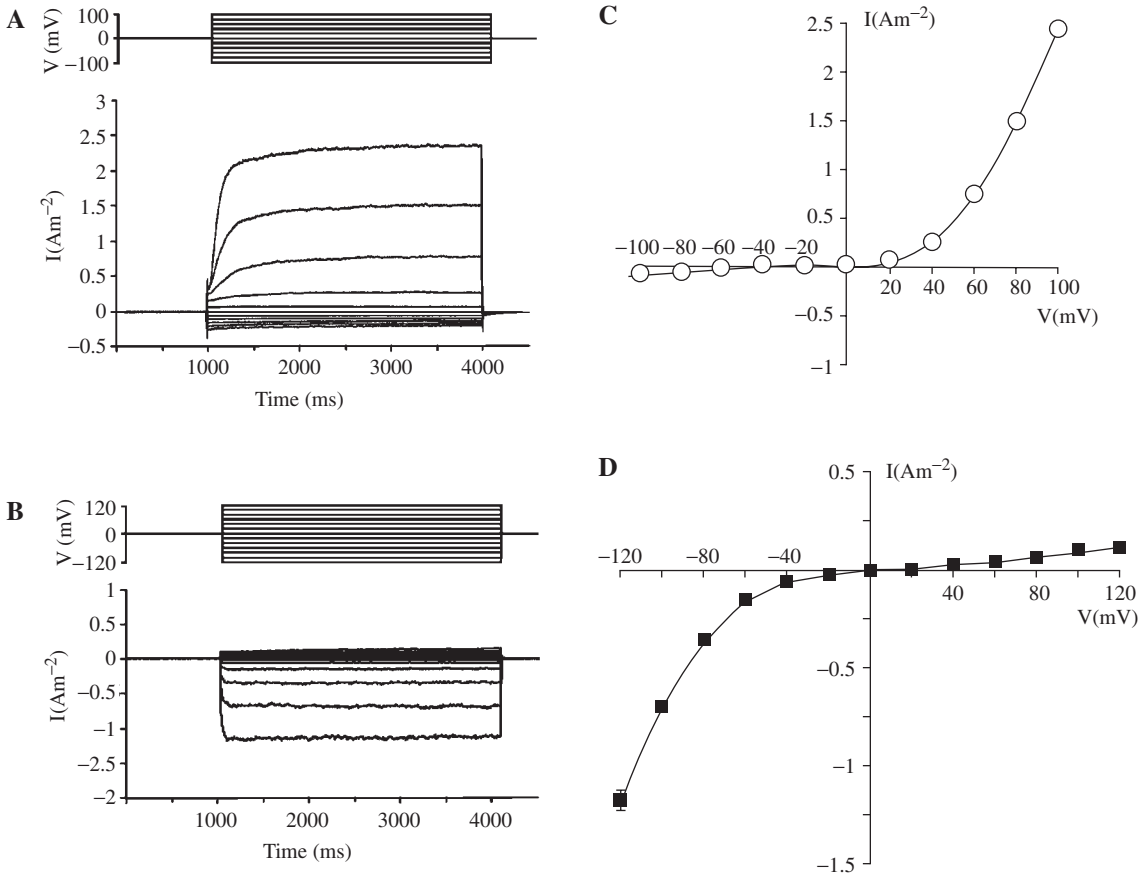

Fig. 1 Whole-vacuole currents from *Conocephalum conicum* vacuoles. (A) Positive currents (SV) were activated in a bath solution (cytosolic side) containing: 100 mM KCl and 2 mM$CaCl_2$. (B) $MgCl_2$ at 50 mM caused activation of large negative currents. Bath solutions were buffered by 15 mM HEPES, pH 7.2. Pipet solution (vacuolar side) contained 100 mM KCl, 2 mM $CaCl_2$, 15 mM MES, pH 5.85 in both cases. The voltage protocol is given at the top of the figures. Current amplitudes were normalized to the surface of the vacuole using the measured membrane capacitance and a ratio of 0.01 F m^{-2}. Vacuoles were isolated in the solution containing 100 mM KCl, 2 mM $CaCl_2$, 15 HEPES/Tris, pH 7.2, 300 mosmol kg^{-1}. (C, D) Current–voltage characteristics of currents displayed in A and B. The averaged current amplitude during the last second of the voltage pulse was plotted as a function of applied voltage. Note the 2-fold expanded ordinate in D. Average of 10 traces with SE as error bars in C and six traces in D.

i.e. 50 mM $MgCl_2$. Starting at about −50 mV, with increasing negative potentials increasing single-channel activity was observed (Fig. 5A). Occasionally, single-channel openings appeared at high positive voltages, but we currently have no evidence of whether this activity is caused be the same or a different ion channel. All-points amplitude histograms of single-channel currents were fitted by a sum of equidistant Gaussian distributions (Fig. 5B) to estimate single-channel current amplitudes and open probabilities. Fig. 5C presents the resulting single-channel I–V curve. The open channel displayed a rectification, i.e. larger unitary currents at more negative potentials. This in part explains the rectification of the whole-vacuole currents. The unitary channel conductance at −100 mV is 32.3 pS. To calculate open probabilities (P_O) from all-points amplitude histograms, we assumed independent channel gating, and introduced the equations describing a binomial distribution into the fit routine. This reduces the number of fit parameters and yields P_O directly as a fit parameter. The resulting open probabilities are graphed in Fig. 5D (open circles). This was compared with the voltage dependence of the average number of open channels in whole vacuoles ($N \times P_O$). $N \times P_O$ was calculated by dividing (background current corrected) whole-vacuole current amplitudes by single-channel current amplitudes. P_O and $N \times P_O$ displayed the same voltage dependence (Fig. 5D). The value of N, the number of channels per μm^2, was estimated as 1.38 by minimizing the sum of the quadratic deviations between P_O and $N \times P_O$ (see different y-axes in Fig. 5D which differ by a factor of 1.38). The voltage dependence of P_O and $N \times P_O$ was mathematically described assuming a simple Boltzmann distribution (solid line). The rectification of the whole vacuole currents (Figs. 1, 2) is caused by both a rectification of the open

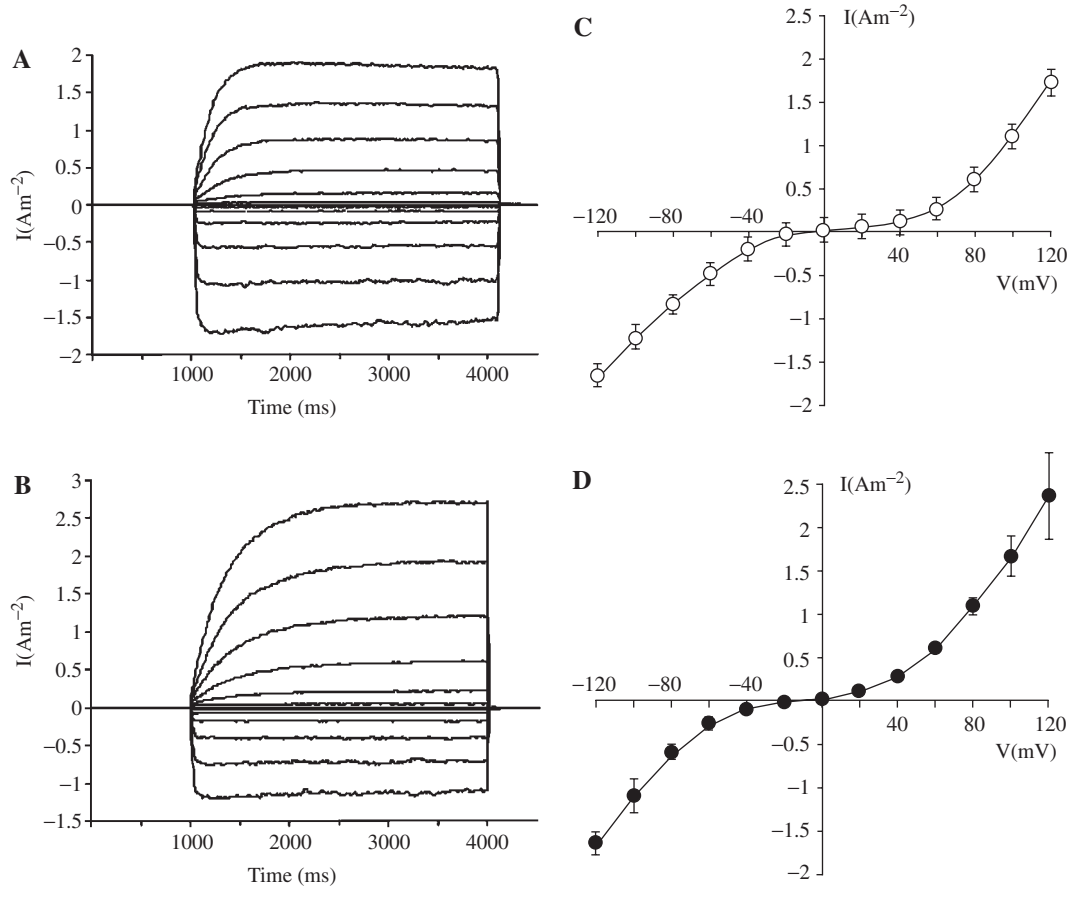

Fig. 2 Whole-vacuole currents in vacuoles of *Conocephalum conicum*. (A) Addition of 2 mM $CaCl_2$ to the bath already containing 50 mM $MgCl_2$ caused activation of positive currents (SV) in addition to negative currents (at negative potentials). (C) Corresponding I–V curve. Average of four traces with SE as error bars. (B) Both negative and positive currents persisted after substituting 50 mM $MgCl_2$ with 100 mM KCl in the bath. (D) Corresponding I–V curve. Average of four traces with SE as error bars. Voltage protocol as in Fig. 1B.

single channel (Fig. 5C) and a voltage-dependent open probability (Fig. 5D).

To investigate the selectivity of the magnesium-activated currents at negative potentials, the $MgCl_2$ concentration in the bath was decreased from 100 to 10 mM by bath perfusion (with 100 mM $MgCl_2$ in the pipet). As a result, negative currents decreased in amplitude (Fig. 6), and the *x*-axis intercept of the I–V curve shifted to negative potentials, of about −50 mV. The pronounced decrease in current amplitude, from >2,000 to <200 pA at −120 mV, is caused by two factors. First, a decrease of Cl^- from 200 to 20 mM is expected to decrease the amplitude of an anion current by up to a factor of 10. A 10-fold decrease indicates a large K_m value for Cl^-. Secondly, the shift in reversal potential by about −50 mV decreases the electrical driving force and thus the amplitude. A 10-fold $MgCl_2$ gradient between the bath and the pipet results in an equilibrium (Nernst) potential for Cl^- of $E_{Cl} = -52$ mV and for Mg^{2+} of $E_{Mg} = +23$ mV (for the calculation of equilibrium potentials activity coefficients were taken into account). It is obvious from Fig. 6 that the reversal potential comes very close to the Nernst potential of Cl^-, clearly indicating selectivity for anions. Summarizing, the data from Fig. 6 indicate that the negative currents were carried by strongly rectifying anion channels conducting Cl^- from the bath (cytoplasmic side) into the vacuole.

To corroborate further that anion channels were activated by Mg^{2+}, chloride in the bath solution was substituted by gluconate (Fig. 7), an anion impermeable for most anion channels. This was done in the following way. After anion currents had been activated with magnesium (see above), $MgCl_2$ was substituted by KCl in the bath (compare Fig. 2). Very low Ca^{2+} in the bath solution (0.2 μM) prevented activation of SV channels (compare Figs. 1 and 2), and only currents at negative potentials carried by Cl^- were recorded (Fig. 7A). The slower activation kinetics observed in Fig. 7A compared

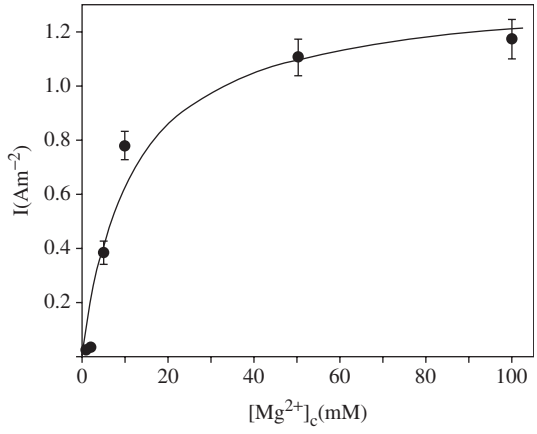

Fig. 3 Activation of negative currents by different Mg^{2+} concentrations in the bath (1, 2, 5, 10, 50 and 100 mM). A constant Cl^- concentration was kept by addition of KCl: 198, 196, 190, 180, 100 and 0 mM, respectively. pH was buffered at 7.2 with 15 mM HEPES. The composition of the pipet solution was: 100 mM KCl, 2 mM $CaCl_2$, 15 mM MES, pH 5.85. Average data of 4–7 vacuoles with SE as error bars. Data points were fitted assuming a simple binding isotherm resulting in $K_m = 11.4$ mM and $I_{Max} = 1.3$ A m^{-2}.

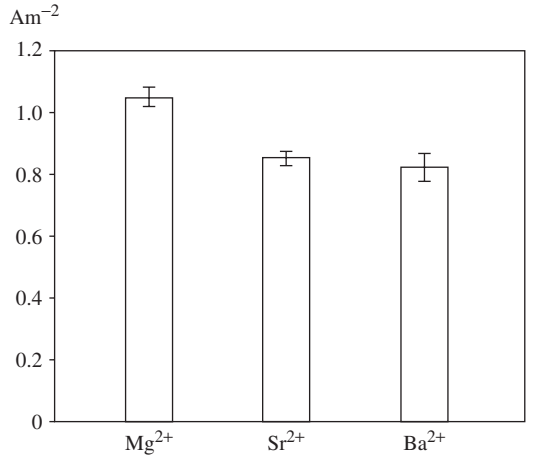

Fig. 4 Negative current densities in cytoplasm-out macropatches of *Conocephalum conicum* vacuoles at −100 mV command voltage obtained in bath solutions containing different divalent cations: 50 mM $MgCl_2$, 50 mM $SrCl_2$ or 50 mM $BaCl_2$. Bath solutions were buffered by 15 mM HEPES, pH 7.2. The pipet solution consisted of 100 mM KCl, 2 mM $CaCl_2$, 15 mM MES, pH 5.85. Average data of 4–12 vacuoles with SE as error bars.

with fast activation in Figs. 1 and 2 show two extremes of the natural variation of the biphasic activation kinetics that were observed during the recordings. Finally, KCl in the bath was substituted by K-gluconate by bath perfusion, which resulted in the complete disappearance of anion currents at negative potentials (Fig. 7B). I–V curves (Fig. 7C) show that gluconate blocked currents at negative potentials. This corroborates that these currents were carried by anion flux into the vacuole.

To investigate further the selectivity of the anion channel, different anions were perfused in the bath medium. Substituting for chloride, NO_3^-, SO_4^{2-} and malate^{2-} were tested. Fig. 8 compares current amplitudes at −100 mV command potential with different anions on the cytoplasmic side.

Addition of malate to media—instead of Mg^{2+}—was not sufficient to activate anion currents in *C. conicum*. This is different from *Kalanhoë daigremontiana* or *Arabidopsis thaliana* (Cerana et al. 1995, Pantoja and Smith 2002, Hafke et al. 2003), where malate was shown to activate anion currents.

Addition of 4,4′-diisothicyanatostilbene-2,2′-disulfonic acid (DIDS; 1 mM), anthracene-9-carboxylic acid (A9C; 2 mM) or ethacrinic acid (0.5 mM), known inhibitors of anion channels, caused substantial reduction of the anion currents (Fig. 9).

The calcium dependence of the anion currents was examined after their activation by a high magnesium content in the bath. This was done at two different vacuolar free Ca^{2+} concentrations and a variety of cytosolic free Ca^{2+} concentrations (Fig. 10). Concentrations of 5×10^{-5} and 2×10^{-3} M free Ca^{2+} in the pipet were taken to reflect the extreme values reported for vacuolar free Ca^{2+} (Felle 1988, Bethmann et al. 1995). In the bath, 0 ($\sim 10^{-9}$), 2×10^{-7}, 2×10^{-6}, 2×10^{-5}, 2×10^{-4} and 2×10^{-3} M free Ca^{2+} were perfused to test different cytosolic Ca^{2+} levels. Unlike SV currents, which nearly vanish after reducing free Ca^{2+} from 2×10^{-3} to 2×10^{-4} M Ca^{2+} in the bath (not shown), anion channels were active in the whole $[Ca^{2+}]_{cyt}$ range investigated (Fig. 10). The anion currents exhibited no significant dependence on vacuolar Ca^{2+}. Cytosolic free Ca^{2+} had a weak effect within the physiological range (up to 2×10^{-5} M). At higher free Ca^{2+}, a significant increase in anion currents was observed. This Ca^{2+} effect—in contrast to the Mg^{2+} activation—was entirely reversible.

In addition to ions, different metabolites, which in intact cells may influence ion channel activity, were examined. ATP (1 mM) did not affect anion currents. In guard cells of *Vicia faba*, malate currents were registered in the presence of a calcium-dependent protein kinase (CDPK; Pei et al. 1996). Here we tested the effect of protein kinase A (PKA) on vacuolar currents. PKA (0.4 U) had no significant effect on the vacuolar currents carried by anions. cAMP, known to activate animal ion channels (Hille 2001), did not affect anion currents in vacuoles of *C. conicum* when added at 10 μM concentration (not shown).

Discussion

The results shown above give the first characterization of negative currents in isolated vacuoles of the

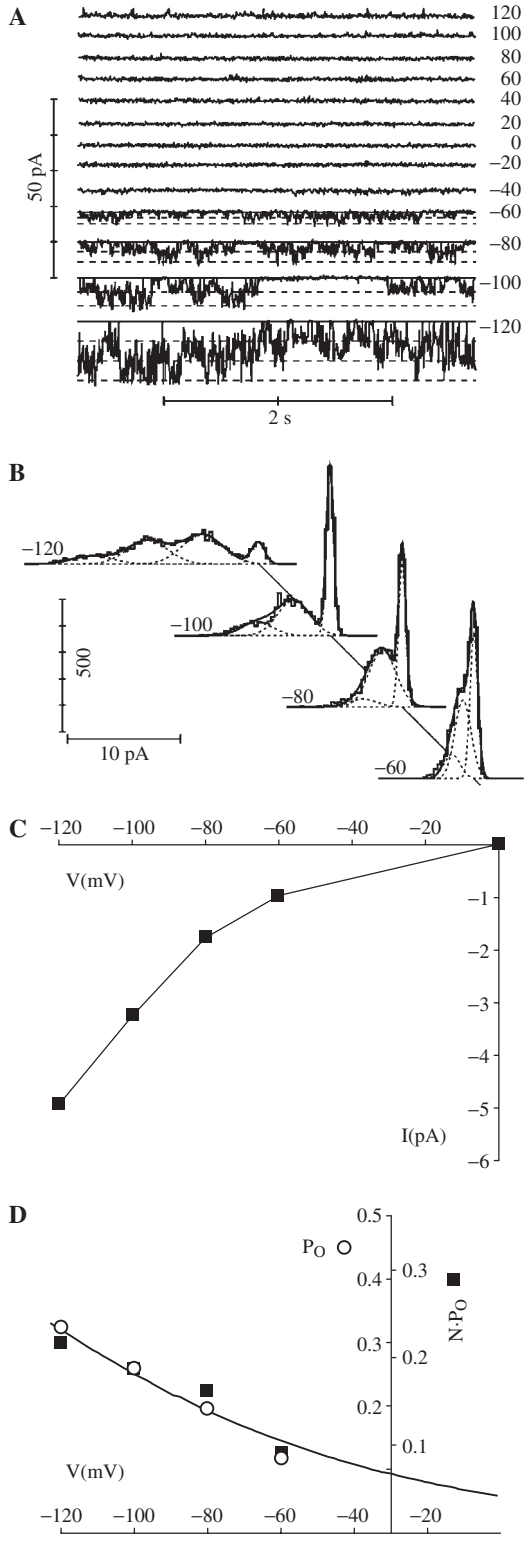

Fig. 5 (A) Single-channel traces obtained with cytoplasm-out patches excised from *Conocephalum* vacuoles. Pipet and bath solutions were as in Fig. 1B. Holding potentials (in mV) are given on the right side of the traces. Continuous horizontal lines indicate the closed state; dashed lines indicate one or more open channels. (B) All-points amplitude histograms at four different voltages indicate the number of sample points with a certain current amplitude (the vertical bar corresponds to 500 sample points). The data are from the single-channel traces shown in A. All-points amplitude histograms were fitted by a sum of equidistant Gaussian distributions (sum curve indicated by the solid line; individual Gaussian distributions indicated by the dashed lines). The closed state is indicated by the diagonal solid line connecting all four histograms. (C) Single-channel current–voltage relationship obtained from all-points amplitude histograms (distance between Gauss curves) in B. (D) Comparison of the voltage dependence of single channels (open circles) and of whole vacuole currents (filled rectangles). The open probability of single channels (P_O) was calculated from 31 s of recording by all-points amplitude histograms as shown in B. All-points amplitude histograms were fitted by a sum of equidistant Gaussian distributions assuming independent gating (i.e. a binomial distribution) resulting in $P_O = 0.324 \pm 0.004$ at -120 mV, $P_O = 0.259 \pm 0.0034$ at -100 mV, $P_O = 0.195 \pm 0.0052$ at -80 mV, and $P_O = 0.117 \pm 0.0026$ at -60 mV. To calculate the voltage dependence of whole-vacuole currents, the whole-vacuole I–V curve (from Fig. 1D) was corrected for the background I–V curve, which was calculated by linear regression from -20 to $+80$ mV (were no single-channel activity was observed). The resulting whole-vacuole current amplitudes were divided by single-channel current amplitudes (from B) at the corresponding voltage. In this way, the average number of open channels per μm^2 ($N \times P_O$) was calculated, which corresponds to the open probability (P_O) multiplied by the number of channels per μm^2 (N). The data for P_O (filled rectangles) and $N \times P_O$ (open circles) were fitted (solid line) simultaneously assuming a voltage dependence described by a Boltzmann function, yielding $z = -0.437 \pm 0.052$ for the gating charge and $V_0 = -164.3 \pm 8.2$ mV for the midpoint potential.

liverwort *C. conicum*. These currents are strongly inward rectifying (Fig. 1), i.e. are carried by anions flowing from the cytosol to the vacuole. A current analysis under asymmetrical $MgCl_2$ (Fig. 6) showed a reversal potential, which did not significantly deviate from the Nernst potential of Cl^-. This indicates that anion flux from the cytosol into the vacuole is carrying the observed inward currents. This was corroborated by (i) a substantial reduction of current amplitudes at reduced cytoplasmic Cl^- concentrations (Fig. 6); (ii) a disappearance of negative currents after substituting cytoplasmic Cl^- by gluconate (Fig. 7)—an impermeant anion; and (iii) an inhibition of negative currents by anion channel inhibitors (Fig. 9).

The inward currents were activated when the bath solution on the cytoplasmic side of the vacuole contained a high concentration of divalent cations while Ca^{2+} was low or virtually absent (with EGTA added). No comparable effect of divalent cations was observed on the luminal side of the vacuolar membrane. The probability of current activation significantly increased when isolation of vacuoles was carried out with 50 mM Mg^{2+} and no Ca^{2+} added. This points out that Mg^{2+} can replace Ca^{2+} in a putative regulation domain of the anion channel. Moreover, Mg^{2+} seems to bind quite firmly to the channel

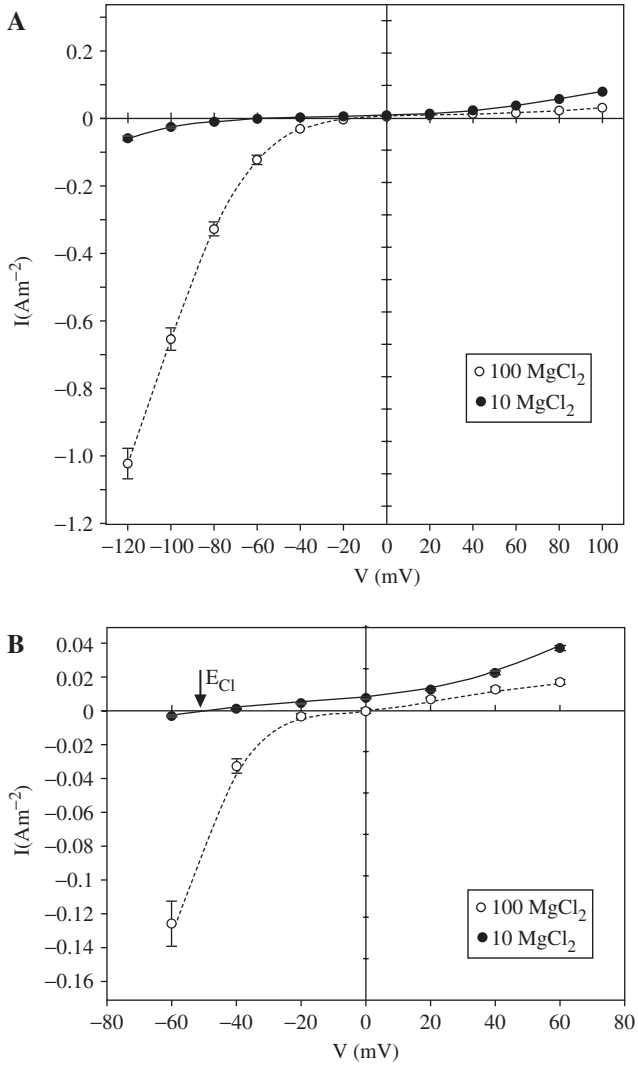

Fig. 6 Current–voltage characteristics obtained with a cytoplasm-out macropatch at symmetric (open circles) and asymmetric $MgCl_2$ (filled circles) concentrations. The pipet solution contained 100 mM $MgCl_2$, 15 mM MES, pH 5.85. The bath solution consisted of: 100 mM $MgCl_2$, 15 mM HEPES, pH 7.2 or 10 mM $MgCl_2$, 15 mM HEPES, pH 7.2. The voltage pulse protocol was the same as in Fig. 1B. (B) A fragment of the I–V curve shown in A in expanded scale. An arrow indicates the equilibrium (Nernst) potential for Cl^- (E_{Cl}) at asymmetric $MgCl_2$ concentrations. Average of five traces with SE as error bars.

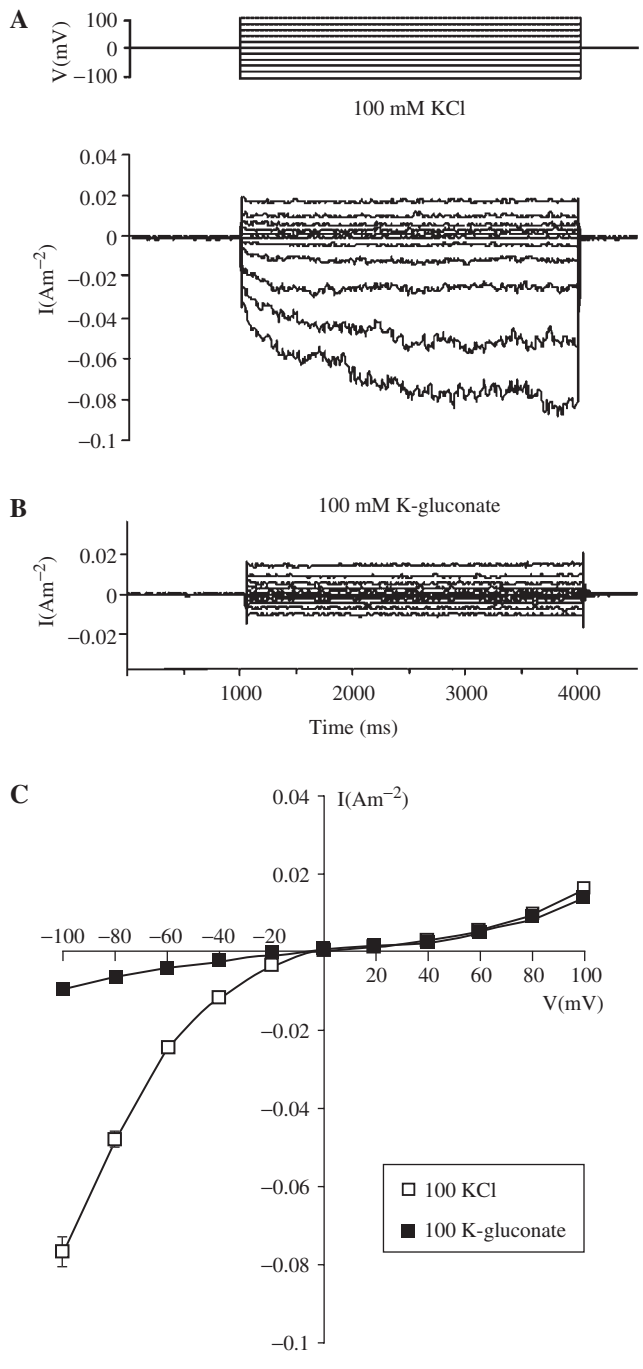

Fig. 7 Mg^{2+}-activated currents at negative potentials in *Conocephalum conicum* vacuoles are inhibited by replacing chloride by gluconate. Records were carried out in a cytoplasm-out macropatch. The pipet solution contained 100 mM KCl, 2 mM $CaCl_2$, 15 mM MES, pH 5.85. Bath solution in (A) consisted of: 100 mM KCl, 2×10^{-4} mM $CaCl_2$ (200 nM) buffered with 5 mM EGTA, 15 mM HEPES, pH 7.2, and in (B) 100 mM K-gluconate, 2×10^{-4} mM $CaCl_2$, 15 mM HEPES, pH 7.2. Voltage protocol is given at the top of the figure. (C) Current–voltage relationship before (open squares) and after replacing KCl in the bath with K-gluconate (filled squares). Average of four traces with SE as error bars.

protein because substituting magnesium with potassium and even addition of Ca^{2+} to the medium did not abolish the activation (at least within approximately 1 h). We demonstrate here that in addition to Mg^{2+}, other divalent cations Sr^{2+} and Ba^{2+} are able to activate the inward current, although, in contrast to magnesium, they seem to have rather minor physiological relevance. An alternative explanation of the mechanism that activates the anion channels may rely on interaction of divalent cations

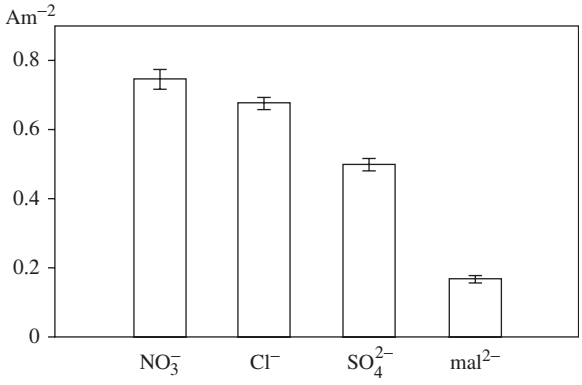

Fig. 8 Negative current densities in cytoplasm-out macropatches of *Conocephalum conicum* vacuoles at –100 mV command voltage obtained in bath solutions containing different anions. Bath solutions contained 50 mM $Mg(NO_3)_2$, 50 mM $MgCl_2$, 50 mM $MgSO_4$ or 50 mM K_2malate. pH 7.2 was buffered by 15 mM HEPES. The pipet solution consisted of 100 mM KCl, 2 mM $CaCl_2$, 15 mM MES, pH 5.85. Average data of 3–10 vacuoles ± SE.

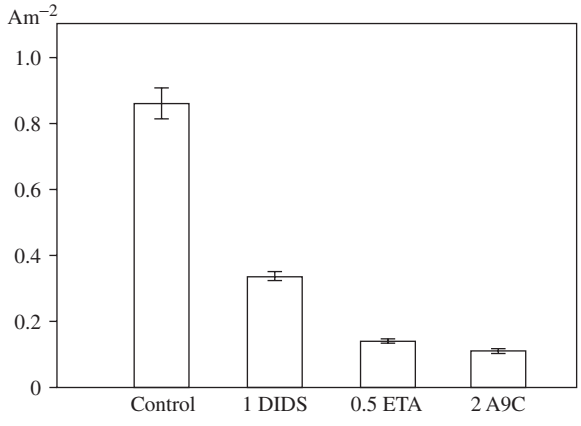

Fig. 9 Effects of anion channel inhibitors on negative currents at −100 mV in cytoplasm-out macropatches of *Conocephalum conicum* vacuoles. The bath solution contained 50 mM $MgCl_2$ (control) and $MgCl_2$ supplemented with 1 mM DIDS, 2 mM A9C or 0.5 mM ethacrinic acid (ETA). Bath solutions were buffered by 15 mM HEPES, pH 7.2. The pipet solution consisted of 100 mM KCl, 2 mM $CaCl_2$, 15 mM MES, pH 5.85. Average data of 4–6 vacuoles ± SE.

with a putative regulatory subunit of the channel. Many ion channels are regulated, and thus can be blocked by interacting subunits. The binding of these regulatory subunits usually is via hydrogen bonds and other non-covalent interactions. High concentrations of divalent cations may reduce the strength of non-covalent interactions by screening negative surface charges. A high concentration of divalent cations could 'wash off' an inhibitory subunit, which would explain why Mg^{2+} can be replaced by another ion once the channel is activated.

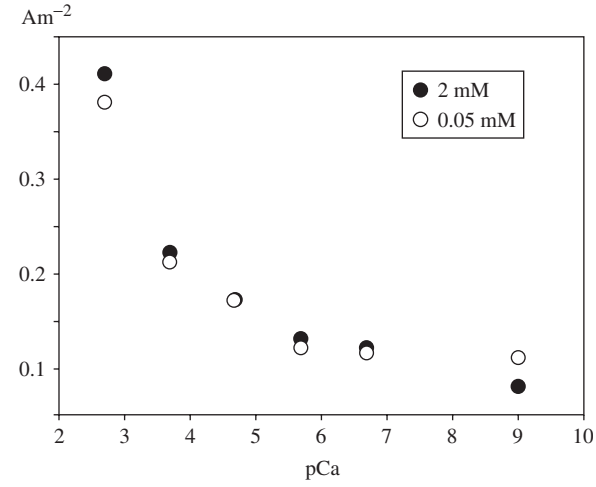

Fig. 10 Ca^{2+} dependence of vacuolar anion channels of *Conocephalum conicum*. With two different free Ca^{2+} concentrations in the pipet (vacuolar side), 0.05 or 2 mM, the free Ca^{2+} concentration in the bath (cytosolic side) was changed by bath perfusion: 0 ($\sim 10^{-9}$), 2×10^{-7}, 2×10^{-6}, 2×10^{-5}, 2×10^{-4} and 2×10^{-3} M. Average data of 4–8 traces ± SE.

Routinely, 50 mM cytosolic Mg^{2+} was used to activate negative vacuolar currents. While 5 mM Mg^{2+} was still sufficient to activate negative currents, no activation was observed at 2 mM Mg^{2+} (Fig. 3). The loss of additional regulatory factors during vacuole isolation may explain why 2 mM Mg^{2+}, which comes close to the cytosolic free Mg^{2+} concentration (Yazaki et al. 1988, Brüggemann et al. 1999), did not activate negative vacuolar currents. Formally, the Mg^{2+} effect can be described by a simple binding isotherm with a K_m of 11.4 mM (Fig. 3). A concentration of 11.4 mM Mg^{2+} is clearly above the estimated free cytosolic Mg^{2+} concentrations of 1.0–2.0 mM (Yazaki et al. 1988, Brüggemann et al. 1999), which means that, without taking into account as yet unknown additional regulatory factors, half-activation of the anion channels by Mg^{2+} is not possible. In already activated channels, increasing cytosolic free Ca^{2+} had little effect in the physiological range, while higher cytosolic free Ca^{2+} concentrations ($>10^{-5}$ M) significantly and reversibly increased anion currents (Fig. 10). Interestingly, in nearly all reports concerning anion currents in plant vacuoles, magnesium was present in the medium at millimolar concentration. Pei et al. (1996) reported that anion channels in *V. faba* guard cell vacuoles are activated by CDPK, acting in concert with Ca^{2+} (in the micromolar range) and ATP. To check whether phosphorylation—in addition to the activation by cytosolic Mg^{2+}—is a component of anion channel regulation in *Conocephalum* vacuoles, we applied PKA. The catalytic subunit of PKA, which is a pre-activated form of the kinase that requires

no cAMP, did not cause additional activation of the inward currents. The effect of cAMP alone on the inward currents was also negligible. Whether CDPK affects the inward currents in *Conocephalum* remains to be tested.

Single-channel activity recorded under conditions favoring the activation of negative vacuolar currents (Fig. 5; high $MgCl_2$ on the cytoplasmic side) demonstrated that the anion flux into the vacuole is carried by a channel of 32 pS unitary conductance at −100 mV. This anion channel showed a pronounced voltage-dependent gating with increasing open probability at more negative potentials. (Fig. 5D). This voltage dependence observed at the single-channel level together with the open channel rectification explains the inward rectification of the negative currents registered in whole-vacuole recordings (Fig. 1). Measurements with ion-selective microelectrodes and anion chromatography show that Cl^- is accumulated inside the vacuole of *Conocephalum* (Trebacz et al. 1994). The Cl^- activity is 7.4 mM in the cytoplasm and 43.7 mM in the vacuole (Trebacz et al. 1994). An opening of anion channels at vacuolar membrane potentials less negative than about –40 mV would allow an equilibration of this Cl^- concentration gradient. Due to its pronounced voltage-dependent gating (Figs. 1, 5, 6), the anion channel is only gated open at negative potentials large enough to drive Cl^- uptake into the vacuole against the existing concentration gradient.

The data from Figs. 1 and 2 clearly show that the negative currents characterized here are not carried by the SV channel. Time-dependent inward currents that were occasionally recorded from *V. faba* guard cell vacuoles, and which were attributed to the SV channel (Pei et al. 1999), may well have been anion currents comparable with those presented here.

Anion channels of the tonoplast of *Conocephalum* resemble VMAL channels of higher plants. Among the similarities are the multiphasic kinetics, the inward rectification (Allen and Sanders 1997) and the blockage by anion channel inhibitors (Pantoja et al. 1992). Anion channels of *Conocephalum* tonoplast once activated by Mg^{2+} (Sr^{2+}, Ba^{2+}) at low $[Ca^{2+}]_{cyt}$ remain active after replacing magnesium with potassium and increasing the $[Ca^{2+}]_{cyt}$. This resembles the behavior of VMAL channels in *Arabidopsis*, which are activated by mal^{2-} but continue operating after substitution of malate with chloride (Cerana et al. 1995). The anion channels in *Conocephalum* are weakly calcium dependent, like anion channels in higher plants (Iwasaki et al. 1992, Cerana et al. 1995, Cheffings et al. 1997). They are able to conduct malate although with lower permeability as compared with inorganic ions (Cl^-, NO_3^- and SO_4^{2-}). A cytoplasmic malate concentration as high as 50 mM does not activate the anion channels in *Conocephalum*. One may speculate that *C. conicum*, which is not a CAM plant, does not require high malate fluxes for stomata operation. Simply because it has no stomata, it did not develop special malate channels. In CAM plants, VMAL channels probably cooperate with other transporters of high homology to mammalian Na^+/bicarbonate symporters, found in *A. thaliana* (Emmerlich et al. 2003). It was shown that mutants lacking the tonoplast malate transporter gene, *At*tDT, exhibited virtually unchanged VMAL channel activity (Hurth et al. 2005). Whether such a co-transporter exists in *C. conicum* tonoplast in addition to the anion channels remains to be elucidated.

In contrast to higher plants and *C. conicum*, anion channels from *Chara* cells possess quite different characteristics. They conduct Cl^- preferentially from the vacuole to the cytosol in response to an increase of $[Ca^{2+}]_{cyt}$ (Tyerman and Findlay 1989, Berecki et al. 1999, Berecki et al. 2001). This allowed the postulation that these channels play a role in the tonoplast action potential (Kikuyama 1986 and Berecki et al. 1999). *Conocephalum*, like *Chara*, is an excitable plant. The channels described here are unlikely to be involved in APs. They conduct Cl^- in the opposite direction to that expected during the AP. However, as yet unknown factor(s) may shift the voltage dependence of the channel to more positive potentials, allowing anion efflux during the AP. The vacuolar anion channel is active at physiological $[Ca^{2+}]_{cyt}$, and an increase in the cytosolic free Ca^{2+} concentration to values occurring during APs (Trebacz et al. 1994) caused little further activation. This would, however, be expected if $[Ca^{2+}]_{cyt}$ was the signal to trigger the tonoplast AP.

The liverwort *Conocephalum*, belonging phylogenetically to the oldest terrestrial plants (*Embryophyta*), possesses vacuolar ion channels closely resembling those of higher plants and qualitatively different from *Characean* algae. This makes *Conocephalum* a suitable, simple model organism to study the principal properties of vacuolar ion channels in early land plants (*Embryophyta*).

Materials and Methods

Plant material

Conocephalum conicum L. collected in a natural habitat, i.e a forest near Zwierzyniec, Poland, was cultivated in a greenhouse. Plants grew in flat pots covered by transparent foil to reduce transpiration. Plants were watered every day with tap water. No additional illumination was applied during the growth period. The temperature was 23–25°C in the day and 16–18°C in the night. The humidity was kept between 70 and 90%.

Isolation of vacuoles

Vacuoles were obtained by a surgical method described by Trebacz and Schönknecht (2000). Fresh thalli were cut into squares of 5–8 mm. The fragments were then cut with a razor blade parallel to the main plane to expose 1–3 cell layers. The slices

were plasmolyzed in a medium containing (in mM) either: 100 KCl, 2 $CaCl_2$, 15 HEPES/Tris, pH 7.2, 500 sorbitol; or 50 $MgCl_2$, 15 HEPES/Tris, pH 7.2, 500 sorbitol. Applying 50 mM $MgCl_2$ during the isolation of vacuoles resulted in a reliable activation of the anion currents studied here and was therefore routinely used in experiments aimed at characterizing the anion channel. For experiments aimed at exploring the activation of anion currents, 100 mM KCl and 2 mM $CaCl_2$ was used for vacuole isolation. After 1 h incubation, tissue fragments were cut perpendicular to the main plane. Most of the protoplasts along the line of incision were destroyed, but a few emerged during a stepwise deplasmolysis from the cut open cell walls. Lowering the osmolality of the perfusion solution to 300 mosmol kg^{-1} by reducing the sorbitol content resulted in protoplasts rupturing and releasing vacuoles. Most of the vacuoles remained attached to the fragments of ruptured cells, which allowed identification of their origin.

Patch-clamp experiments

Patch-clamp measurements were performed as described by Trebacz and Schönknecht (2000) either in the 'whole-vacuole' configuration, analogous to the 'whole-cell' configuration, or with excised patches in the 'cytoplasmic-side-out' configuration with the cytoplasmic face of the vacuolar membrane facing the bath (Hamill et al. 1981). An Ag/AgCl reference electrode was connected to the bath solution via a 2% agar bridge filled with 100 mM KCl solution. The convention of current and voltage signs was according to Bertl et al. (1992). Ca^{2+} activities in the presence of different ions and buffers (EGTA, HEDTA, NTA) were calculated using the software WinMaxc v.1.78 written by Chris Patton, Stanford University. Liquid junction potentials were calculated using JPCalc written by P. H. Barry. GraFit (Robin J. Leatherbarrow) was used for non-linear regression analysis.

Acknowledgments

This work was supported by the State Committee for Scientific Research (Poland) 3P04C 05325 and the National Science Foundation (MCB-0212663, G.S.).

References

Allen, G.J. and Sanders, D. (1997) Vacuolar ion channels of higher plants. *Adv. Bot. Res.* 3914: 217–252.

Barkla, B.J. and Pantoja, O. (1996) Physiology of ion transport across the tonoplast of higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 159–184.

Berecki, G., Varga, Z., Van Iren, F. and Van Duijn, B. (1999) Anion channels in *Chara corallina* tonoplast membrane: calcium dependence and rectification. *J. Membr. Biol.* 172: 159–168.

Berecki, G., Eijken, M., Van Iren, F. and Van Duijn, B. (2001) Tonoplast anion channel activity modulation by pH in *Chara corallina*. *J. Membr. Biol.* 184: 131–141.

Bertl, A., Blumwald, E., Coronado, R., Eisenberg, R., Findlay, G.P., et al. (1992) Electrical measurements on endomembranes. *Science* 258: 873–874.

Bethmann, B., Thaler, M., Simonis, W. and Schönknecht, G. (1995) Electrochemical potential gradients of H^+, K^+, Ca^{2+}, and Cl^- across the tonoplast of the green alga *Eremosphaera viridis*. *Plant Physiol.* 109: 1317–1326.

Brüggemann, L.I., Pottosin, I.I. and Schönknecht, G. (1999) Cytoplasmic magnesium regulates the fast activating vacuolar cation channel. *J. Exp. Bot.* 50: 1547–1552.

Cerana, R., Giromini, L. and Colombo, R. (1995) Malate-regulated channels permeable to anions in vacuoles of *Arabidopsis thaliana*. *Aust. J. Plant Physiol.* 22: 115–121.

Cheffings, C.M., Pantoja, O., Ashcroft, F.M. and Smith, J.A.C. (1997) Malate transport and vacuolar ion channels in CAM plants. *J. Exp. Bot.* 48: 623–631.

Dziubinska, H., Paszewski, A., Trebacz, K. and Zawadzki, T. (1983) Electrical activity of the liverwort *Conocephalum conicum*. The all-or-nothing law, strength–duration relation, refractory periods and intracellular potentials. *Physiol. Plant.* 57: 279–284.

Emmerlich, V., Linka, N., Reinhold, T., Hurth, M., Traub, M., Martinoia, E. and Neuhaus, H.E. (2003) The plant homolog of human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. *Proc. Natl Acad. Sci. USA* 16: 11122–11126.

Felle, H. (1988) Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: interaction of Ca^{2+} and pH?. *Planta* 176: 248–255.

Hafke, J.B., Hafke, Y., Smith, J.A., Lüttge, U. and Thiel, G. (2003) Vacuolar malate uptake is mediated by an anion-selective inward rectifier. *Plant J.* 35: 116–128.

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

Hedrich, R., Barbier-Brygoo, H., Felle, H., Flügge, U.I., Lüttge, U., et al. (1988) General mechanisms for solute transport across the tonoplast of plant vacuoles: a patch-clamp survey of ion channels and proton pumps. *Bot. Acta* 101: 7–13.

Hedrich, R. and Neher, E. (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. *Nature* 329: 833–835.

Hille, B. (2001) Ion Channels of Excitable Membranes. Sinauer Associates, Sunderland, MA.

Hurth, M.A., Suh, S.J., Kretzschmar, T., Geis, T., Bregante, M., Gambale, F., Martinoia, E. and Neuhaus, H.E. (2005) Impaired pH homeostasis in *Arabidopsis* lacking the vacuolar dicarboxylate transporter and analysis of carboxylic acid transport across the tonoplast. *Plant Physiol.* 137: 901–910.

Iwasaki, I., Arata, H., Kijima, H. and Nishimura, M. (1992) Two types of channels involved in the malate ion transport across the tonoplast of a Crassulacean acid metabolism plant. *Plant Physiol.* 98: 1494–1497.

Kikuyama, M. (1986) Tonoplast action potential of *Characeae*. *Plant Cell Physiol.* 27: 1461–1468.

Krol, E., Dziubinska, H. and Trebacz, K. (2003) Low-temperature induced transmembrane potential changes in the liverwort *Conocephalum conicum*. *Plant Cell Physiol.* 44: 527–533.

Krol, E. and Trebacz, K. (2000) Ways of ion channel gating in plant cells. *Ann. Bot.* 86: 449–469.

Pantoja, O., Gelli, A. and Blumwald, E. (1992) Characterization of malate and K^+ channels under physiological conditions. *Plant Physiol.* 100: 1137–1141.

Pantoja, O. and Smith, J.A.C. (2002) Sensitivity of the plant vacuolar malate channel to pH, Ca^{2+} and anion channel blockers. *J. Membr. Biol.* 186: 31–42.

Paszewski, A., Dziubinska, H., Trebacz, K. and Zawadzki, T. (1982) Electrical activity of the liverwort *Conocephalum conicum*: method of investigation and general characteristics of excitation. *Physiol. Plant.* 54: 83–87.

Pei, Z.M., Ward, J.M., Harper, J.F. and Schroeder, J.I. (1996) A novel chloride channel in *Vicia faba* guard cell vacuoles activated by the serine/threonine kinase, CDPK. *EMBO J.* 15: 6564–6574.

Pei, Z.M, Ward, J.M. and Schroeder, J.I. (1999) Magnesium sensitizes slow vacuolar channels to physiological cytosolic calcium and inhibits fast vacuolar channels in fava bean guard cell vacuoles. *Plant Physiol.* 121: 977–986.

Ping, Z., Yabe, I. and Muto, S. (1992) Identification of K^+, Cl^-, and Ca^{2+} channels in the vacuolar membrane of tobacco cell suspension cultures. *Protoplasma* 171: 7–18.

Plant, P.J., Gelli, A. and Blumwald, E. (1994) Vacuolar chloride regulation of an anion-selective tonoplast channel. *J. Membr. Biol.* 140: 1–12.

Pottosin, I.I. and Schönknecht, G. (2007) Vacuolar calcium channels. *J. Exp. Bot.* 58: 1559–1569.

Qiu, Y.L., Cho, Y.R., Cox, J.C. and Palmer, J.D. (1998) The gain of three mitochondrial introns identifies liverworts as the earliest land plants. *Nature* 394: 671–674.

Trebacz, K. and Schönknecht, G. (2000) Simple method to isolate vacuoles and protoplasts for patch-clamp experiments. *Protoplasma* 213: 39–45.

Trebacz, K. and Schönknecht, G. (2001) Ion channels in the tonoplast of the liverwort *Conocephalum conicum*. *In* Plant Membrane Biology. The 12th International Workshop. Madison. Program and Abstracts.

Trebacz, K., Simonis, W. and Schönknecht, G. (1994) Cytoplasmic Ca^{2+}, K^+, Cl^-, and NO_3^- activities in the liverwort *Conocephalum conicum* L. at rest and during action potentials. *Plant Physiol.* 106: 1073–1084.

Trębacz, K., Stolarz, M., Dziubińska, H. and Zawadzki, T. (1997) Electrical control of plant development. *In* Travelling Shot on Plant Development. Edited by Greppin, H., Pennel, C. and Simon, P. pp. 163–179. University of Geneva, Geneva.

Trebacz, K. and Zawadzki, T. (1985) Light-triggered action potentials in the liverwort *Conocephalum conicum*. *Physiol. Plant.* 64: 482–486.

Tyerman, S.D. and Findlay, G.P. (1989) Current–voltage curves of single Cl^- channels which coexist with two types of K^+ channel in the tonoplast of *Chara corallina*. *J. Exp. Bot.* 40: 105–117.

Ward, J.M. and Schroeder, J.I. (1994) Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell* 6: 669–683.

Yazaki, Y., Asukagawa, N., Ishikawa, Y., Ohta, E. and Kata, M. (1988) Estimation of cytoplasmic free Mg^{2+} levels and phosphorylation potentials in mung bean root tips by in vivo ^{31}P NMR spectroscopy. *Plant Cell Physiol.* 29: 919–924.

(Received September 19, 2007; Accepted October 23, 2007)