Fast-activating cation channel in barley mesophyll vacuoles. Inhibition by calcium

Lioubov I. Tikhonova, Igor I. Pottosin, Karl-Josef Dietz and Gerald Schönknecht
Lehrstuhl Botanik I, Universität Würzburg, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany

Summary
In contrast to the vacuolar ion channels which are gated open by an increase of cytosolic Ca \textsuperscript{2+} the vacuolar ion currents at resting cytosolic Ca \textsuperscript{2+} are poorly explored. Therefore, this study was performed to investigate the properties of the so-called fast-activating vacuolar (FV) current which dominates the electrical characteristics of the tonoplast at physiological free Ca \textsuperscript{2+} concentrations. Patch-clamp measurements were performed on whole barley (Hordeum vulgare) mesophyll vacuoles and on excised tonoplast patches. Single ion channels were identified, which, based on their selectivity, activation kinetics, Ca \textsuperscript{2+} - and voltage-dependence, carry the whole-vacuole FV current. Reversal potential determinations indicated a K \textsuperscript{+} over Cl\textsuperscript{-} permeability ratio of about 30. Both inward and outward whole-vacuole currents as well as the activity of single FV channels were inhibited by an increase of cytosolic Ca \textsuperscript{2+}, with a K\textsubscript{d} \approx 6 \mu M. At physiological vacuolar Ca \textsuperscript{2+} activities, the FV channel is an outward-rectifying potassium channel. The FV channel was activated in less than a few milliseconds both by negative and positive tonoplast potentials, having a minimal activity that is 40 mV negative of the K \textsuperscript{+} equilibrium potential. It is proposed that transport of K \textsuperscript{+} through this cation channel controls the electrical potential difference across the tonoplast.

Introduction
There are two major ion currents in plant vacuoles, the so-called slow-activating (SV) and fast-activating (FV) vacuolar currents. The SV channel is activated by cytosolic free Ca \textsuperscript{2+} concentrations in the micromolar range and by positive tonoplast potentials relative to the cytoplasm (Hedrich and Neher, 1987; Reifarth et al., 1994; Schulz-Lessdorf and Hedrich, 1995). The FV current is an outward-rectifying potassium channel. The FV channel was activated in less than a few milliseconds both by negative and positive potential steps, having a minimal activity that is 40 mV negative of the K \textsuperscript{+} equilibrium potential. It is proposed that transport of K \textsuperscript{+} through this cation channel controls the electrical potential difference across the tonoplast.

Results
Dependence of whole vacuolar currents on cytosolic calcium
Whole-vacuole currents were recorded with 2 mM Ca \textsuperscript{2+} inside the vacuole, and the free Ca \textsuperscript{2+} concentration at the cytosolic side was changed from < 1 nM (referred to as 0 Ca \textsuperscript{2+}) to 0.5 mM. The instantaneous component of the whole-vacuole current under these conditions displayed an outward rectification as shown in Figure 1(a). There was large current flow at positive potentials (relative to the cytoplasm), i.e. efflux of cations from the cytoplasm into the vacuole or influx of anions from the vacuole into the cytoplasm. Increasing the cytosolic Ca \textsuperscript{2+} concentration from 0 to 5 \mu M resulted in a twofold decrease of the outward rectifying instantaneous currents. It should be noted that the relative small instantaneous inward currents at 2 mM vacuolar Ca \textsuperscript{2+} also decreased at increasing cytosolic Ca \textsuperscript{2+} (Figure 1a). A further increase of cytosolic Ca \textsuperscript{2+} to 50 \mu M resulted in a further decrease of the amplitude of the instantaneous currents, and in the activation of a slowly-activating vacuolar current at positive potentials above +50 mV. The amplitudes of the instantaneous currents were plotted as a function of the applied membrane voltage at different levels of cytosolic Ca \textsuperscript{2+} (Figure 1b). The resulting current–voltage (I / V) relationships show that the amplitude of the current decreased proportionally over the whole voltage range with increasing cytosolic...
Ca²⁺ activities. The relative current amplitudes at +100 mV were plotted as a function of the cytosolic Ca²⁺ activity in Figure 1(c). The blockage of the instantaneous currents by cytosolic Ca²⁺ could be described by a Ca²⁺ binding with an affinity of 6 μM and a Hill coefficient of 0.7. At the end of each experiment, the 0 Ca²⁺ solution was returned to the measuring chamber. Even after more than one hour’s perfusion, only a small rundown of the fast vacuole currents was detected, never exceeding 20% of the initial currents (Figure 1a).

Outward rectification of the fast vacuolar current (Figure 1a and b) was observed with 2 mM Ca²⁺ in the vacuole and 0 Ca²⁺ in cytoplasm, under otherwise symmetrical ion conditions (100 mM KCl at the both membrane sides). As can be seen in Figure 2(a), with 0 Ca²⁺ on both sides of the tonoplast, the instantaneous currents had comparable amplitudes at positive and negative potentials. An increase of cytosolic Ca²⁺ decreased both the outward and the inward instantaneous currents. The I/V relationships of the instantaneous currents at different levels of cytosolic Ca²⁺ activity are displayed in Figure 2(b). The relative amplitudes of the fast activating current at +100 and −100 mV were plotted as a function of cytosolic free Ca²⁺ (Figure 2c). Both sets of data can be fitted by the same curve, with a Kₐ of 6 μM and a Hill coefficient of 0.7.

We attempted to resolve the activation–deactivation kinetics of the fast vacuolar currents, but it was obviously faster than the time course which can be resolved by our measuring set-up. The half-rise times were less than 1 ms (data from 10 separate vacuoles).

Dependence of the whole-vacuole current on vacuolar calcium

To analyse the effect of vacuolar Ca²⁺ on the FV current in more detail, three intermediate vacuolar free Ca²⁺ concentrations, namely 5 μM, 18 μM and 50 μM, were examined. With 0 Ca²⁺ on the cytosolic side, the free Ca²⁺ concentration in the pipette filling solution was fixed to different levels, and 31 vacuoles were examined. The resulting I/V relationships of the instantaneous whole-vacuole current are shown in Figure 3(a). It can be seen that for up to 50 μM Ca²⁺ inside the vacuole, the outward current (at positive potentials) was unaffected, while with 2 mM Ca²⁺ in the vacuole, the outward current was decreased by 37% compared with 0 Ca²⁺ at +100 mV. At the same time, even at 5 μM Ca²⁺, there is a significant decrease of the inward current (at negative potentials). To describe the effect of vacuolar Ca²⁺ on the inward current quantitatively, the relative current at −100 mV was plotted against the free Ca²⁺ concentration inside the vacuole (Figure 3b). The data points can be fitted assuming Ca²⁺ binding at the lumenal side of the membrane, with a Kₐ for Ca²⁺ of 14 μM and a Hill coefficient of 0.5. Despite this relative high Ca²⁺ binding...
affinity from the vacuolar side, a small inward current remained even at millimolar Ca$^{2+}$. For instance, at 2 mM vacuolar Ca$^{2+}$, the inward current at -100 mV had an amplitude of about 8% compared with 0 Ca$^{2+}$ (Figure 3b). This was significantly larger than the non-specific leak current, which was about -0.024 A m$^{-2}$ at -100 mV as an average, or 3% of the maximum current. The leak current was estimated by linearly extrapolating the current measured with 2 mM Ca$^{2+}$ inside the vacuole (Figure 3a) from the high-resistance region around -40 mV to more negative potentials.

**Ion selectivity of the fast vacuolar current**

Starting with symmetrical 100 mM KCl, we decreased the KCl concentration in the bath to 10 mM to determine the ion selectivity of the fast vacuolar current. The resulting I/V curves with 2 mM Ca$^{2+}$ inside the vacuole (Figure 4a) and the I/V curves with 0 Ca$^{2+}$ at both membrane sides (Figure 4b) displayed the same reversal potentials. The 10-fold decrease of the KCl concentration shifted the reversal potential by 50 mV to more positive voltages. The reversal potential of about +50 mV came close to the equilibrium potential for K$^+$ of +56 mV (after correcting for ion activities) suggesting a high K$^+$/Cl$^-$ permeability ratio. This result indicates that the fast vacuolar current is carried by monovalent cations, although a small contribution of Cl$^-$ cannot be ruled out. Accordingly, the 10-fold decrease of the KCl concentration in the bath resulted in a decrease of the outward current, i.e. the efflux of K$^+$ from the bath into the vacuole. A threefold increase of the KCl concentration in the bath from 100 mM to 300 mM resulted in an increase of the outward current by about 35% at +100 mV, as shown in Figure 4. Thus, the fast vacuolar current tended to saturate at some hundred millimoles of K$^+$. The slope conductance determined from the linear portion of I/V relationships for the outward whole vacuolar current was 4.2 ± 1.0, 12.6 ± 0.6, 18.6 ± 0.9, 23.3 ± 0.2, and 22.0 ± 0.8 S m$^{-2}$ for 10, 30, 100, 300 and 1000 mM cytosolic K$^+$, respectively (n = 6, 2, 6, 3 and 4 vacuoles). These data points were fitted assuming a Michaelis-Menten type of

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**Figure 2. Effect of increasing cytosolic Ca$^{2+}$ concentration on fast-activating vacuolar currents at zero vacuolar Ca$^{2+}$.**

(a) Whole-vacuole currents (I in nA) at different levels of cytosolic Ca$^{2+}$ (Ca$_{cy}$). All experiments were performed in symmetrical 100 mM KCl and 0 Ca inside the vacuole. From a holding potential of 0 mV, the electrical potential across the tonoplast was first stepped to -100 mV for 0.5 s to deactivate slow vacuolar channels, then the voltage was changed for 4.9 s in 20 mV steps from -100 to +100 mV. By bath perfusion, the external solution was changed from 0 Ca$_{cy}$ (○) to 5 μM (△) and 50 μM (△) free Ca$^{2+}$. The whole-vacuole currents at 50 μM Ca$_{cy}$ are displayed on an expanded time scale. This shows that after the deactivation of SV channels by a -100 mV pulse, the small instantaneous currents can unambiguously be separated from the slowly activating SV current. In this experiment, a vacuole with a capacitance of 17 pF was used.

(b) The amplitude of the instantaneous vacuolar currents shown in (a) as a function of membrane voltage (V in mV) for three different cytosolic Ca$^{2+}$ concentration. Different symbols reflect different Ca$^{2+}$ concentrations as in (a). The current amplitudes were normalized to the vacuolar membrane surface and expressed in A m$^{-2}$. Solid lines are low-order polynomials fitted to the data. (c) The dependence of fast vacuolar currents on the cytosolic Ca$^{2+}$ concentration. Whole-vacuole currents were measured at -100 mV (○) and at +100 mV (△) and normalized to the current at 0 Ca$_{cy}$. Each data point represents the mean ± SD of three separate vacuoles. The data points of both data sets were fitted simultaneously by a Hill equation, yielding a Hill coefficient of 0.68 ± 0.02 and log(Kd) = -5.20 ± 0.02, i.e. Kd = 6.3 μM.
binding, resulting in a Michaelis constant for $K^+$ of $K_m = 33.5 \pm 9.3$ mM and maximal conductance of $23.8 \pm 1.5$ S m$^{-2}$. The slope conductance determined from the linear portion of the $I/V$ relationships of the inward whole-vacuole current was $33.2 \pm 1.9$ and $40.5 \pm 6.9$ S m$^{-2}$ for 100 and 1000 mM vacuolar K$^+$, respectively ($n = 6$ and 4 vacuoles). The slope conductance was about twofold higher for the inward current compared with the outward current. An increase from 100 to 1000 mM on both sides of the vacuolar membrane resulted in a small increase (20 to 30%) of the measured currents, indicating comparable K$^+$ affinities on both membrane sides (data not shown).

Under symmetrical ion conditions with 0 Ca$^{2+}$ and 100 mM KCl on both sides of the membrane, the $I/V$ relationship of the whole-vacuole current was not linear but slightly asymmetrical. It displayed a characteristic high resistance region at about $-40$ mV (Figures 2b, 3a and 4). This observation was consistently reproduced with more than 200 vacuoles. An increase of cytosolic K$^+$ to 300 mM
0 Ca\textsubscript{vac} mV
50 \mu M Ca\textsubscript{vac}

Figure 5. Fast-activating currents in excised vacuolar side-out membrane patches.

Instantaneous voltage-dependent activation and blockage by vacuolar calcium of current fluctuations in excised patches. From a holding potential of -30 mV, steps of 130 ms duration were applied to voltages as indicated. The records were filtered at 2.5 kHz and sampled at 10 kHz. The currents on the left-hand side were measured at symmetrical 100 mM KCl, 0 Ca\textsuperscript{2+}. On the right-hand side, the vacuolar free Ca\textsuperscript{2+} was 50 \mu M. No leak subtraction was applied, the seal resistance was about 13 GΩ.

If the free Ca\textsuperscript{2+} concentration to 50 \mu M, by bath perfusion with a vacuolar side-out patch, caused an outward rectification of the fast-activating currents in isolated tonoplast patches (Figure 5). The flickering current at negative voltages was greatly reduced, almost to the leak current level, whereas current fluctuations at positive voltages were not affected.

Due to the high activity, the fast-activating currents in isolated membrane patches could often hardly be resolved at the single-channel level. To resolve single-channel currents at large negative and at positive potentials, recordings were performed with small patch pipettes (diameter < 1 µm) to reduce the membrane surface of the patch. From long recordings, we selected short stretches with lower channel activity where the single-channel current amplitudes could be resolved for further analysis. Figure 6(a) shows single-channel activity recorded in this way from an cytoplasmic side-out patch bathed in symmetrical 100 mM KCl, 0 Ca\textsuperscript{2+}. The first indication that the ion channel shown in Figure 6(a) shares the properties of the fast vacuolar current comes from its voltage dependence. At -40 mV, only rare channel openings were observed, whereas at positive and more negative voltages, the activity increased. Single-channel currents displayed a slight outward rectification under symmetrical ion conditions, with a slope conductance of 25.1 ± 0.8 pS and 17.5 ± 0.4 pS (n = 14 patches) at negative and positive potentials, respectively (Figure 6b, open circles). This partly explains the different slope conductance of the whole-vacuole current at positive and large negative potentials (Figure 4) but not the existence of the high-resistance region. The latter is obviously caused by the voltage-dependence of the single-channel open probability (P\textsubscript{o}) which was analysed as shown in Figure 6(c). All-points amplitude histograms of recordings lasting some seconds were fitted by a sum of Gaussian distributions. Assuming independent gating of FV channels, the equations describing a binomial distribution were introduced into the fit routine which reduced the number of fit parameters and yielded P\textsubscript{o} as a fit parameter. With three active channels (in some patches only two), all histograms were perfectly described assuming a binomial distribution (Figure 6c), and the single-channel current amplitudes resulting from these fits were the same as those measured directly from the single channel traces. P\textsubscript{o} had a minimum at -40 mV. This was compared with the voltage-dependence of the average number of open channels in whole vacuoles (N.P\textsubscript{o}) which was calculated by dividing whole-vacuole currents by single-channel currents. P\textsubscript{o} and N.P\textsubscript{o} displayed exactly the same voltage-dependence (Figure 6d). This voltage-dependence was mathematically described assuming three states, one closed and two open, occupied at large negative and positive potentials (Figure 6d, solid line). The equilibrium between each open state and the closed one was...
described by a Boltzmann distribution. This analysis yielded $N = 1.75$ for the mean number of channels per $\mu\text{m}^2$.

To analyse the ion selectivity of the fast-activating vacuolar channels, we changed the bath solution with cytosolic side-out patches from 100 to 10 mM KCl, and repeated single-channel measurements under this 10/100 mM KCl gradient. The resulting $I / V$ relationship is shown in Figure 6(b) (filled circles). The reversal potential of the single-channel current under a 10/100 mM KCl gradient was +50 mV which came close to the Nernst potential for $K^+$. This was in perfect agreement with reversal potential determinations in the whole-vacuole mode (Figure 4).

Using the Goldmann–Hodgkin–Katz equation, a relative $K^+/Cl^-$ permeability of about 30 can be estimated from the reversal potential. Replacement of 100 mM KCl in the bath
by 10 mM KCl caused a shift of the voltage-dependence of the fast-activating vacuolar channel in a positive direction (n = 3 patches). Now at -30 mV, the activity was comparable to that observed at -80 mV in symmetrical 100 mM KCI, and at +20 mV only rare openings of single channels were observed (data not shown). This shift of the voltage-dependence upon changing the cytosolic K+ concentration was similar to that observed for the whole-vacuole current (Figure 4). The application of a 10/100 mM KCI gradient caused a rectification of the single-channel I/V relationship, the single-channel conductance at positive potentials decreased implying a smaller K+ efflux from the cytosolic side, whereas at negative potentials, the I/V relationship asymptotically approaches the I/V curve for symmetrical conditions (Figure 6b). An opposite rectification was observed with a vacuolar side-out patch when 100 mM KCl were replaced by 10 mM KCl (Figure 6b, asterisks). With this 100/10 mM KCl gradient, the reversal potential of the single channel current was about -50 mV. This implies that the channel opening at negative and positive potentials, had the same K+/Cl- selectivity.

The single-channel conductance in 10 mM KCl was estimated from measurements with 10 mM KCI in the bath. Under these conditions, the outward current of cytosolic side-out patches and the inward currents of vacuolar side-out patches mainly reflected the K+ flow from the membrane side facing 10 mM KCI. Combining these two data sets, the single-channel conductance at 10 mM KCl can be estimated by linear regression (Figure 6b, dashed line). This results in a slope conductance of 4.4 ± 0.3 pS. Compared with a value of 20.5 pS in symmetrical 100 mM KCl as an average for positive and negative potentials, this implies an increase of the single-channel conductance by a factor of 4.7 with a tenfold increase of the K+ concentration. This result is in good agreement with the increase of the outward whole-vacuole current, from 4.2 to 16.6 S m⁻², for the same change of cytosolic K+ (Figure 4).

**Cytosolic calcium inhibits the fast vacuolar channel**

The effect of cytosolic Ca²⁺ on single-channel currents was measured in cytosolic side-out patches. A control measurement (Figure 7a, top) shows the single-channel activity at 0 Ca²⁺ at both membrane sides. The bath was consecutively replaced by solutions containing 5 and 50 µM free Ca²⁺ which resulted in a progressive decrease of single-channel activity (Figure 7a, lower traces). To give a quantitative description of the effect of cytosolic Ca²⁺, we estimated the time-averaged channel-mediated current, which is the total time-averaged ion current across the membrane patch minus the leak current. The leak current is given by the level when all channels are closed. The data for six separate patches, normalized to the control at zero cytosolic Ca²⁺ are presented in Figure 7(b). It can be seen that an increase of the cytosolic free Ca²⁺ concentration caused a decrease of inward and outward currents by approximately 50% at 5 µM Ca⁺ and by 75-80% at 50 µM Ca²⁺. This is in good agreement with our whole-vacuole measurements where an increase of free cytosolic Ca²⁺ concentration caused a decrease of both outward and

![Figure 7](image-url)
Vacuolar calcium reduces the single-channel current

To analyse the effect of vacuolar Ca\(^{2+}\), we performed measurements with vacuolar side-out patches. Again, control measurements were done at 0 Ca\(^{2+}\) at both membrane sides. The single-channel traces and resulting I/V relationships are shown in Figure 8 (open circles). The replacement of the bath solution by a solution with 5 \(\mu\)M free Ca\(^{2+}\) caused a decrease of single-channel current amplitudes at negative potentials (Figure 8b, open squares). At -100 mV, a decrease of 40% was measured. The whole-vacuole current was reduced by 35% under 5 \(\mu\)M vacuolar Ca\(^{2+}\) (Figure 3). A bath solution with 50 \(\mu\)M free Ca\(^{2+}\) caused a large decrease of single-channel currents at negative potentials, and at the same time the very rapid channel gating seemed to be slowed down, as shown in Figure 8 (filled squares). At -100 mV, a decrease of 78% was measured. The whole-vacuole current was reduced by 65% under these conditions (Figure 3). The single-channel currents at positive potentials were unaffected at up to 50 \(\mu\)M Ca\(^{2+}\) at the vacuolar side (Figure 8). A further increase of vacuolar Ca\(^{2+}\) to 2 mM resulted in completely 'silent' patches at negative voltages – only the leak current was recorded (data not shown). This was not surprising, as the whole-vacuole current under these conditions was decreased to about 5% of the control. At positive voltages, single-channel activity was slightly lower and single-channel currents decreased by about 20% to 14.3 ± 0.3 pS (n = 6) at 2 mM vacuolar Ca\(^{2+}\) (data not shown). This explains the observed 37% decrease of the whole-vacuole current at positive potentials with 2 mM Ca\(^{2+}\) in the vacuole compared to 0 Ca\(^{2+}\) (Figure 3a).

In about 10% of all patches, the activity of another ion channel was observed, as depicted in Figure 9. This ion channel displayed slow kinetics, i.e., prolonged openings and closures, and had a single-channel conductance of about 30 pS (in 100 mM KCI). This channel was almost voltage-independent. Due to the infrequent appearance and short periods of channel activity, we were not able to examine this channel in more detail.

Discussion

Using the patch-clamp technique, we characterized the fast-activating currents in barley mesophyll vacuoles at
the whole-vacuole and the single-channel level. These instantaneously activating currents dominated at low cytosolic Ca\(^{2+}\). In accordance with Hedrich and Neher (1987) who were the first to observe these currents in sugarbeet (Beta vulgaris) taproot vacuoles, we will call them FV currents (fast vacuolar) and the corresponding ion channel the FV channel.

**Identification of the fast vacuolar ion channel (FV channel)**

Both whole-vacuole and single-channel measurements showed a relative high selectivity for K\(^+\) over Cl\(^-\), \(P_K / P_C = 30\), as shown in Figures 4 and 6. Although the FV channel binds Ca\(^{2+}\) with a high affinity from both membrane sides, there was no indication of a significant Ca\(^{2+}\) permeability. Even with large Ca\(^{2+}\) gradients, we never observed a shift of the reversal potential of whole-vacuole currents (compare Figures 1 and 2; Figure 4) or single-channel currents (Figures 7 and 8). The dependence of whole-vacuole and single-channel currents on K\(^+\) concentration yields a half-saturation at about 35 mM for inward and outward currents, indicating comparable binding affinities for K\(^+\) on both membrane sides.

The voltage-dependence of whole-vacuole and single-channel currents was exactly the same (Figure 6d). From comparison of the single-channel open probability (\(P_O\)) and the average number of open channels in whole vacuoles (\(N_P\)), a density of nearly two FV channel per \(\mu m^2\) of vacuolar membrane was calculated. The characteristic high-resistance region at about 40 mV negative of the K\(^+\) equilibrium potential (Figure 4b) was explained by a minimum single-channel activity at the corresponding voltage (Figures 5 and 6). The FV channel appears to have at least one closed state (about 40 mV negative of \(E_K\)) and two open states occupied at more positive and more negative potentials. At negative potentials, channel gating was significantly faster (shorter open times) compared with positive voltages (Figures 5 to 8). The two open states of the FV channel differed kinetically and by their unitary conductance. At the same time, they had equal K\(^+\)/Cl\(^-\) selectivities (Figure 5b).

Vacuolar Ca\(^{2+}\) blocked the FV channel in a voltage-dependent manner, i.e. an increase of the vacuolar Ca\(^{2+}\) concentration caused a larger decrease of the inward FV current compared with the outward FV current (Figure 3a and 8). The decrease of the inward FV current by vacuolar Ca\(^{2+}\) was described by a binding isotherm with a \(K_d\) of about 14 \(\mu M\) (Figure 3b). At vacuolar Ca\(^{2+}\) activities in the millimolar range, a decrease of the outward FV current was observed (Figure 3).

Cytosolic Ca\(^{2+}\) blocked the FV channel in a voltage-independent manner (Figure 2 and 7), indicating a Ca\(^{2+}\)-binding site that is located close to the tonoplast surface. The decrease of the FV current was described by a binding isotherm with a \(K_d\) of about 6 \(\mu M\) for cytosolic free Ca\(^{2+}\) (Figure 2c). Inward and outward currents both at the whole-vacuole and at the single-channel level displayed the same sensitivity towards cytosolic Ca\(^{2+}\), corroborating the view that the currents at positive and negative potentials are due to the same type of ion channel, the FV channel. In more than hundred tonoplast patches with 0 Ca\(^{2+}\) inside the vacuole, we never observed FV channel activity only at negative or at positive potentials. Rather, the maximal numbers of simultaneously open FV channels at positive and at large negative membrane potentials always corresponded to each other (Figures 5 to 8), and inward and outward whole vacuole currents always had the same proportion (Figures 2b, 3a and 4).

Summarizing, the selectivity, voltage-dependence and Ca\(^{2+}\) sensitivity of the fast-activating whole-vacuole currents described here can be explained at the molecular level by the properties of a single calcium-inhibited cation-selective ion channel, namely the FV channel.

**Comparison with other vacuolar channels**

Hedrich and Neher (1987) were the first to describe the FV current. The whole-vacuole \(I / V\) relationship published by Hedrich and Neher (1987) showed a high-resistance region slightly negative of the K\(^+\) equilibrium potential similar to the \(I / V\) characteristic shown here (Figures 2–4). The fast-activating whole vacuole current in sugarbeet vacuoles was only observed at Ca\(^{2+}\) concentrations at the cytosolic side below 1 \(\mu M\), comparable to our results (Figures 1 and 2). The selectivity of the whole-vacuole FV current was not investigated by Hedrich and Neher (1987). Single-channel currents with a conductance of about 30 to 40 \(pS\) (in 200 mM KCl) and a cation over anion selectivity (\(P_K / P_C\)) of about 6 were measured under low cytosolic Ca\(^{2+}\) in sugarbeet vacuoles (Hedrich and Neher, 1987). This channel seems to be different from the FV channel described here. It does not display an obvious voltage-dependence of the open probability, has a much lower cation over anion selectivity (6 compared to 30), a higher single-channel conductance and a much slower gating behaviour (Figures 5 and 6). The ion channel described by Hedrich and Neher (1987) is much more similar to the ion channel shown in Figure 9 which has a comparable conductance and gating behaviour. However, we do not think that this channel is responsible for the fast-activating whole-vacuole currents, as this channel was observed only rarely and did not display a significant voltage-dependence.

Colombo et al. (1989) observed instantaneously activating vacuolar currents in Acer pseudoplatanus cultured cells. At 1 mM Ca\(^{2+}\) inside the vacuole and 1 \(\mu M\) Ca\(^{2+}\) at the cytosolic side, these currents display a pronounced outward rectification (referring to the vacuolar side it is...
called an inward rectification in the original publication), reminiscent of the fast-activating currents described here for barley. In contrast to the results of Hedrich and Neher (1987) and the results described here, Colombo et al. (1989) observed no effect of the cytosolic Ca$^{2+}$ concentration on the fast-activating currents. Kolb et al. (1987) described single potassium channels in isolated barley mesophyll vacuoles with a conductance of 18 pS (250 mM KCl) with no Ca$^{2+}$ added. Davies and Sanders (1995) reported a slight increase of instantaneous vacuolar currents at low cytosolic Ca$^{2+}$ (< 500 nM) when 5 mM ATP were added. They argued that this ATP-activated current could provide a shunt conductance for the vacuolar H$^+\text{-ATPase}$.

The K$^+$-selective instantaneous current discovered in Vicia faba guard cells, called VK current (Ward and Schroeder, 1994), is activated by micromolar cytosolic Ca$^{2+}$. The VK channels reveal a more than threefold higher unitary conductance in symmetrical 100 mM KCl (70 pS for VK), compared with the FV channel. Furthermore, the instantaneous nature of the VK current is caused by its largely voltage-independent open probability (Ward and Schroeder, 1994); a constant proportion of VK channels are always open. In contrast, the FV current is clearly voltage-dependent (Figure 6d), and the instantaneous nature is due to a very rapid transition from low to high single channel activity (Figure 5). Therefore, FV and VK channels are clearly different from each other. In contrast to the VK channel, the channel presented in Figure 9 is not Ca$^{2+}$-activated and has a lower single-channel conductance.

Instantaneous currents across the vacuolar membrane of the unicellular green alga Eremosphaera viridis were interpreted to result from two different current components (Linz and Köhler, 1994): a voltage-independent component which was inhibited by increasing cytosolic Ca$^{2+}$ concentrations but not by high vacuolar Ca$^{2+}$ concentrations, and an outward-rectifying component which was not affected by cytosolic Ca$^{2+}$. Both components are clearly different from the FV current.

The FV channel differs from the SV channel by its much faster activation (Hedrich and Neher, 1987) and its Ca$^{2+}$ dependence. Increasing cytosolic Ca$^{2+}$ concentrations shift the voltage dependence of the SV channel to less positive voltages (Reifarth et al., 1994; Schulz-Lessdorf and Hedrich, 1995), while the voltage-dependence of the FV channel was not shifted by cytosolic Ca$^{2+}$ (Figures 1 and 2).

Although there are only very few reports about FV channels, we think that the FV channel is widespread in plant vacuoles. Using the experimental protocols and Ca$^{2+}$-buffered solutions described in this paper, we were able to resolve FV currents in mesophyll vacuoles from Peperomia metallica and rye (unpublished results). The unphysiologically high Ca$^{2+}$ concentration at the cytosolic side used by many researchers is probably the reason why the FV channel was not observed in most patch-clamp studies on plant vacuoles. This inhibition of the FV channel by high cytosolic Ca$^{2+}$ may serve as an identification criterion for this vacuolar ion channel.

**Possible physiological role of the fast vacuolar channel**

At physiological cytosolic Ca$^{2+}$ activities of 100–200 nM (Bush, 1995), the FV channel is expected to be active (Figures 1 and 2). Published values for vacuolar free Ca$^{2+}$ concentrations range from 0.2 mM to 2.3 mM (Bethmann et al., 1995; Felle, 1988; Miller and Sanders, 1987). Interestingly, in barley mesophyll cells, the vacuolar Ca$^{2+}$ concentration is well regulated and kept constant at 1 to 2 mM when the Ca$^{2+}$ concentration in the culture medium is changed 100-fold, from 0.3 to 30 mM (Dietz et al., 1992). Although the inward current through the FV channels is largely suppressed by millimolar Ca$^{2+}$ in the vacuole, it still remains in the range of some 10 pA at 2 mM vacuolar Ca$^{2+}$ (Figure 3). Thus, at physiological conditions, the fast vacuolar channel plays the role of an outward-rectifying K$^+$ channel which allows K$^+$ efflux from the cytoplasm into the vacuole and to a lesser extent K$^+$ influx from the vacuole into the cytoplasm.

If the FV channel gives rise to a high K$^+$ permeability of the vacuolar membrane under physiological conditions, the electrical potential difference across the vacuolar membrane might come close to the equilibrium potential for K$^+$. Published data sets that include tonoplast potentials and K$^+$ gradients seem to corroborate this view. In those cases where the measured tonoplast potential is vanishingly small, vacuolar and cytosolic K$^+$ concentrations do not differ significantly (Bethmann et al., 1995; Trebacz et al., 1994). In those cases where negative tonoplast potentials were measured, ranging from −18 to −27 mV, the vacuolar K$^+$ concentration is significantly smaller than the cytosolic one, and in each case the resulting driving force of the K$^+$ gradient (+22 to +24 mV) nearly completely balances the electrical driving force (Raven, 1967; Rona et al., 1982; Vorobiev, 1967). Thus, under physiological conditions, the tonoplast is likely to have a significant K$^+$ permeability due to the activity of the FV channel, and this results in a tonoplast potential which is close to the equilibrium potential for K$^+$.

Keeping in mind the presence of different electrogenic transporters in the tonoplast, this high K$^+$ permeability may be meaningful. There are two H$^+\text{-phosphohydrolases}$, namely the V-type H$^+\text{-ATPase}$ and the pyrophosphatase which build up the H$^+$ gradient across the tonoplast, and a large number of secondary transport systems which use the H$^+$ gradient to transport other substances – such as anions, cations, sugars and amino acids – across the tonoplast (Martinoia, 1992; Taiz, 1992). Whenever the activity of these electrogenic transport systems is not perfectly
balanced, this gives rise to an increasing tonoplast potential which interferes with electrogenic transport processes. The FV channel clamps the tonoplast potential close to the equilibrium potential for K⁺ and thus prevents large changes of the tonoplast potential. As K⁺ is the most abundant cation in plant cells, with concentrations of 100–200 mM in the cytosol, the K⁺ fluxes needed to electrically compensate other transport processes are probably too small to cause large changes in the K⁺ gradient across the tonoplast.

Conclusion
The FV channel is the only vacuolar ion channel identified to date that is highly selective for monovalent cations (K⁺), gated open at physiological Ca²⁺ activities and at physiological tonoplast potentials, and probably widespread in different plant cells. We propose that the FV channel is responsible for a high K⁺ permeability of the tonoplast clamping the tonoplast potential close to the equilibrium potential for K⁺.

Experimental procedures

Isolation of barley mesophyll protoplasts

Protoplasts were isolated from primary leaves of 7-9-day-old barley plants (*Hordeum vulgare* L.) grown under a 14 h 20°C light/10 h 18°C dark cycle. The preparation procedure was modified from Martinoia *et al.* (1981). The leaves were stripped from their abaxial epidermis and floated on the surface of 500 mM D-sorbitol, 30 mM HEPES-Tris (pH 7.5), 30% Percoll; (2) 500 mM sorbitol, 1 mM CaCl₂, 15 mM MES-Tris (pH 5.5), 0.1% BSA (Roth, Karlsruhe, Germany); 1% cellulose Y-C, 0.01% polylecylate Y23 (both from Seishin Pharmaceutical, Tokyo, Japan) and 0.5% macerozyme R-10 (Serva, Darmstadt, Germany). After 1 h incubation at 30°C, the released protoplasts were filtered through a 0.5 mm nylon net, collected by centrifugation (20 min, 125 g) and purified by flotation (10 min, 1750 g) through a Percoll (Pharmacia, Upsala, Sweden) density gradient with (1) suspension of protoplasts in 500 mM sorbitol, 30 mM HEPES-Tris (pH 7.5), 30% Percoll; (2) 500 mM sorbitol, 0.8 mM CaCl₂, 12 mM MES plus 6 mM HEPES (pH 6.0 with Tris), 0.04% polyvinylpyrrolidone, 0.08% BSA, 20% Percoll; (3) 500 mM sorbitol, 1 mM CaCl₂, 15 mM MES-Tris (pH 5.5), 0.06% polyvinylpyrrolidone, 0.1% BSA. After centrifugation, the mesophyll protoplasts were collected from the interface between steps 2 and 3, and were kept on ice during the experiment.

Experimental solutions

The standard bath solution contained 100 mM KCl, 5 mM EGTA, 15 mM Tricine-Tris (pH 7.5). Osmolalities of this and all other solutions were adjusted to 650 mOsm with sorbitol and verified by a cryoscopic osmometer (Osmomat 030, Germany). Patch-clamp microelectrodes were filled either with the same solution as the bath, or, alternatively, by one containing 2 mM CaCl₂ instead of 5 mM EGTA. The basic solution for measurements of calcium dependence contained 100 mM KCl, 15 mM Tricine-Tris (pH 7.5), 2 mM EGTA (ethylene glycol bis(β-aminoethyl)-N,N,N',N'-tetra-acetic acid), 2 mM HEDTA (N-(2-hydroxyethyl) ethylenediaminetriacetic acid), 2 mM NTA (nitritriacetic acid), 400 mM sorbitol. Stability constants for complexes of different chelators with calcium were taken from Otto *et al.* (1985). Free calcium concentrations in the range of 1.4 nM to 490 µM were adjusted by variable additions of CaCl₂ to the basic solution. For calculations of free Ca²⁺ concentration, a home-made computer program was used (courtesy of Dr A. Alexeyev, Pushchino). The total (resulting free) Ca²⁺ concentrations (in µM) were: 90 (0.0014), 550 (0.011), 1550 (0.077), 2600 (0.66), 3500 (2.9), 3700 (4.9), 4200 (12.7), 4350 (18.3), 4650 (30.4), 4750 (35.7), 4900 (48.6), 5300 (89.1), 5350 (104) and 6300 (493). Nominally Ca²⁺-free basic solutions containing 100 mM KCl, 15 mM Tricine-Tris (pH 7.5), 2 mM EGTA, 2 mM HEDTA, 2 mM NTA, 400 mM sorbitol or 100 mM KCl, 15 mM Tricine-Tris (pH 7.5), 5 mM EGTA and 410 mM sorbitol were routinely measured by ICP-AES (ion-coupled plasma emission spectroscopy). Total Ca²⁺ concentrations ranged from 3 to 10 µM, which corresponds to free Ca²⁺ concentrations of 0.2 to 0.7 nM. Solutions without added Ca²⁺ containing < 1 nM free Ca²⁺ were referred to as 0 Ca²⁺.

Patch-clamp measurements and data analysis

A single vacuole was mechanically isolated from a protoplast within the measuring chamber. For this, a glass micro-electrode filled with the standard bath solution was attached to a protoplast and the plasma membrane was ruptured by application of a suction pulse. By this quick and simple procedure, a new vacuole was isolated for each measurement. Within a few minutes after the release of the vacuole, the patch-clamp measurement was initiated with a fresh glass micro-electrode.

Patch pipettes were made from Kimax-51 glass capillaries (Kimble Products, Vineland, NJ) using a two-step pulling protocol (electrode puller L/M-3P-A, List Medical, Darmstadt, Germany). The tip diameters of patch pipettes after fire-polishing were 2–3 µm and about 1 µm for whole-vacuole and single-channel measurements, respectively. When the electrodes were filled with the standard solution, this resulted in resistances of 2–5 MΩ and 10–15 MΩ for the two types of micro-electrodes. For high-resolution single channel recordings, the tip of the glass pipette was coated with silgarde (Silicones RT615, General Electric Co., Waterford, NY).

After achievement of a high-resistance seal (10–20 GΩ) of the patch pipette with the tonoplast, the measurements of vacuolar ion currents were performed under voltage-clamp conditions using one of three different geometries (Hamill *et al.*, 1981): whole-vacuole (analogous to whole-cell) cytosolic side-out excised patch (cytoplasmic side of the membrane faces the bath; technically an outside-out patch) and vacuolar side-out excised patch (vacuolar side faces the bath; technically an inside-out patch). Whole-vacuole measurements were started 20–30 min after rupturing the vacuolar membrane inside the pipette to ensure complete equilibration of the pipette solution with the interior of the vacuole. During this time, protoplast remains were completely washed away. The membrane capacitance of vacuoles used in this study was 7–28 pF, which corresponds to diameters between 15 and 30 µm. The capacitance and the access resistance were monitored and compensated throughout the experiment and this compensation was controlled each time before an I/V curve was recorded. Measurements were performed under continuous bath perfusion.
References


Linz, K.W. and Köhler, K. (1994) Vacular ion currents in the primitive green alga *Eremosphaera viridis*: the electrical properties are suggestive of both the Characeae and higher plants. Protoplasma, 179, 34–45.


