

Voltage-Dependent Channels Permeable to K^+ and Na^+ in the Membrane of *Acer pseudoplatanus* Vacuoles

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Summary. The patch-clamp technique in whole-cell configuration was used to study the electrical properties of the tonoplast in isolated vacuoles from *Acer pseudoplatanus* cultured cells. In symmetrical KCl or K_2 malate solutions, voltage- and time-dependent inward currents were elicited by hyperpolarizing the tonoplast (inside negative), while in the positive range of potential the conductance was very small. The specific conductance of the tonoplast at -100 mV, in 100 mM symmetrical KCl was about $160 \mu S/cm^2$. The reversal potentials (E_{rev}) of the current, measured in symmetrical or asymmetrical ion concentrations (cation, anion or both) were very close to the values of the K^+ equilibrium potential. Experiments performed in symmetrical or asymmetrical NaCl indicate that Na^+ too can flow through the channels. Neither E_{rev} nor amplitude and kinetics of the current changed by replacing NaCl with KCl in the external solution. These results indicate the presence of hyperpolarization-activated channels in tonoplasts, which are permeable to K^+ as well as to Na^+ . Anions such as Cl^- or malate seem to contribute little to the channel current.

Key Words cation channel · patch-clamp · plant vacuole

Introduction

In plant cells, the transport of ions and metabolites between cytoplasm and vacuole plays an important role in the regulation of cell turgor and in cytoplasmic homeostasis (Matile, 1978; Leigh, 1983).

In recent years the use of isolated vacuoles and tonoplast-enriched vesicle preparations has allowed much progress in the study of transport mechanisms in tonoplasts. The occurrence of a tonoplast H^+ -ATPase has been established in such preparations (Sze, 1985), and the existence has been suggested of symports or antiports, driven by the H^+ electrochemical gradient built up by the ATPase (see Marin, 1987). Direct evidence concerning passive flow of ions—which might be associated with the H^+ pump activity—has been lacking until the patch-clamp technique was applied to directly mea-

sure the ionic exchanges in isolated vacuoles. This method has recently allowed to show hyperpolarization-activated K^+ channels in isolated vacuoles from *Acer pseudoplatanus* cultured cells (Colombo, Lado & Peres, 1987), from barley mesophyll (Hedrick, Flügge & Fernandez, 1986; Kölb, Köhler & Martinoia, 1987) and from sugarbeet (Coyaud et al., 1987). In barley and sugarbeet vacuoles these channels seem to be poorly selective, as they appear to be partially permeable to anions (Hedrick et al., 1986; Coyaud et al., 1987).

In the present work, by using the patch-clamp technique in the whole-cell (whole-vacuole) recording mode in isolated vacuoles from *Acer pseudoplatanus* cultured cells, we further examined the currents activated by hyperpolarization, carefully investigating the nature of the ions carrying the current.

Our results indicate the presence of hyperpolarization-activated channels in tonoplasts, which are permeable to K^+ as well as to Na^+ . Anions such as Cl^- or malate seem to contribute little to the channel current.

Materials and Methods

PREPARATION OF VACUOLES

Cell suspension cultures of *Acer pseudoplatanus*, kindly gifted by Prof. J. Guern, were grown in the liquid medium described by Bligny (1977). Protoplasts were prepared from 7-day-old cultures by enzymatic digestion. Cells (about 10 g fresh wt) were incubated under low agitation (40 rpm) at $25^\circ C$ in 20 ml of 25 mM Tris_L-MES_L buffer (pH 5.5) containing 0.7 M mannitol, 1% cellulase RS and 0.1% pectolyase Y23. After 100 min of digestion the suspension was filtered through 100 μm pore nylon cloth and centrifuged for 5 min at $160 \times g$ at $4^\circ C$. The pelleted protoplasts were resuspended in 25 mM Tris_L-MES_L buffer (pH 7.5) containing 0.7 M mannitol.

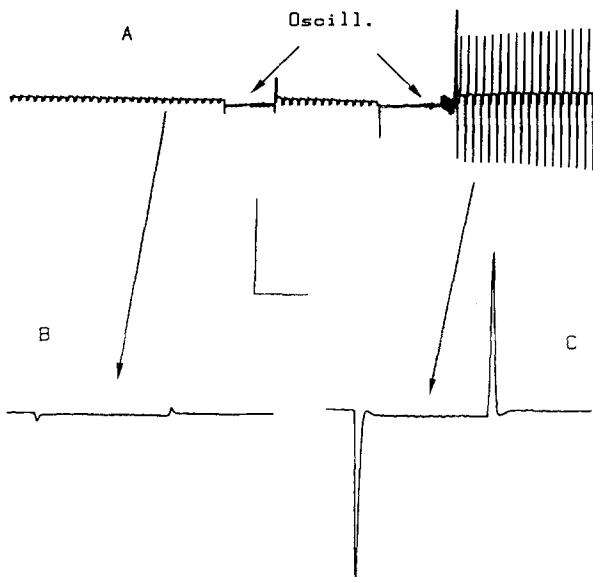


Fig. 1. (A) Transition from vacuole-attached to whole-vacuole configuration; the first oscillation was ineffective; successful rupture of the patch was achieved during the second oscillation as indicated by the large increase in the capacitive transients (signal filtered at 400 Hz). (B) Example of a single pulse in vacuole-attached mode. (C) Single pulse after breaking into the vacuole. Signals in (B) and (C) were filtered at 4 kHz. Calibration bars represent 0.5 nA and 250 msec for (A) and 1 nA and 4 msec for (B) and (C)

Vacuoles were isolated from protoplasts by osmotic shock. Protoplasts (4×10^6 /ml) were diluted 1:0.9 with 25 mM Tris_L-MES_L buffer pH 7.5 containing 2 mM EDTA, 2 mM DTT and incubated under low agitation at room temperature. After 13 min, 7 volumes of a solution containing 8% Ficoll® 400, 0.55 M mannitol (pH 7.5) were added, thus ending the osmotic shock. This suspension (about 12 ml) was filtered through 100 μ m pore nylon cloth and poured into centrifuge tubes; 0.8 ml of 2 mM Tris_L-MES_L buffer (pH 7.5) containing 0.6 M mannitol were stratified on the top. After 15 min of centrifugation at 160 \times g at 4°C, the vacuoles were collected at the interface. A drop of the vacuole suspension was laid in the glass recording chamber and left undisturbed for a few minutes to allow the vacuoles to settle down and stick to the bottom before flushing with the desired solution. Vacuoles with a diameter between 40 and 60 μ m were used in the experiments.

RECORDING

The whole-cell (whole-vacuole) configuration of the patch-clamp technique was used throughout (Marty & Neher, 1983). Pipettes had resistances between 4 and 6 M Ω . The patch-clamp amplifier was designed by Prof. E. Wanke, Milan, and built in our electronics shop. Current and voltage signals were fed to a pulse code modulator (Sony PCM 601 ESD) and stored on a video cassette recorder. They were subsequently digitally converted and analyzed with an Olivetti M24 personal computer equipped with a Labmaster board.

The expression "membrane voltage" (V), used throughout the paper, refers to the potential difference between the inside

and the outside of the isolated vacuole. The sign of the current (I) is conventionally taken as positive when the current flows in the outward direction.

An agar bridge containing the same solution used to fill the pipette was used to connect the bath to an Ag-AgCl pellet which was in turn connected to the ground of the patch-clamp amplifier. Residual liquid junction potentials were compensated electronically before establishing contact with the vacuole.

As previously reported (Colombo et al., 1987), the formation of a gigaseal between pipette and tonoplast was a very quick process. However, the establishment of the whole-vacuole condition was very difficult to achieve because suction pulses frequently led to the destruction of the vacuole. In addition, after a successful rupture, the membrane often tended to reseal obstructing the pipette tip.

A substantial improvement in establishing the whole-vacuole condition was obtained by making the pipette potential oscillate at high frequency in order to disrupt the patch. Figure 1 illustrates a typical passage from the "vacuole-attached" to the "whole-vacuole" condition. Repetitive stimulation with 10 msec-10 mV pulses was applied to the pipette. Stray capacities were almost completely subtracted and brief pulses of oscillating potential were applied. As illustrated in Fig. 1(A), a sudden increase in the capacitive transients signalled a patch rupture; however, the patch often resealed. Several oscillation pulses were usually necessary to reach a stable "whole-vacuole" condition.

The maintenance of a low resistance connection to the vacuole interior (R_s) can be evaluated from the membrane capacity (C) and from the time constant (τ) of the capacitive transients. R_s can be approximated by τ/C (see Marty & Neher, 1983). Tonoplast capacities were obtained by calculating the current time integral of the capacitive transient after breaking into the vacuole. Typical values were between 50 and 100 pF. Time constants were computed from the same transients and were of the order of some tenths of msec. With these values R_s was between 5 and 10 M Ω . In practice, the time course of the transient decay was checked by eye and when the transients appeared to slow down, further pulses of oscillation were applied to reopen the patch.

No leakage subtraction was performed. In all the I-V plots the current values at positive potentials are uncorrected final values (i.e. at the end of the 1-sec pulses); for negative potentials, the initial instantaneous jump of current has been subtracted from the final value. At negative potentials, therefore, the I-V plots represent only the time-dependent component of the current.

SOLUTIONS

The bath solutions are listed in Table 1. The pipette was filled with: 100 mM KCl and 10 mM HEPES when the external solutions were A, B or C; 100 mM K⁺, 56 mM malate when the external solutions were D, E or F; 100 mM NaCl and 10 mM HEPES when the external solutions were G, H or I. The osmolarity was adjusted to 0.54 osM with mannitol. The pH was 5.3.

For the sake of comparison, the physiological *Acer* vacuolar concentration of the ions present in the pipette was: K⁺ around 100 mM, Na⁺ around 2 mM, Cl⁻ and malate around 10 mM and the pH was around 5.5 (A. Kurkdjian, *personal communication*).

The equilibration of ions between the pipette solution and the vacuole interior appeared to be fast (Marty & Neher, 1983). The hyperpolarization-elicited currents and E_{rev} measured soon

Table 1. External solutions^a

	K	Na	TMA	Cl	Malate	Gluconate	Sulphate	HEPES	Tris
A	103.5	—	—	100	—	—	—	10	—
B	13.5	—	90	10	—	—	45	10	—
C	103.5	—	—	10	—	90	—	10	—
D	100	—	—	—	56	—	—	—	8.5
E	10	—	70	—	5.6	—	35	—	0.8
F	100	—	—	—	5.6	—	45	—	0.8
G	—	103.8	—	100	—	—	—	10	—
H	—	13.8	90	10	—	—	45	10	—
I	—	13.8	90	100	—	—	—	10	—

^a All concentrations in mM. All solutions contained 1 mM $CaSO_4$ and 2 mM $MgSO_4$. The osmolarity was adjusted to 0.6 osM with mannitol. The pH was 7.2.

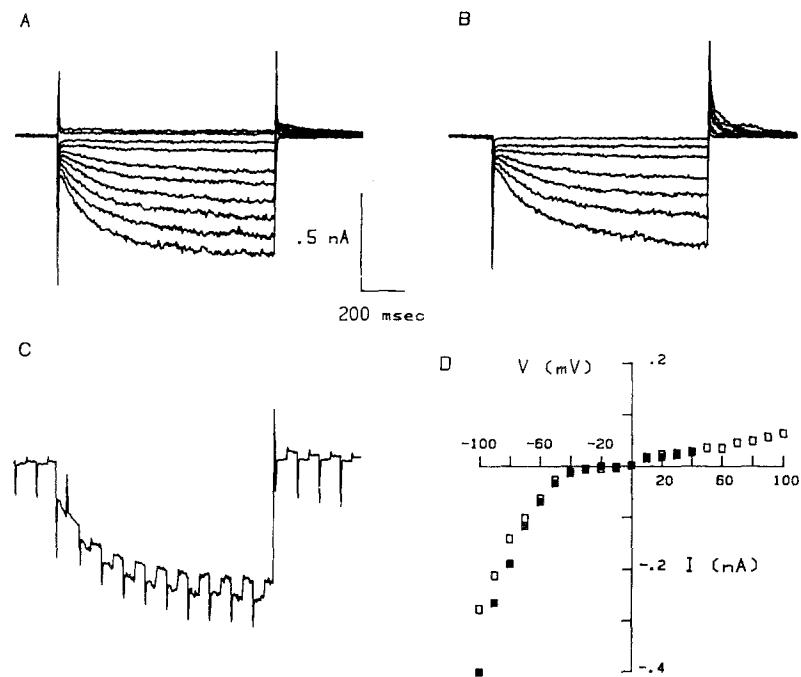


Fig. 2. (A) Currents with symmetrical K^+ concentrations (solution C) activated by voltage pulses from $V_h = 0$ mV to $+50$, $+30$, -30 , -50 , -70 , -80 , -90 , -100 , -110 , -120 mV. (B) Currents activated by hyperpolarizing steps from $V_h = +50$ mV to $+30$, -30 , -50 , -70 , -80 , -90 , -100 mV. In (D) current *vs.* voltage relationships from (A) (open squares) and from (B) (filled squares) are shown; leakage subtraction protocol as described in Materials and Methods. In (C) a train of 10 mV-50 msec steps was superimposed on a hyperpolarizing voltage step from $V_h = 0$ to -100 mV. It can be seen that corresponding current displacements (ΔI) are approximately proportional to the total current (I).

after the establishment of the whole-cell configuration did not change significantly during the recording (up to 30 min).

All solutions were filtered through 0.22 μ m millipore filters just before use. The temperature during the experiments was kept at 20–25°C.

Results

INWARD CURRENTS ACTIVATED BY HYPERPOLARIZATION

As recently reported (Hedrick et al., 1986; Colombo et al., 1987; Coyaud et al., 1987; Kolb et al., 1987), the tonoplast conductance is strongly nonlinear, showing low values upon depolarizations and in-

creasingly higher values upon hyperpolarizations (Fig. 2). Upon hyperpolarization there is an instantaneous jump of current followed by a slow and sigmoidal increase of the current in the inward direction. Figure 2 shows typical currents in response to various voltage pulses from $V_h = 0$ mV (A) and $V_h = +50$ mV (B) when symmetrical K^+ concentrations were maintained on both sides of the membrane (solution C, same vacuole). In Fig. 2(D) the current *vs.* voltage plot shows the same voltage dependence for (A) and (B). In Fig. 2(C) a train of small hyperpolarizing voltage pulses (ΔV) was superimposed on a hyperpolarizing pulse from $V_h = 0$ to -100 mV to monitor changes in membrane conductance. It can be seen that the corresponding current displacements (ΔI) are approximately propor-

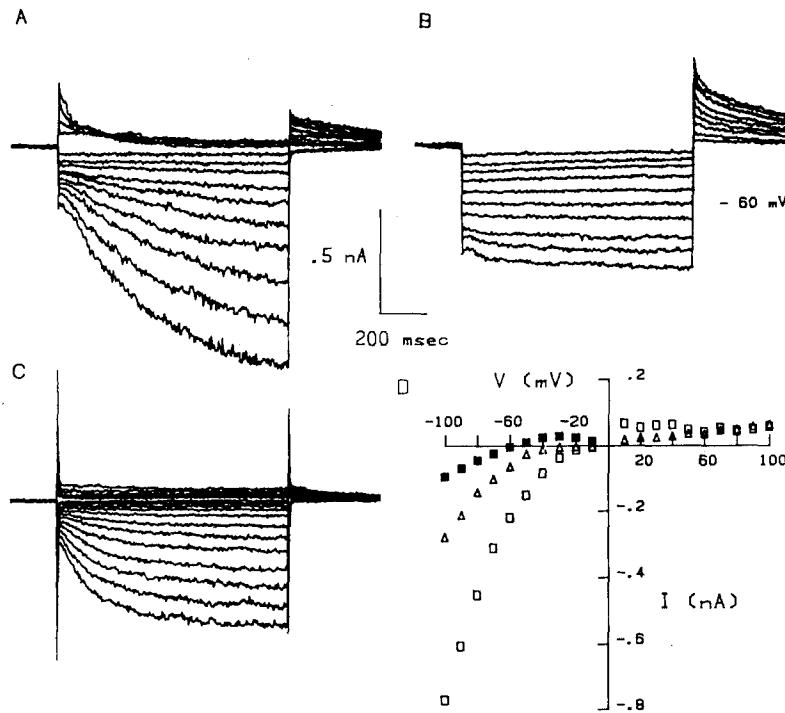


Fig. 3. Currents evoked by various voltage pulses from $V_h = 0$ mV with symmetrical KCl concentrations (A, solution A), with asymmetrical KCl concentrations (B, solution B) and with symmetrical K^+ and reduced external Cl^- concentrations (C, solution C). In (A) the voltage pulses ranged from +20 to +100 mV (steps of 20 mV) and from -10 to -100 mV (steps of 10 mV). In (B) the voltage pulses ranged from -10 to -100 mV (steps of 10 mV). In (C) the voltage pulses ranged from +20 to +100 mV (steps of 20 mV) and from -10 to -120 mV (steps of 10 mV). In (D) current *vs.* voltage relationships are shown from (A) (open squares), (B) (filled squares) and (C) (open triangles)

tional to the total current (I), thus suggesting that the increase in inward current during hyperpolarization is due to an increase in membrane conductance.

THE CURRENT IN KCl SOLUTIONS

To examine the ionic nature of this current we used ion substitution experiments. Holding potential (V_h) was 0 mV. In the experiments shown in Fig. 3 the external K^+ and Cl^- concentrations varied, while the internal concentrations were kept constant. In Fig. 3(A) K^+ and Cl^- concentrations were the same on both sides of the membrane; in (B) both Cl^- and K^+ were reduced from 100 and 103.5 mM to 10 and 13.5 mM, respectively. In (C) K^+ was symmetrical, while Cl^- was reduced to 10 mM by substitution with gluconate.

The instantaneous current jumps were not consistently affected by the solution changes, their amplitudes being different in different vacuoles and they were therefore considered due to leakage and neglected.

We concentrated our attention on the time-dependent component of the current (i.e. the total current minus the instantaneous jump).

Figure 3(C) shows that by reducing the external Cl^- concentration the time-dependent components of the hyperpolarization-activated current are al-

ways inward as under conditions of symmetrical KCl (Fig. 3A), while following a reduction in the external concentration of both Cl^- and K^+ (Fig. 3B), the time-dependent components of the current become much smaller and show a different behavior around -60 mV. For potentials more positive than -60 mV a decreasing inward current is detected, whereas for voltage steps more negative than -60 mV the inward current is increasing. The experiment of Fig. 3(B) suggests that the reversal potential (E_{rev}) of the current is at about -60 mV, close to the K^+ equilibrium potential (E_K).

The current *vs.* voltage relationship is illustrated in Fig. 3(D). To determine E_{rev} , tail currents were measured by repolarizing to different membrane potentials following activating pulses of constant amplitude. Tail currents should change direction around the E_{rev} value.

As shown in Fig. 4, under conditions of symmetrical KCl concentration (A) and under symmetrical K^+ concentration and reduced Cl^- concentration (C), E_{rev} is close to 0 mV. For reduced K^+ and Cl^- concentrations (Fig. 4B) when repolarizations are more positive than -50 mV the tails are outward. At more negative voltages, the tails appear flat: this may be expected as it is probable that the number of open channels does not change significantly within this range of potentials. This experiment allows us to say that E_{rev} must be more negative than -40 mV, in agreement with Fig. 3(B). It, therefore, appears that E_{rev} is always close to E_K ,

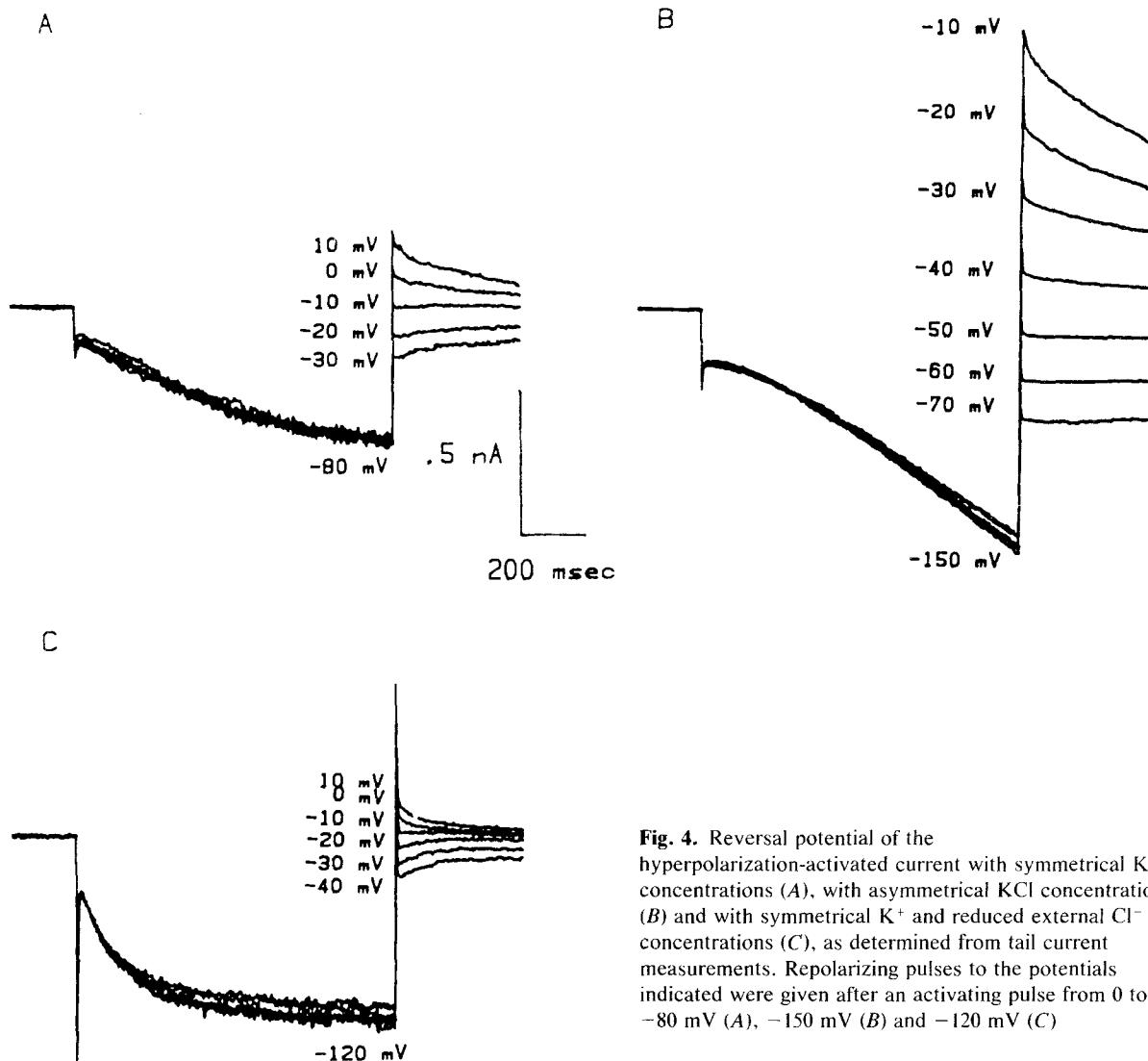


Fig. 4. Reversal potential of the hyperpolarization-activated current with symmetrical KCl concentrations (A), with asymmetrical KCl concentrations (B) and with symmetrical K⁺ and reduced external Cl⁻ concentrations (C), as determined from tail current measurements. Repolarizing pulses to the potentials indicated were given after an activating pulse from 0 to -80 mV (A), -150 mV (B) and -120 mV (C)

being not significantly affected by changes in the Cl⁻ concentration.

As shown in Fig. 3(A), transient outward currents were sometimes observed upon depolarizing pulses from $V_h = 0$ mV. This indicates that some channels are already open around 0 mV. The presence (or the absence) of these outward decaying currents was not correlated with any particular composition of the solutions (see also Figs. 5B, 6A and 6B).

THE CURRENT IN K₂ MALATE SOLUTIONS

In their pioneering study, Hedrick et al. (1986) suggested that the hyperpolarization-activated channel they observed in barley mesophyll vacuoles is permeable both to K⁺ and to malate ions. We have tested the permeability to malate in our preparation.

Figure 5 shows the currents evoked by different voltage pulses from $V_h = 0$ mV. In Fig. 5(A) symmetrical K₂malate was used; in (B) the external K⁺ and malate concentrations were reduced about ten-fold; in (C) external malate was reduced while keeping K⁺ symmetrical. Figure 5(D) shows the current vs. voltage curves obtained under the three different experimental conditions described.

It appears that the results shown in Fig. 5 are similar to those of Fig. 3. Tail currents experiments were performed under these conditions as well, and the results are shown in Fig. 6: (A) symmetrical K₂malate, (B) asymmetrical K₂malate, (C) symmetrical K⁺ and asymmetrical malate.

Again, E_{rev} is close to zero when K⁺ is symmetrical and, when there is a cation gradient, between -50 and -60 mV (Figs. 5B and 6B) while it is far from the values of E_{malate} (see Table 2).

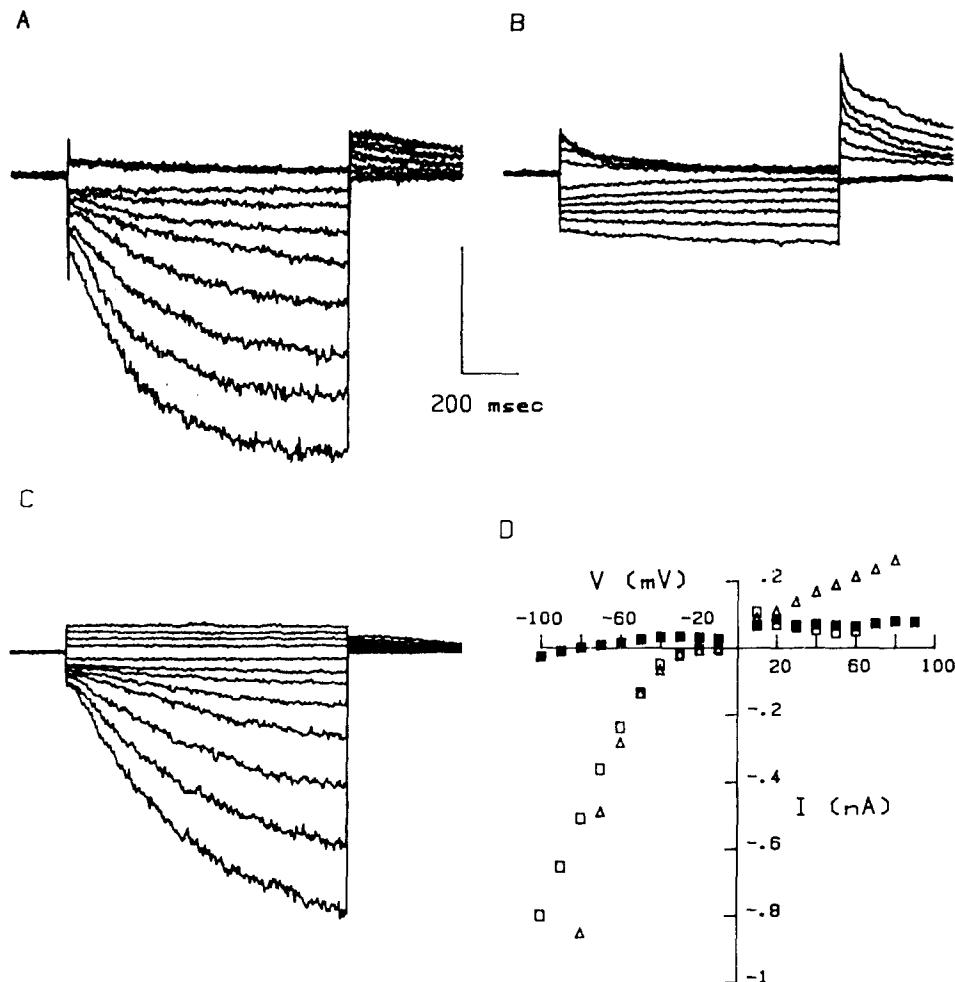


Fig. 5. Currents in response to various voltage pulses from $V_h = 0$ mV with symmetrical K₂malate concentrations (A, solution D), with asymmetrical K₂malate concentrations (B, solution E) and with symmetrical K⁺ and reduced external malate concentrations (C, solution F). In (A) the voltage pulses were to +60, +40, +20, -20, -40, -50, -60, -70, -80, -90, -100 mV. In (B) the voltage pulses ranged from +80 to -120 mV (steps of 20 mV). In (C) the voltage pulses were to +80, +60, +40, +20, -20, -40, -50, -60, -70, -80, -90, -100 mV. Calibration bar represents 0.5 nA for (A) and (B) and 1 nA for (C). In (D) current *vs.* voltage relationships are shown from (A) (open squares), (B) (filled squares) and (C) (open triangles)

THE CURRENT IN NaCl SOLUTIONS

In this set of experiments we tested whether Na⁺ ions could carry the current. Figure 7 shows the results obtained by using NaCl instead of KCl and with different combinations of Na⁺ and Cl⁻ concentrations inside and outside the vacuole.

As in the experiments with K⁺ solutions, time-dependent inward currents are elicited by hyperpolarizations with symmetrical NaCl concentrations (Fig. 7A), while with asymmetrical Na⁺ concentrations, independently of the Cl⁻ gradient, decreasing inward currents were observed with potentials more positive than -60 mV and increasing inward currents were detected when more negative potentials were applied (Fig. 7B and C). Figure 7(D)

shows the current *vs.* voltage relationship under the three experimental conditions described. E_{rev} of the current is close to zero under conditions of symmetrical NaCl concentrations (Fig. 7E) and it shifts to negative potentials (around -60 mV) in the presence of a Na⁺ gradient (Fig. 7B,C and Fig. 7F for tail current experiment). These results indicate that Na⁺ too can flow through these channels while Cl⁻ does not contribute significantly to the current.

It is important to assess the relative permeabilities of K⁺ and Na⁺ through these channels. In order to do so, we have performed a number of experiments in which we measured the current with NaCl on both sides of the membrane first and subsequently after replacing the external solution with one containing KCl. No significant changes in the

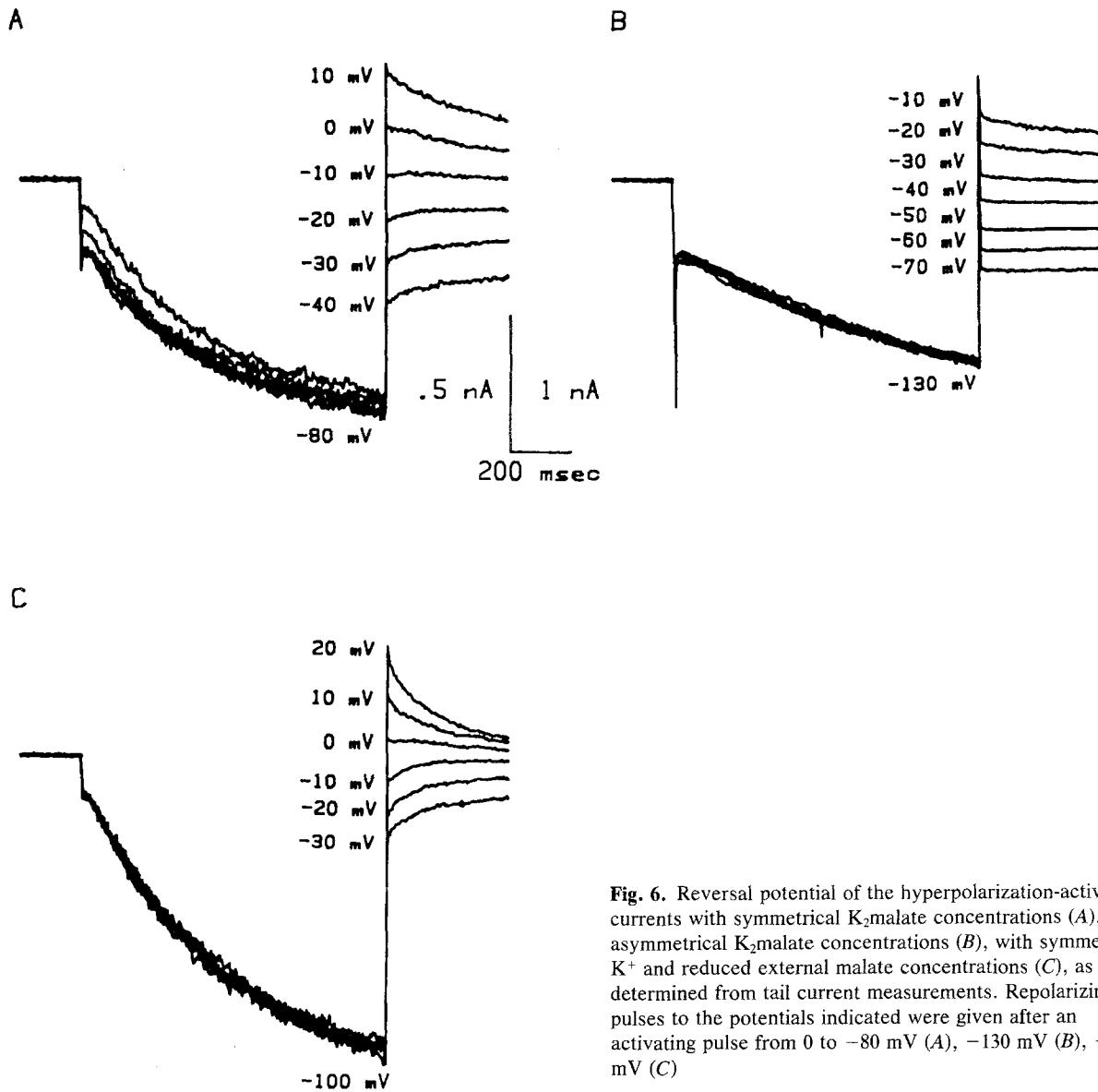


Fig. 6. Reversal potential of the hyperpolarization-activated currents with symmetrical $K_2\text{malate}$ concentrations (A), with asymmetrical $K_2\text{malate}$ concentrations (B), with symmetrical K^+ and reduced external malate concentrations (C), as determined from tail current measurements. Repolarizing pulses to the potentials indicated were given after an activating pulse from 0 to -80 mV (A), -130 mV (B), -100 mV (C)

current amplitude or kinetics were seen (*data not shown*). Moreover, the reversal potential of the current measured with 100 mM NaCl inside the vacuole and 100 mM KCl outside was found to be close to 0 mV (Fig. 8), thus indicating an approximately equal permeability of the tonoplast channel to both K^+ and Na^+ .

COMPARISON BETWEEN EQUILIBRIUM AND REVERSAL POTENTIAL

Table 2 compares the computed equilibrium potentials of the various ions with the measured reversal potentials. The experimental protocol allowed to determine a 10 mV range for E_{rev} . All determina-

tions in each condition fell within a 10 mV range. It can be seen that there is good agreement between reversal potential and K^+ (or Na^+) equilibrium potential.

Discussion

Our data show the presence of a strongly inward rectifying current in the tonoplast of isolated *Acer* vacuoles. The current is activated by hyperpolarizations, while within the range of positive voltages it is very small. These findings indicate the presence of ionic channels, thus confirming the data previously obtained by us in this material (Colombo et

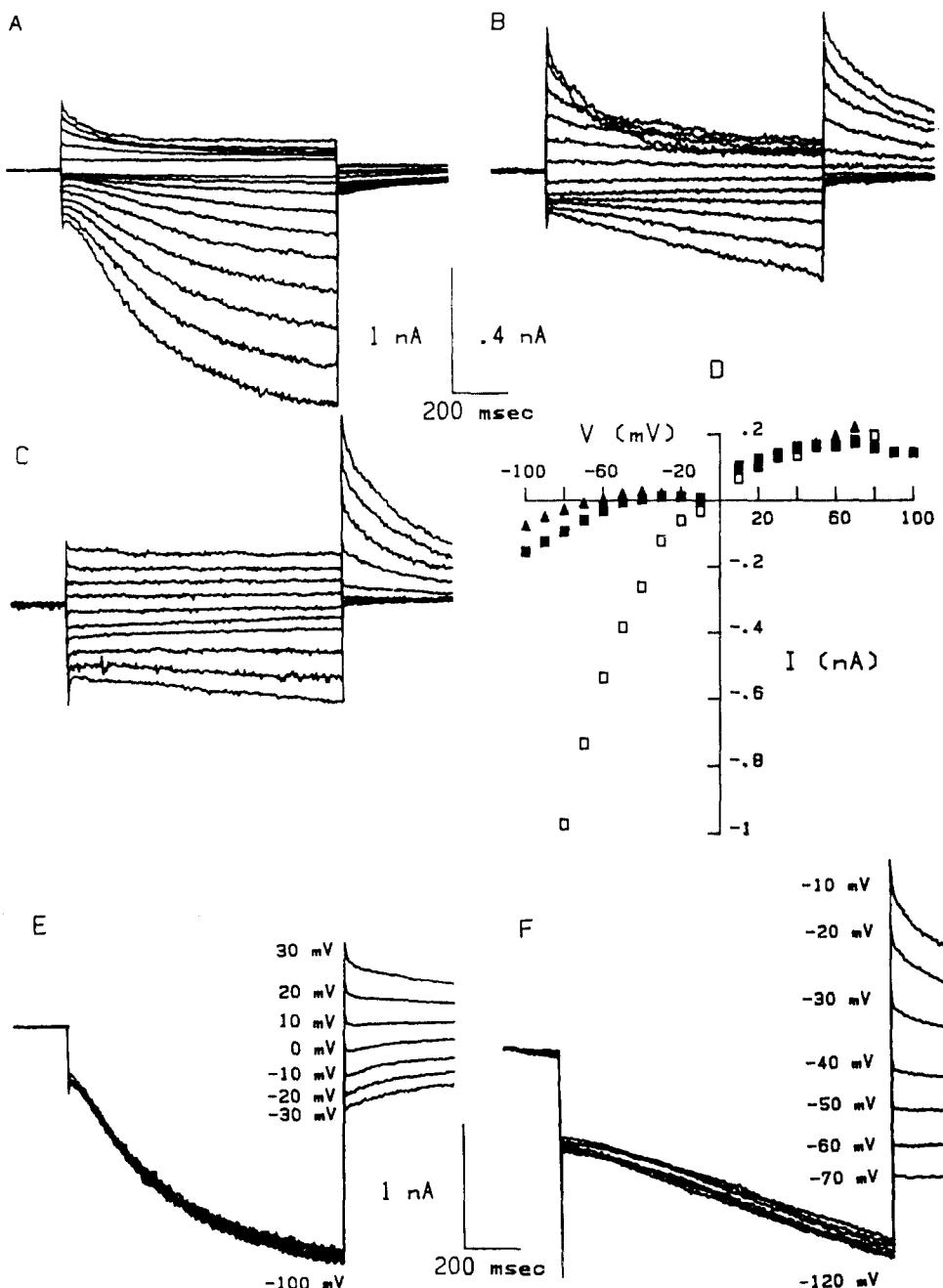


Fig. 7. Currents in response to various voltage pulses from $V_h = 0$ mV with symmetrical NaCl concentrations (A, solution G), with asymmetrical NaCl concentrations (B, solution H) and with symmetrical Cl^- and reduced external Na^+ concentrations (C, solution I). In (A) the voltage pulses ranged from +20 to +100 mV (steps of 20 mV) and from -10 to -100 mV (steps of 10 mV). In (B) the voltage pulses ranged from +120 to -120 mV (steps of 20 mV). In (C) voltage pulses ranged from +70 to -110 mV (steps of 20 mV). Current *vs.* voltage relationships from (A) (open squares), (B) (filled squares) and (C) (filled triangles) are shown in (D). In (E) reversal potential with symmetrical NaCl concentrations. In (F) reversal potential with asymmetrical Na^+ and symmetrical Cl^- concentrations

al., 1987) and by Hedrich et al. (1986), Kolb et al. (1987) and Coyaud et al. (1987) in other materials.

The specific chord conductance of the membrane measured at -100 mV potential in 100 mM symmetrical salts is around 160 $\mu S/cm^2$.

The reversal potentials of the current, mea-

sured in symmetrical or asymmetrical ion concentrations (cation, anion or both), are in all conditions very close to the K^+ equilibrium potential. Our data, therefore, show that K^+ is the major carrier of the current, whereas the contribution of the two anions tested seems negligible. The presence of

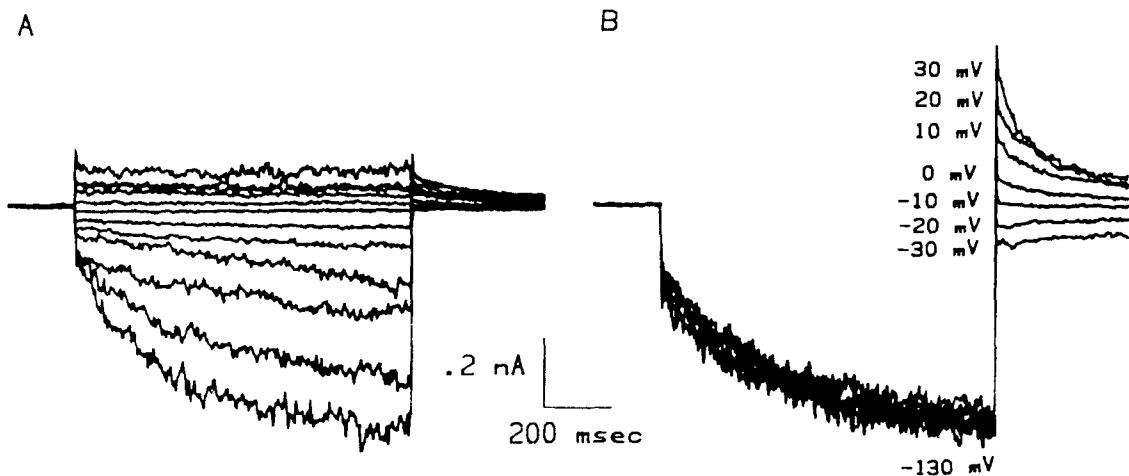


Fig. 8. (A) Currents in response to voltage pulses (+90 to -130 mV, steps of 20 mV) from $V_h = 0$ mV with mM NaCl inside the vacuole and 100 mM KCl outside. (B) Reversal potential determination in the same conditions (same vacuole)

Table 2. Comparison of equilibrium and reversal potential^a

Concentrations (mM)				Equilibrium potential (mV)		Reversal potential range (mV)	
in		out		E_K	E_{Cl}		
K^+	Cl^-	K^+	Cl^-				
100	100	103.5	100	+0.9	0	0 to -10	
100	100	103.5	10	+0.9	+59	0 to -10	
100	100	13.5	10	-51.3	+59	-50 to -60	
K^+	Mal^-	Mal^{2-}	K^+	Mal^-	Mal^{2-}	E_K	E_{Mal^-}
100	21.6	33.4	100	0.50	55.5	0	+96
100	21.6	33.4	100	0.05	5.55	0	+155
100	21.6	33.4	10	0.05	5.55	-59	+23
Na^+	Cl^-		Na^+	Cl^-		E_{Na}	E_{Cl}
100	100		103.8	100		+0.9	0
100	100		13.8	10		-50.7	+59
100	100		13.8	100		-50.7	0

^a Malate⁻ and malate²⁻ concentrations were computed using $pK_{a1} = 3.54$ and $pK_{a2} = 5.11$. At least three vacuoles were tested in each condition.

channels selective for cations seems reasonable inasmuch as it allows a specific regulation of cation transport. It is worthwhile pointing out that cation selectivity is very common in membrane channels of animal cells. Other authors (Hedrich et al., 1986; Coyaud et al., 1987) suggested that in barley and sugarbeet vacuoles, voltage-dependent K^+ channels may partially carry anions. This difference does not seem to depend on the experimental conditions (media composition, pH, applied voltage), while it

might depend on the kind of tissue. The flow of malate between cytoplasm and vacuole is more important in photosynthetic tissues and might be different from that occurring in cultured cells. However, as the transport of malate across the tonoplast is a common feature to all plant cells and it is involved in many functions (growth, movement of stomata, salt uptake, see Lüttge & Smith, 1985) the problem requires further investigation.

Our results show that Na^+ too can pass through

the voltage-dependent channels and that the permeability of the tonoplast for the two cations is about the same. A similar result has been reported for sugarbeet vacuoles (Coyaud et al., 1987).

The fact that a flow of K⁺ occurs through ionic channels, would fit well with the generally accepted view that the distribution of K⁺ reflects a situation of equilibrium between cytoplasm and vacuole (see Lüttge & Higinbotham, 1979). The question arises of why the K⁺ channels open at a negative potential, while the electric potential across the tonoplast is considered slightly positive with respect to the cytoplasm (Bates, Goldsmith & Goldsmith, 1982; Gibrat et al., 1985). However, the threshold for the opening of these channels is around 0 mV, as revealed by the finding of transient outwards currents upon depolarizations starting from 0 mV. We might therefore speculate that the opening of these channels is regulated by changes of potential within a small range around 0 mV. For example, such shifts of potential could depend upon a change in the activity of the H⁺-ATPase or upon a downhill carrier-mediated anion influx (Lüttge & Smith, 1985). On the other hand, it is likely that other factors might modulate the opening of these voltage-dependent channels.

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