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Outward K^+ channels in *Brassica chinensis* pollen protoplasts are regulated by external and internal pH

L.-M. Fan**, **Y.-F. Wang,** and **W.-H. Wu***

Department of Plant Sciences, State Key Laboratory in Plant Physiology and Biochemistry College of Biological Sciences, China Agricultural University, Beijing

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Summary. Patch-clamp whole-cell and single-channel recording techniques were used to investigate the regulation of outward K^+ channels by external and internal protons in *Brassica chinensis* pollen protoplasts. Outward K^+ currents and conductance were insensitive to external pH (pH_o) except at pH 4.5. Maximal conductance (G_{max}) for the outward K^+ currents was inhibited at acidic external pH. Half-activation voltage ($E_{1/2}$) for the outward K^+ currents shifted to more positive voltages along with the decrease in pH_o. $E_{1/2}$ can be described by a modified Henderson–Hasselbalch equation expected from a single titratable binding site. The activation kinetics of the outward K^+ channels was largely insensitive to pH_o. An internal pH (pH_i) of 4.5 significantly increased outward K^+ currents and conductance. G_{max} for the outward K^+ currents decreased with elevations in pH_i. In contrast to the effect of pH_o, $E_{1/2}$ was shifted to more positive voltages with elevations in pH_i. The outward K^+ currents, G_{max} and $E_{1/2}$ can be described by the modified Henderson–Hasselbalch equation. Furthermore, acidifying pH_i accelerated the activation of the outward K^+ currents significantly. The differences in electro-physiological properties among previously reported and currently described plant outward K^+ channels may reflect differences in the structure of these channels.

Keywords: *Brassica chinensis*; K^+ channel; Patch clamp; pH; Pollen protoplast.

Abbreviations: $E_{1/2}$ half-maximal activation voltage; E_{rev} reversal potential; G conductance; I current; pH_i, pH_o internal and external pH; pK ionization constant, pH at which half of the binding sites are protonated; $t_{1/2}$ half-activation time; V_m (or V) membrane potential.

Introduction

Pollen germination has been regarded as an ideal model for the study of polarized cell growth (Heslop-Harrison 1987, Mascarenhas 1993, Feijó et al. 1995, Taylor and Hepler 1997). It has been demonstrated that external K^+ is required for both pollen germination and subsequent tube growth (Brewbaker and Kwack 1963, Weisenseel and Jaffe 1976, Feijó et al. 1995). Recently, Mouline et al. (2002) cloned and characterized a K^+ channel of the Shaker family (*SPIK*) from *Arabidopsis thaliana* and showed that disruption of the *SPIK* coding sequence strongly affected inwardly rectifying K^+ channel activity in the pollen plasma membrane, resulting in impaired pollen tube growth. On the other hand, a decrease in K^+ concentration in the cytoplasm during pollen hydration is a prerequisite for protein synthesis and subsequent onset of pollen germination (Bashe and Mascarenhas 1984). It has been suggested that efflux of K^+ through the outward K^+ channels may play a role in the regulation of K^+ concentration in pollen (Obermeyer and Blatt 1995). However, there is little information on the properties of the outward K^+ channels in the pollen plasma membrane and their role in pollen germination. To date, outward K^+ channels from pollen have been reported only twice in lily pollen plasma membrane through patch-clamp or conventional voltage-clamping techniques (Obermeyer and Kolb 1993, Obermeyer and Blatt 1995). It is well established that the external pH (pH_o) is an important regulatory factor for pollen germination and pollen tube growth in vitro (Tupý and Øihová 1984, Southworth 1983, Shivanna and Sawhney 1995), although the optimum pH_o for in vitro pollen germination varies for different species. In general, the optimum pH_o for in vitro pollen

* Correspondence and reprints: Department of Plant Sciences, College of Biological Sciences, China Agricultural University, Beijing 100094, People's Republic of China.
** Present address: Biology Department, Pennsylvania State University, University Park, Pennsylvania, U.S.A.

germination is approximately 6.0 (Brewbaker and Kwack 1963, Southworth 1983) except for *Brassica napus*, where the optimal pH_o for germination is 8.0 (Shivanna and Sawhney 1995).

The regulation of K^+ channels by protons has been reported for many plant cell types (Blatt 1992, Ilan et al. 1994, 1996, Giromini et al. 1997, Hoth et al. 1997, Amtmann et al. 1999). However, it remains unclear whether outward K^+ channel activity in pollen protoplasts can be regulated by protons. The aim of this study is to investigate the regulation of outward K^+ channels in *Brassica chinensis* pollen plasma membranes by external and internal protons.

Material and methods

Isolation of pollen protoplasts

Mature *Brassica chinensis* (var. *chinensis*) pollen protoplasts were isolated as described previously (Fan et al. 1999) with modifications. Briefly, mature pollen grains were collected from ten flowers, washed in standard solution containing 1 mM KNO_3, 0.2 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM KI, 0.1 μM $CuSO_4$, 5 mM $CaCl_2$, 5 mM morpholineethanesulfonic acid-Tris (MES-Tris) (pH 5.8), 500 mM glucose and sorbitol (osmolality, 1.5 osmol/kg) before enzymatic digestion. After filtration through a nylon mesh (pore diameter, 80 μm) and centrifugation at 160 **g** for 5 min, pollen grains were incubated in 2 ml of enzyme solution at 28 °C for about 1 h to release pollen protoplasts. The enzyme solution was prepared with standard solution and contained 1% (w/v) cellulase R-10 (Yakult Honsha Co., Tokyo, Japan), 0.08% (w/v) pectolyase Y-23 (Yakult Honsha Co.), 0.2% (w/v) potassium dextran sulphate (Cal-Biochem, La Jolla, Calif., U.S.A.), and 0.2% (w/v) bovine serum albumin. The mixture was centrifuged at 160 **g** for 5 min and the pellet was resuspended with 2 ml of standard solution. This centrifugation-resuspension cycle was conducted 3 times to completely remove bovine serum albumin, enzymes, and wall debris. The pollen protoplasts were finally resuspended in standard solution and kept on ice before use in patch clamp experiments.

Patch-clamp experiments

Patch-clamp whole-cell and single-channel recordings were conducted at room temperature (20 ± 2 °C) in dim light. For whole-cell recordings, the control bath solution contained 5 mM MES-Tris (pH 5.8), 10 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM potassium glutamate (osmolality at 1.5 mol/kg adjusted with sorbitol). The control pipette solution contained 5 mM HEPES-Tris (pH 7.2), 1 mM 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 100 mM potassium glutamate, and 1 mM $MgCl_2$ (osmolality at 1.5 mol/kg adjusted with sorbitol). The whole-cell recordings conducted under these conditions were taken as controls. Variations in solution composition for different experiments are indicated in the text. Cell capacitance was measured for each cell with the capacity compensation device of the amplifier. All data were acquired 5 min after achieving whole-cell configuration. Whole-cell currents were measured with an Axopatch-200A amplifier (Axon Instruments, Foster City, Calif., U.S.A.), and pCLAMP (version 6.0.4; Axon Instruments) software was used to acquire and analyze the whole-cell currents. After obtaining whole-cell configuration, the membrane potential (V_m) was clamped to –82 mV (holding potential). Whole-cell current data were filtered at 1 kHz and leak currents of whole-cell recordings were subtracted from steady-state currents. The final time-dependent whole-cell currents were normalized as current per unit capacitance (pA/pF) to account for variations in cell surface area. All data are given as means with standard errors. Liquid junction potential was calculated by Clampfit 8.0 (Axon Instruments) and corrected for all whole-cell recordings. Data were analyzed by Student's t-test.

Cell-attached configuration was used for single-channel recordings. Pipette and bath solutions used for single-channel recordings were identical to the control bath solutions used for the whole-cell recordings. Data were acquired for 50 s at each membrane potential indicated in the figure legends, filtered at 1 kHz, and digitized at 8 kHz. pCLAMP 6.0.4 software was used for data analysis. Single-channel current amplitude was obtained from Gaussian fitting of amplitude histograms derived from the data recorded.

Chemicals

All chemicals were obtained from Sigma (St. Louis, Mo., U.S.A.) unless otherwise indicated.

Results

Characterization of B. chinensis pollen outward K^+ channels

Whole-cell outward currents were elicited under control conditions (pH 5.8) when the plasma membrane was depolarized from –82 mV to the voltages more positive to –62 mV (Fig. 1 A). Tail current analysis was used to determine the major ionic species contributing to the recorded whole-cell outward currents (Fig. 1 B). Under control conditions with 10 mM K^+ in the bath and 100 mM K^+ in the pipette solutions, the measured E_{rev} value for the outward currents was nearly -44 ± 1.7 mV ($n = 6$). This value is in close approximation to –56 mV, the theoretical equilibrium potential for K^+. The theoretical equilibrium potentials for Ca^{2+}, Mg^{2+}, Cl^-, and glutamate ions are approximately +177 mV, +2 mV, –62 mV, and +57 mV, respectively. This result suggests that the recorded outward whole-cell currents were mainly carried through outward K^+ channels and/or inward Cl^- channels. Ionic selectivity was determined by substituting $CaCl_2$ with Ca-gluconate. No change was observed in both outward currents and E_{rev}, demonstrating that the K^+ efflux accounted mainly for the recorded whole-cell outward currents (data not shown). Additionally, the more positive E_{rev} relative to E_K suggests that some other ions may slightly contribute to the recorded outward whole-cell currents. This ion was most likely Ca^{2+} since the equilibrium potential for Ca^{2+} was +177 mV.

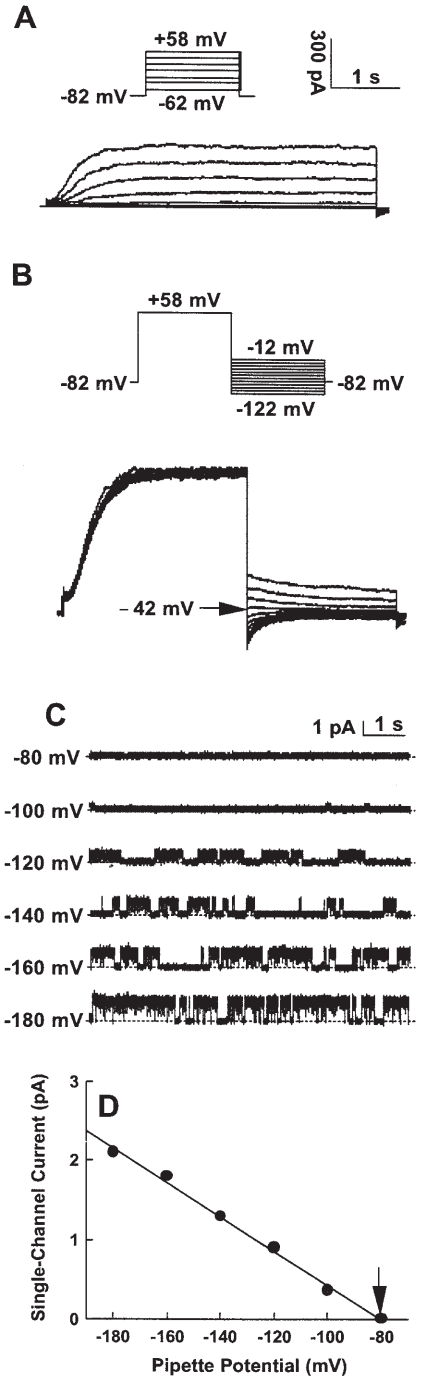

Fig. 1 A–D. Characterization of outward whole-cell K^+ currents in *B. chinensis* pollen protoplast. **A** Typical whole-cell patch-clamp recording; **B** tail current analysis. **C** Typical single-channel recordings in cell-attached configuration under the control conditions; **D** corresponding *I-V* relationship of single-channel recordings. The arrow in **B** indicates the reversal potential of the whole-cell currents. The voltage values indicated on the left of the recordings in **C** are pipette potentials, and the arrow in **D** indicates the reversal pipette potential. Base levels of channel currents in **C** are shown in dotted lines

In cell-attached configuration under control conditions, the measured resting membrane potential of Brassica pollen protoplasts was –79 ± 2.8 mV (n = 67), while the measured reversal pipette potential of the currents was –71 ± 5.5 mV (n = 10). According to $V_m = (V_c - V_p) + V_L$ (V_m, membrane potential; V_c, cell resting potential; V_p, command pipette potential; V_L, liquid junction potential), the reversal membrane potential should be approximately +14 mV. Assuming an intracellular K^+ concentration of 120 mM (Obermeyer and Kolb 1993, Obermeyer and Blatt 1995), the Nernst equilibrium potential for K^+ across the clamped membrane patch would be approximately –4 mV, which is close to and less positive than the above calculated reversal membrane potential. This result is in agreement with data obtained from whole-cell recordings. Figure 1 C and D shows typical cell-attached recordings and the corresponding *I-V* relationship of the outward K^+ currents. Taken together, the results demonstrate that K^+ efflux through outward K^+ channels in pollen plasma membrane accounts mainly for the recorded outward currents.

Effects of protons on the outward K^+ currents and conductance

Figure 2 shows the effects of the external pH (pH_o) on whole-cell outward K^+ currents and conductance. Outward K^+ currents and conductance were insensitive to pH_o except at pH 4.5 (Fig. 2 A–C). The whole-cell current and conductance at +58 mV decreased by about 50% with acidification from 5.8 to 4.5 (Fig. 2 A, B). This result is consistent with those obtained from cell-attached recordings (Fig. 3). The single-channel current remained largely unchanged over time when the pH of the pipette solution was 5.8 (Fig. 3 A), whereas a time-dependent reduction of the current was observed at pH 4.5 (Fig. 3 B). This is likely to be due to a gradual equilibration of the pH of the cytoplasm with acidifying pipette solution. Because the pH of the bath solution was 5.8, the diffusion of the pipette solution at pH 4.5 could result in an acidic-going external milieu outside the attached membrane patch. Thus, the currents at 1 min and 5 min at pH 4.5 could be considered as being recorded in different pH_o environments beginning at pH 5.8 and ending at a more acidic pH (Fig. 3 B).

Figure 4 illustrates the effect of pH_i on the whole-cell outward K^+ currents and conductance. An internal pH (pH_i) of 4.5 significantly increased outward K^+ cur-

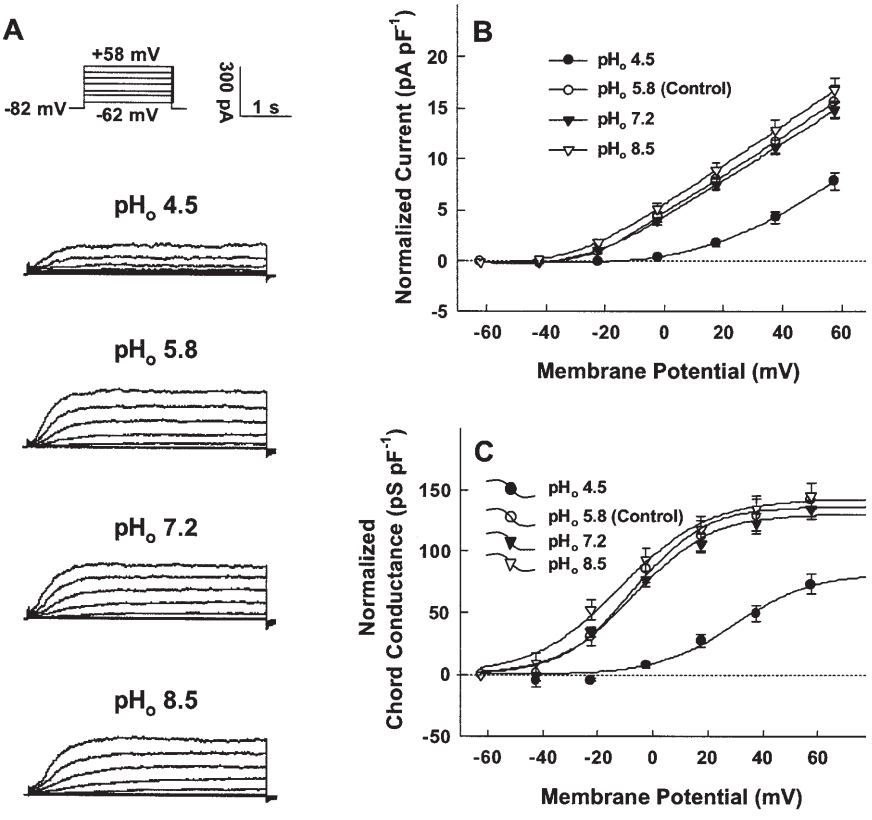

Fig. 2 A–C. Effects of pH_o on outward whole-cell K^+ currents and conductance of *B. chinensis* pollen protoplasts. **A** Whole-cell recordings at various pH_o; **B** *I-V* relationships at various pH_o; **C** *G-V* relationships at various pH_o. Values are means with standard errors for n = 18 for pH 4.5, 5.8, and 8.5 and for n = 16 for pH 7.2

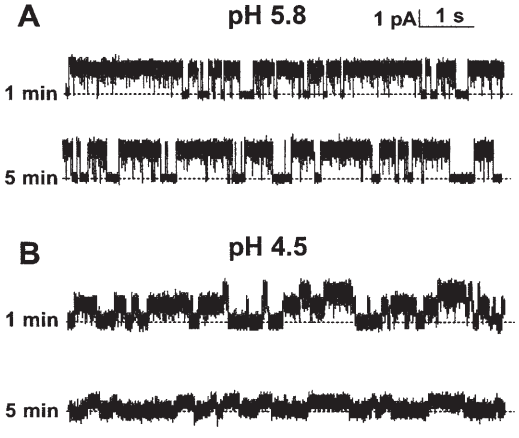

Fig. 3. Single-channel recordings in cell-attached configuration at pH_o 5.8 (**A**) and pH_o 4.5 (**B**) of the pipette solution at a pipette potential of –180 mV. The time on the left of each recording represents the time period after achieving cell-attached configuration. Base levels of channel currents are shown in dotted lines

rents and conductance (Fig. 4 A, B). The whole-cell current at +58 mV increased by about 66% with acidification from 5.8 to 4.5 (Fig. 4 A), while the outward K^+ conductance increased by about 56% (Fig. 4 B).

Effects of protons on the steady-state properties of the outward K^+ currents

The effects of pH on voltage-independent maximal conductance (G_{max}) and the half-maximal activation voltage ($E_{1/2}$) of the outward K^+ currents were obtained by Boltzmann fitting of the *G-V* relationships of the outward K^+ currents. G_{max} decreased significantly at pH_o 4.5 compared to that at pH_o 5.8 (Fig. 5 A). This result is consistent with pH regulation of the outward K^+ currents and conductance presented in Fig. 2. $E_{1/2}$ shifted to more positive voltages along with the decrease in pH_o (Fig. 5 B).

The following function (Amtmann et al. 1999) was used to obtain titration p*K* values for the relationships of the outward current vs. pH_o, G_{max} vs. pH_o, and $E_{1/2}$ vs. pH_o:

$$X = X_{min} + (X_{max} - X_{min})/(1 + 10^{pH-pK}), \quad (1)$$

where X is the current or G_{max} or $E_{1/2}$, X_{min} is the basal current or G_{max} or $E_{1/2}$ in absence of protonation, and p*K* is the pH at which half of the binding sites are protonated. This is a modified version of the Henderson–Hasselbalch equation and is based on the

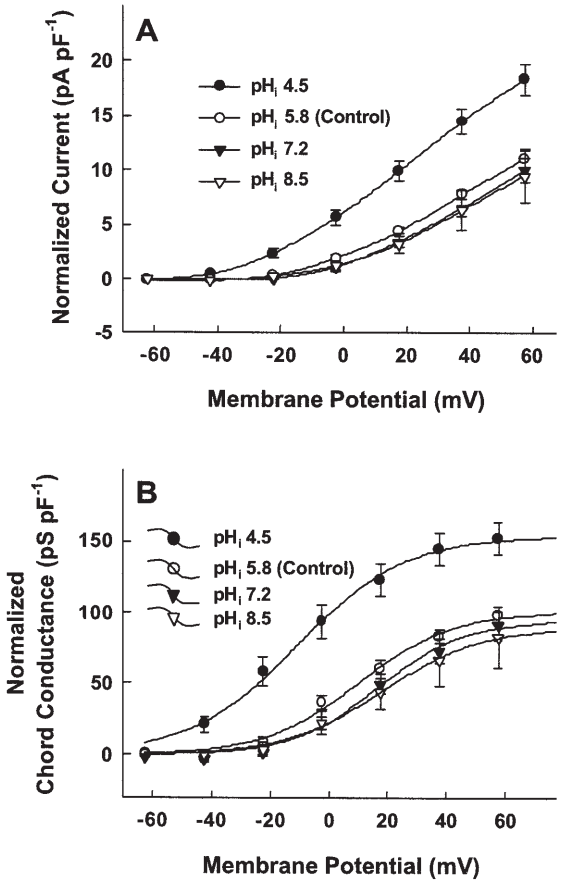

Fig. 4 A, B. Effects of pH_i on outward whole-cell K^+ currents and conductance. **A** *I-V* relationships at various pH_i; **B** *G-V* relationships at various pH_i. Values are means with standard errors for n = 7 for pH 4.5, n = 6 for pH 5.8 and 7.2, and n = 5 for pH 8.5

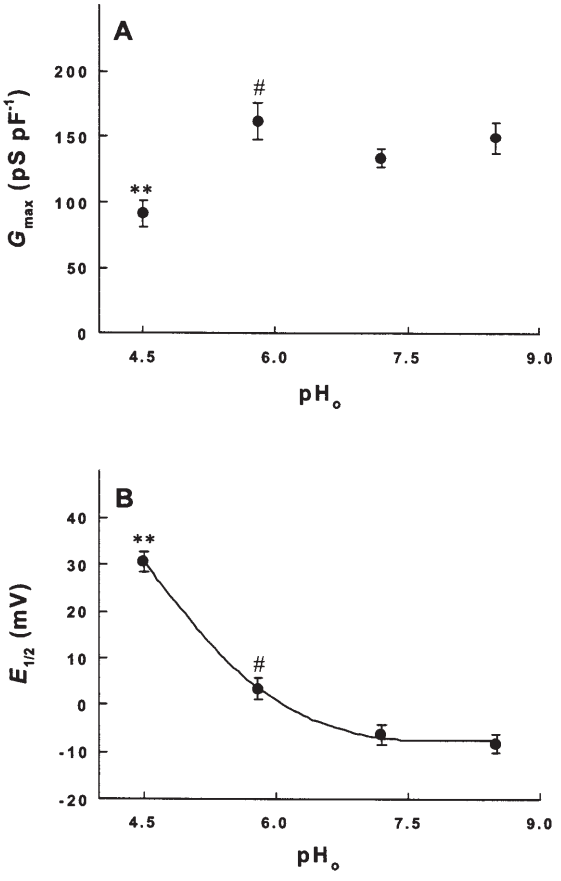

Fig. 5. Titration of pH_o effects on steady-state properties of outward whole-cell K^+ currents. The symbols represent the raw data (means with standard errors), and the solid line is the fit with Eq. (1). The numbers of replicates for each treatment are the same as in Fig. 2 B and C. # Control; ** significantly different from the control by t-test, $P \leq 0.01$

assumption that there exists a single titratable binding site of proton for each parameter tested.

The pH_o titration of the above parameters shows that the outward whole-cell currents and G_{max} did not follow Eq. (1) (Fig. 5 A), whereas $E_{1/2}$ could be well fitted (Fig. 5 B). The resulting p*K* for the relationship of $E_{1/2}$ to pH_o of the outward K^+ currents was 5.33.

In contrast to the effect of pH_o, $E_{1/2}$ was shifted to more positive voltages with elevations in pH_i. The outward whole-cell currents, G_{max}, and $E_{1/2}$ vs. pH_i fitted Eq. (1) very well (Fig. 6 A–C). The resulting p*K*s for G_{max} vs. pH_i, and $E_{1/2}$ vs. pH_i relationships were 4.94 and 5.30, respectively, and the resulting p*K*s for the relationship of *I* to pH_i of the outward K^+ currents at different membrane potentials are shown in Table 1. The p*K* values for the latter remained largely consistent and were approximately 4.90.

Table 1. p*K* values for the outward currents vs. pH_i at various membrane potentials

Membrane potential (mV)	p*K*
–42	4.56
–22	4.09
–2	4.97
+18	4.92
+38	4.95
+58	4.91

Effects of protons on the activation kinetics of the outward K^+ currents

The outward K^+ currents activated with a sigmoidal time course and can be fitted by a Hodgkin–Hux-

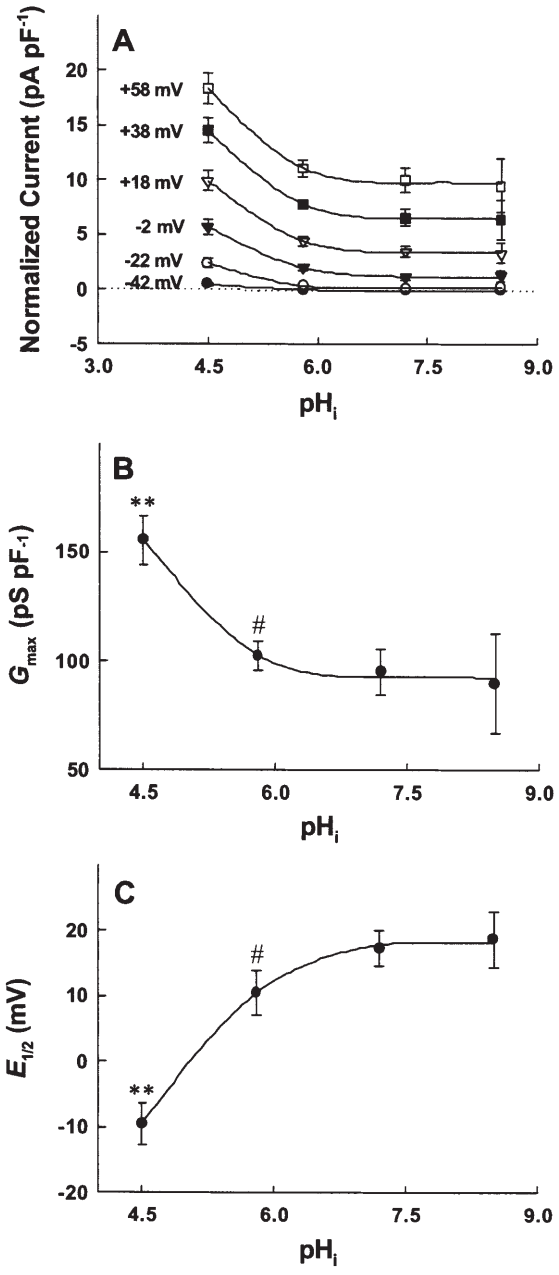

Fig. 6. Titration of pH_i effects on magnitude (**A**) and steady-state properties (B and C) of outward whole-cell K^+ currents. The symbols represent the raw data (means with standard errors), and solid lines are the fits with Eq. (1). The numbers of replicates for each treatment in A, B, and C are the same as in Fig. 4 A and B. # Control; ** significantly different from the control by t-test, $P \le 0.01$

ley-type model (Hodgkin and Huxley 1952), according to the following equation:

$$I = I_L + I_\infty[1 - \exp(-t/\tau)]^p, \quad (2)$$

where p is the number of independent membrane bound gating particles that control the opening of the channel, I_L is a leak component and I_∞ is the steady-state current after activation, τ is the activation time constant. For most cases the outward whole-cell currents were best fitted (using the least-squares fitting method) when p was set to 3 (Fig. 7 A).

Figure 7 B and C shows the effects of pH_o and pH_i on the activation kinetics of the outward K^+ currents at +58 mV, respectively. The half-activation time ($t_{1/2}$) of the outward K^+ currents exhibited no regular response to pH_o but was the longest at pH_o 4.5 (Fig. 7 B), corresponding to the inhibitory effect of pH_o 4.5 on the outward K^+ currents and conductance (Fig. 2). The response of the activation time constants to pH_o was similar to that of the half-activation time.

Changes in pH_i affected $t_{1/2}$ of outward K^+ currents significantly. The more acidic the pH_i, the faster was the activation of the outward K^+ currents. The activation time constant of the currents at +58 mV increased with alkalinization of pH_i (Fig. 7 C). The increase in the time constant could account for the increase in the half-activation time of the outward currents with the elevation of pH_i.

Discussion

Responses of outward K^+ channels to protons in different cell types

The regulation of outward K^+ channels in higher-plant cells by pH_o was reported only for *Vicia faba* guard cells (Blatt 1992, Blatt and Amstrong 1993, Grabov and Blatt 1997), *V. faba* guard cell protoplasts (Ilan et al. 1994), suspension-cultured *A. thaliana* cell protoplasts (Giromini et al. 1997), and heterologously expressed SKOR (an Arabidopsis depolarization-activated K^+-selective channel) in *Xenopus laevis* oocytes (Lacombe et al. 2000) and GORK (Ache et al. 2000). Current through the outward K^+ channels in *V. faba* guard cells (Blatt 1992) was reported to be largely unaffected by pH_o. In addition, the outward K^+ current was also reported to be insensitive to pH_o in suspension-cultured Arabidopsis cell protoplasts (Giromini et al. 1997). In contrast, acidifying pH_o strongly inhibited the outward K^+ channel currents in *V. faba* guard cell protoplasts (Ilan et al. 1994). Acidification of pH_o also inhibited the currents of heterologously expressed SKOR (Lacombe et al. 2000) and GORK (Ache et al. 2000). The response of Brassica pollen outward K^+ channels to pH_o presented in this

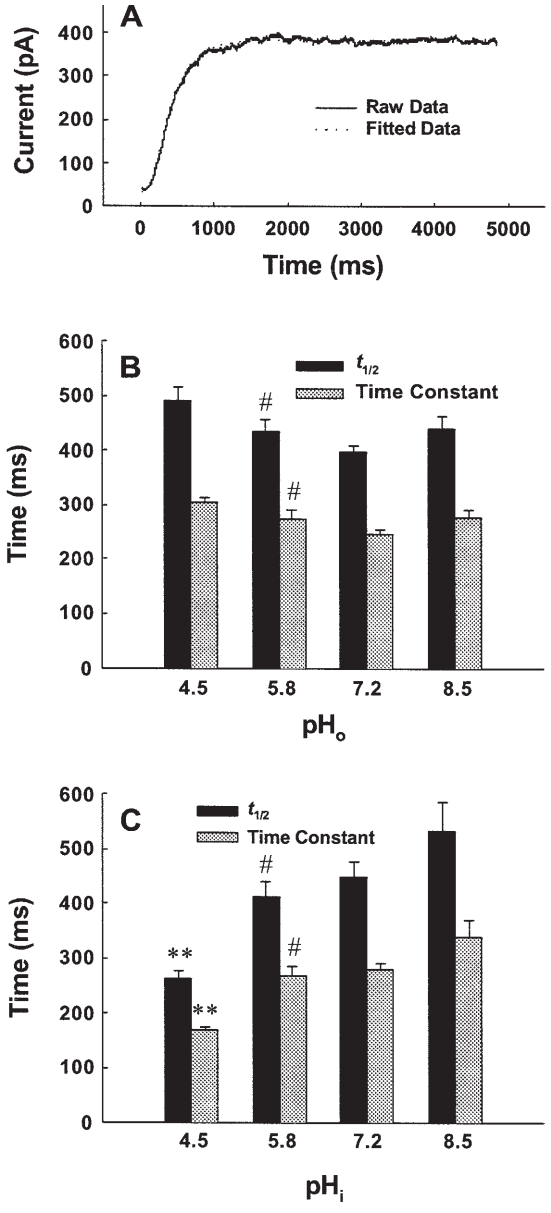

Fig. 7 A–C. Effects of pH_o and pH_i on activation kinetics of outward whole-cell currents. **A** Representative of the fittings of the activation of the outward whole-cell currents at +58 mV under the control conditions; **B** dependence of $t_{1/2}$ and activation time constant on pH_o; **C** dependence of $t_{1/2}$ and activation time constant on pH_i. Note, $t_{1/2}$ and activation time constants in **B** and **C** were derived from the fittings of the outward whole-cell currents at +58 mV for various pH_o or pH_i, respectively. The numbers of replicates for each treatment in B are the same as in Fig. 2 B and C, while the numbers of replicates for each treatment in C are the same as in Fig. 4 A and B. Values in B and C are expressed as means with standard error. # Control; ** significantly different from the control by t-test, $P \leq 0.01$

work was similar to the results from *V. faba* guard cell protoplasts (Ilan et al. 1994).

The inhibitory effect of acidifying pH_i on the outward K^+ channels was reported for *V. faba* guard cells (Blatt 1992, Blatt and Amstrong 1993, Miedema and Assmann 1996, Grabov and Blatt 1997), suspension-cultured Arabidopsis cell protoplasts (Giromini et al. 1997), and heterologously expressed SKOR (Lacombe et al. 2000). In contrast, an acidic pH_i 4.5 significantly increased the outward K^+ currents and conductance in Brassica pollen protoplasts and promoted the activation kinetics. The differences in electro-physiological properties among the previously reported and currently described plant outward K^+ channels may reflect a difference in the structure of these channels.

Hypothetical mechanisms of pH modulation of outward K^+ channels

The inhibitory effect of pH_o on the macroscopic conductance of the outward K^+ channel in *V. faba* guard cell protoplasts was attributed to the number of available channels but not the single-channel conductance (Ilan et al. 1994). The effect of pH_o on $E_{1/2}$ (V_{50}) reflects proton-dependent modulation of channel gating (Amtmann et al. 1999). The data of Ilan et al. (1994, 1996) suggest that the effect of external protons on channel gating is due to the decrease of the negative surface charge density in the vicinity of the channel gates. An analysis performed on KST1, an inward K^+ channel from potato, suggested that a His residue situated in the outer-pore region of this inward channel is crucial for the protonation on $E_{1/2}$ (V_{50}) (Hoth et al. 1997). This observation was then supported by the result that the pK of 6.5 derived from the pH titration of $E_{1/2}$ (V_{50}) of the inward K^+ currents in root cell protoplasts was similar to the pK of His titration (Amtmann et al. 1999). However, the pK of 5.33 for the relationship of $E_{1/2}$ (V_{50}) to pH_o was determined with the same fitting method in the present study. Such a pK value is characteristic of a β or γ carboxyl group of an Asp or Glu in a nonpolar environment (Segel 1975), indicating that an Asp or Glu residue would be a low-affinity protonation site responsible for the voltage-dependence of Brassica pollen outward K^+ channel.

It is unclear how pH_i regulates the activity of the outward K^+ channels in plant cells. Armstrong and Blatt (1993) suggested that the pH_i sensitivity of the outward K^+ current in *V. faba* guard cells can be attributed to proton binding of the channel or regulatory factors, and that the steep pH_i dependence is unlikely to reflect a simple titration of a single site. The mech-

anism by which pH_i regulates the outward K^+ channels in Brassica pollen protoplasts is likely to be different since there are at least three differences in the properties between these two channel types. First, the acidification of pH_i significantly increased outward K^+ current and conductance in Brassica pollen protoplast, while the opposite is true for Vicia guard cells. Second, $E_{1/2}$ of the outward K^+ channel in Brassica pollen protoplast was sensitive to pH_i, while $E_{1/2}$ of outward K^+ channel in *V. faba* guard cells was not. Third, the macroscopic outward K^+ currents in Brassica pollen protoplast can be described by a modified Henderson–Hasselbalch equation, which is based on the assumption that there exists a single titratable binding site of proton for each parameter. On the basis of the resulting p*K* value of 5.30 derived from the titration of $E_{1/2}$ vs. pH_i, it is reasonable to assume that there is a protonation site, Asp or Glu residue, within the channel responsible for the control of its voltage dependence. These differences suggest that outward K^+ channel in Brassica pollen may be structurally different from those reported previously in *V. faba* guard cells (Blatt 1992, Armstrong and Blatt 1993).

Possible physiological significance of the outward K^+ channels in pollen plasma membranes

Maintenance of a relatively constant K^+ level in the cytoplasm is generally accepted as a prerequisite for normal biochemical and osmotic processes in plant cells (Walker et al. 1996). However, alterations in cytoplasmic K^+ level in pollen occur during pollen hydration and germination and are required for the onset of germination (Bashe and Mascarenhas 1984). Dehydration of pollen grains prior to anther desiccation (Heslop-Harrison 1987) results in an increase of cytoplasmic K^+ concentration, with concentrations as high as 280 mM in *Tradescantia paludosa* pollen grains (Bashe and Mascarenhas 1984). It has been shown that concentrations of K^+ above 220 mM completely inhibited in vitro protein translation (Weber et al. 1978). Therefore, pollen grains at the time of release from the anther are likely to be in a quiescent state. Rehydration and swelling during germination result in K^+ concentrations suitable for initiation of protein synthesis, which is required for pollen germination and pollen tube growth (Bashe and Mascarenhas 1984, reviewed by Mascarenhas 1993). Moreover, excessive external K^+ supply is more harmful to pollen germination than to pollen tube growth (Fan et al. 2001). These studies suggest that an excessive K^+ influx is unnecessary and even harmful to the initiation of pollen germination, particularly at the early stage of pollen germination. The outward K^+ channels in the plasma membrane may provide a conduit for the efflux of K^+ from the cytoplasm, a process dependent on a trans-membrane electro-chemical potential gradient. It has been reported that membrane potentials in germinated lily pollen grains or tubes were always more positive compared to ungerminated or nongerminating pollen grains (Weisenseel and Wensich 1980, Malhó et al. 1995). Obermeyer and Blatt (1995) observed that membrane potentials were highly variable in nongerminating pollen grains, although some pollen grains exhibited a V_m more positive than or around the corresponding equilibrium potentials for K^+ in given K^+ conditions. The rehydration and swelling of a pollen grain result in an increase in surface area and thereby an increase in cell membrane capacitance. Assuming the net quantity of electric charge of a pollen grain is kept constant, i.e., there is no net ion flux across the cell membrane, the membrane potential will shift to a less negative value, according to the following equations:

$$V_1 = Q/C_1,\ V_2 = Q/C_2, \quad \text{thus,} \quad V_2 = V_1C_1/C_2,$$

where V_1 and V_2 are the membrane potentials before and after swelling of the pollen grain, respectively; C_1 and C_2 are the cell membrane capacitances before and after swelling of the pollen grain, respectively; and Q is the net quantity of electric charge of the pollen grain.

Under these circumstances, the outward K^+ channels in the pollen plasma membrane may be activated, thereby contributing to the hyperpolarization of the membrane along with the activation of H^+-ATPase. Considering the existence of inward K^+ channels (Obermeyer and Kolb 1993, Obermeyer and Blatt 1995, Fan et al. 1999, 2001, Fan and Wu 2000) and of hyperpolarization- and stretch-activated calcium-permeable channels (Y.-F. Wang and W.-H. Wu, China Agricultural University, Beijing, unpubl. data) in the pollen plasma membrane, the hyperpolarization of the membrane and the germinating pore-localized stretch of the plasma membrane would drive calcium influx and potassium influx at the site of pollen tube emergence, which have been suggested to be involved in establishment of polarization and maintenance of turgor pressure in pollen grain (Feijó et al. 1995). Since protons regulate pollen germination and tube growth

and outward K^+ channel activity, it is likely that protons affect pollen growth behavior at least partly via their impact on the outward K^+ channels in pollen. However, to directly demonstrate the physiological significance of the K^+ efflux via the outward K^+ channels requires further investigation.

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