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Characterization of Whole-cell K^+ Currents across the Plasma Membrane of Pollen Grain and Tube Protoplasts of *Lilium longiflorum*

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Abstract. Outward and inward currents, mainly carried by K^+, were detected in protoplasts of pollen grains (PG) and pollen tubes (PT) of *Lilium longiflorum* Thunb. by using the whole-cell configuration of the patch-clamp technique. The outward K^+ current (I_{K^+out}) was similar in both protoplast types, while the inward K^+ current (I_{K^+in}) was higher in pollen tube protoplasts. In PT but not in PG protoplasts, inward K^+ currents were already detectable at negative membrane voltages usually monitored in lily pollen. I_{K^+in} consisted of a slow and a fast current component, as revealed by fitting a sum of two exponential functions to the time-dependent current. The contribution of the fast component to the total inward current was higher in PT than in PG protoplasts, which was even more evident at acidic pH of the external medium. Therefore, based on the measured characteristics, the I_{K^+in} of PT protoplasts may contribute to the endogenous K^+ currents surrounding a growing pollen tube.

Key words: Ion channel — K^+ channel — Lily pollen — Patch clamp — Pollen tube — Tip growth

Introduction

Pollen grain germination and pollen tube growth are essential steps in reproduction of flowering plants. Upon landing on a compatible stigma, pollen grains, the male gametophytes of higher plants, germinate, grow a pollen tube through the female tissue, and finally deliver the sperm cells to the ovaries, where fertilization takes place. This specialized way of polar cell elongation, the so-called tip growth, is achieved by several cellular components and processes, e.g., cytoskeleton, signal transduction pathways, ion gradients and currents, forming a sensitive network that is responsible for the fast growth and the orientation of the pollen tube (*see* recent reviews of Franklin-Tong, 1999; Zheng & Yang, 2000; Hepler, Vidali & Cheung, 2001). In this regulatory network, an extracellular oscillating electrical field surrounding the germinating pollen grain and the growing pollen tube, may serve as a coordination system helping to determine the direction of tube growth, and may also synchronize cellular processes (Feijó, Malhó & Obermeyer, 1995; Feijó et al., 2001). First measurements indicated that ion currents generate the electrical field around a growing pollen tube across the plasma membrane, carried mainly by an influx of K^+ along the tube, an active extrusion of H^+ at the grain, and an influx of Ca^{2+} at the extreme tube tip (Weisenseel, Nuticelli & Jaffe, 1975; Weisenseel & Jaffe, 1976; Kühtreiber & Jaffe, 1990). Local and temporal fluctuations in the tip-localized Ca^{2+} influx are probably responsible for the speed and the direction of tube growth (Malhó et al., 1994; Malhó & Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli & Robinson, 1997; Messerli, Danuser & Robinson, 1999) and in general, oscillations of one or more of the ion fluxes across the plasma membrane may create a positive feedback loop involved in the generation of morphological patterns, e.g., organization of the cytoskeleton, cell-wall deposition and organelle distribution (Feijó et al., 2001).

To understand the molecular basis of these oscillations, the respective ion currents and the regulation of the ion transporters carrying these currents have to be characterized. Proton-translocating ATPases of the plasma membrane were identified, localized and sequenced from *Arabidopsis* (Houlné & Boutry, 1994), tobacco (Oufattole, Arrango & Boutry, 2000; Zhao et al., 2000) and lily pollen (Obermeyer et al., 1992; Pertl et al., 2001; Gehwolf

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Abbreviations: BS, bath solution; BTP, bis-Tris-propane; MES, 2-*N*-morpholinoethane sulfonic acid; V_{act}, activation voltage; V_M, membrane voltage; E_{rev}, reversal potential; I_{K^+in}, inward K^+ current; I_{K^+out}, outward K^+ current; PG, pollen grain; PT, pollen tube; PM, pipette medium

et al., 2002), and activation of the plasma membrane H^+ ATPases was suggested as an essential step to enable the influxes of K^+ and water (Feijó et al., 1995; Pertl et al., 2001). Recently, a hyperpolarization-activated K^+ channel, which is strongly expressed in pollen grains of *Arabidopsis thaliana*, was identified (Mouline et al., 2002). In other pollen species, K^+ channels and inward- as well as outward-rectifying K^+ currents were observed and partially characterized. In protoplasts obtained from pollen grains of *Brassica* (Fan, Wu & Yang, 1999) and *Arabidopsis* (Fan, et al., 2001) K^+ currents were measured using the whole-cell configuration of the patch-clamp technique. In lily pollen, single K^+ channels (pollen grain protoplasts; Obermeyer & Kolb, 1993) and K^+ currents (intact pollen grains; Obermeyer & Blatt, 1995) were characterized by patch-clamp and 2-electrode voltage-clamp techniques, respectively. Although the biophysical properties of the lily pollen grain K^+ channels and currents have been measured in detail, a contribution of the inward-rectifying K^+ current to the K^+ influx during pollen grain germination and tube growth under physiological conditions could not be postulated from these measurements. At 1 mM K^+ in the germination medium, the inward K^+ current was only observed at voltages more negative than −160 mV (Obermeyer & Blatt, 1995), whereas the average, free running membrane voltage (V_M) of lily pollen grains was ranging between −90 mV (Weisenseel & Wenisch, 1980) and −110 mV (Obermeyer & Blatt, 1995). Therefore, still unknown regulatory factors that shift the activation voltage (V_{act}) of the K^+ inward current to more positive values or not yet identified K^+ current components may be involved in the K^+ uptake during pollen tube growth. In this study, the inward-rectifying K^+ currents of protoplasts from pollen grains and pollen tubes were measured, and a K^+ current component enabling the uptake of K^+ under physiological conditions was detected in pollen tube protoplasts.

Materials and Methods

Plant Material

Lilium longiflorum Thunb. plants were obtained from a local flower shop (Blumen Doll, Salzburg, Austria). Mature, fully dehydrated anthers were collected and used immediately for the patch-clamp experiments or frozen in liquid nitrogen and stored at −70°C for not longer than 3 months. Fresh and frozen pollen grains showed similar activities in germination, pollen tube growth and K^+ currents.

Isolation of Protoplasts

Pollen grain (PG) protoplasts were prepared according to Tanaka, Kitazume & Ito (1987) and Obermeyer & Kolb (1993) with the following modifications. Pollen grains of one anther were washed in isolation medium (in mM: 680 mannitol, 5 $CaCl_2$, 10 KCl, 0.5 ascorbic acid, 10 MES adjusted with BTP to pH 6.0), resuspended in enzyme solution (isolation medium with 24 U ml^{-1} cellulase "Onozuka" (Serva, Heidelberg, Germany), 2 U ml^{-1} pectinase (Sigma) and 2.6 U ml^{-1} macerocyme (Serva)) and incubated in the dark for 2 h with gentle shaking at 21°C. After enzymatic cell wall digestion the protoplasts were washed twice in isolation medium by centrifugation for 5 min at 50× *g* (4°C) and resuspended in isolation medium. Finally, protoplasts were purified using a sucrose step gradient to separate the protoplasts from cell wall and exine material using a 0.8 M sucrose solution, a 1:1 mix of 0.8 M sucrose and isolation medium, and the protoplast suspension. After centrifugation for 11 min at 50× *g* (4°C), the protoplasts banded at the interface between the 0.8 M sucrose and the 1:1 mixture. They were carefully collected and stored on ice to prevent regeneration of the cell wall. Protoplasts were used within 3–4 h.

To obtain pollen tube protoplasts, pollen grains of one anther were washed and suspended in germination medium (10% (w/v) sucrose; in mM: 1.6 H_3BO_3, 1 KCl, 0.1 $CaCl_2$, 5 MES/BTP pH 5.6). After incubation for 4 h at 21°C the germinated pollen grains were separated from ungerminated grains by filtration through a metal net with a mesh width of 120 μM. The germinated pollen grains were resuspended in an enzyme solution (isolation medium with 22 U ml^{-1} cellulase "Onozuka" and 11.2 U ml^{-1} macerocyme and incubated while gently shaken at 21°C for 30 min. After enzymatic cell wall digestion the isolated protoplasts were centrifuged three times for 5 min at 50× *g* (4°C). The pellet was washed twice with 2 ml isolation medium. To increase the concentration of pollen tube (PT) protoplasts after the third centrifugation, the pellet was resuspended in 50 μl isolation medium and stored on ice.

Patch-clamp Experiments

Conventional patch-clamp technique was applied according to Hamill et al. (1981). The experiments were performed in the whole-cell configuration using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Union City, CA, USA).

The protoplasts were placed in standard bath solution (BS-100K, Tab. 1) containing in mM: 10 KCl, 90 K-glutamate, 5 $CaCl_2$, 25 MES adjusted to pH 7.2 with BTP; the osmolality was adjusted with mannitol to about 700 mosmol kg^{-1} for pollen grain (PG) protoplasts and to about 750 mosmol kg^{-1} for pollen tube (PT) protoplasts. Increasing the bath solution osmolality in PT protoplast experiments increased the sealing rate.

The patch capillaries (thick-walled borosilicate glass, Hilgenberg, Malsfeld, Germany) were cleaned with 70% ethanol for 4 h and dried at 80°C before micropipettes were pulled to an outer diameter of 1.5–2 μM (pipette puller L/M-3P-A, List-Medical, Darmstadt, Germany). Micropipettes were backfilled with standard pipette medium (PM-100K) containing (in mM): 450 mannitol, 10 KCl, 90 K-glutamate, 1 EGTA, 2 Mg-ATP, 0.54 $CaCl_2$ (resulting in 240 nM free Ca^{2+}, according to Günzel & Galler, 1990), 25 MES adjusted to pH 7.2 with BTP (osmolality was the same as for the standard bath solution). The filled pipettes were dipped briefly into SigmaCote (Sigma) and used immediately for patch-clamp measurements.

The micro-electrode was attached to the plasma membrane of a pollen protoplast forming a resistance in the GΩ range (2–10 GΩ) and the whole-cell configuration was achieved by gentle suction. Between the application of pulse protocols, the whole-cell parameters, such as access resistance and cell capacity, were monitored to avoid errors in the voltage clamp due to the low membrane resistance resulting from the big surface area of the lily pollen protoplasts (typical protoplast diameter of ca. 80 μM). Usually, the whole-cell configuration was stable after 5 min, and then a voltage pulse protocol was applied. Starting from a holding potential

Table 1. Comparison of the salt concentrations (in mM) in the pipette media (PM) and bath solutions (BS) used in the patch-clamp experiments

	PM-100K	PM-100KCl	BS-100K	BS-100KCl	BS-10K	BS-TEA	BS-BaCl	BS-1K
KCl	10	100	10	100	10	10	10	1
HCl	–	–	–	–	–	–	–	9
K-Glutamate	90	–	90	–	–	–	–	–
Mg-ATP	2	2	–	–	–	–	–	–
EGTA	1	1	–	–	–	–	–	–
$CaCl_2$ (free Ca^{2+})	0.54 (240 nM)	0.54 (240 nM)	5	5	5	5	5	5
Inhibitor	–	–	–	–	–	10 TEACl	10 BaCl	–

Unless otherwise stated, all solutions were buffered with 25 mM MES and adjusted to pH 7.2 with BTP. The osmolality was adjusted with mannitol to 700 mOsm for experiments with PG protoplasts and to 750 mOsm for experiments with PT protoplasts. The free Ca^{2+} concentration was calculated according to Günzel & Galler (1990).

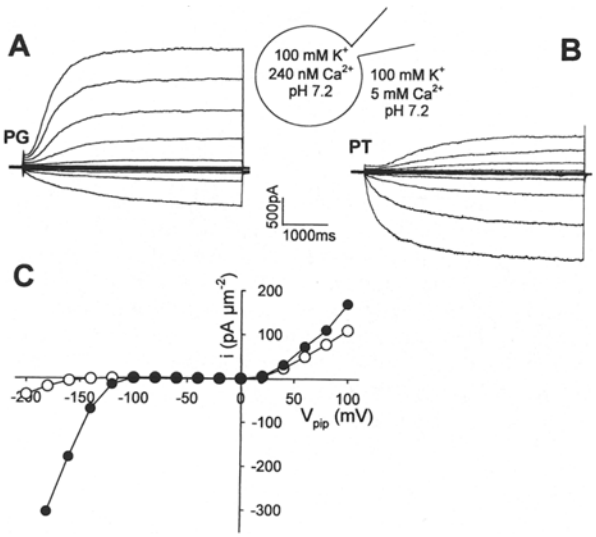

Fig. 1. Typical whole-cell patch-clamp recordings of pollen protoplasts under ionic conditions as indicated. Starting from a holding potential of −20 mV, voltage pulses from +100 to −180 or −200 mV with increments of 20 mV were applied. (*A*) Pollen grain (PG) protoplast with a diameter of 85 µM. (*B*) Whole-cell currents of a pollen tube protoplast (PT) of 40 µM diameter. (*C*) *I-V* curve of the time-dependent current densities of a PG (open circles) and a PT (filled circles) protoplast.

(V_{hold}) of −20 mV (100 mM K^+ in the bath) or −80 mV (1 mM K^+ in the bath), a prepulse of −120 mV and 6 s duration was applied to close all slowly-deactivating, outward-conducting K^+ channels, followed by voltage pulses with a duration of 5 s ranging from −200 or −180 mV to +100 mV in 20 mV increments.

To determine the reversal potentials (E_{rev}), the pipette and bath media contained only 100 mM KCl instead of KCl and K-glutamate (PM-100KCl, BS-100KCl, *see* Table 1). A double-pulse protocol was used: starting from V_{hold}, this protocol consisted of a large positive or negative voltage pulse, which activated the outward or the inward currents, followed by a set of less positive (or negative) potential pulses, which deactivated the outward (or inward) current. The deactivation of the respective current resulted in a transient change in whole-cell current to a new steady-state current. E_{rev} was determined as the voltage value where the potential difference between the instantaneous and the new steady-state current of the second pulse was zero.

For generation and application of the pulse protocols and data recording, the program Clampex (pClamp 6.0.2, Axon Instruments) was used.

Solutions

All patch-clamp experiments started with standard conditions, namely BS-100K and PM-100K as bath and pipette medium, respectively. After recording the current under these symmetrical K^+ conditions, the bath solution was exchanged during the experiments (Tab. 1). Perfusion of the bath chamber was done manually within 30–60 s using 10–15 times the chamber volume.

Data Analysis

For basic analysis the Clampfit software (pClamp 6.0.2) was used. Further data analysis was carried out using Origin 4.1 (Microcal Software, Northampton, MA). In the *I-V* diagrams the time-dependent current was plotted against the pipette voltage (V_{pip}).

To determine the activation potential (V_{act}) of the outward and the inward current, namely the membrane voltage at which the currents became detectable, the respective *I-V* curves were fitted with a 5th-degree polynomial. We defined V_{act} as the voltage where the fitted curve intercepts the x-axis.

Results

Inward and outward K^+ currents of protoplasts obtained from ungerminated pollen grains (PG, diameter = 82.5 ± 6.3 µM, n = 148) and for the first time in protoplasts derived from pollen tubes (PT, diameter = 44.1 ± 10 µM, n = 40) were measured by using the whole-cell configuration of the patch-clamp technique. Typical whole-cell currents under symmetrical K^+ conditions (bath: 100 mM K^+, 20 mM Cl^-, 5 mM Ca^{2+}, pH 7.2; pipette: 100 mM K^+, 11 mM Cl^-, 240 nM Ca^{2+}, pH 7.2) are shown in Fig. 1*A* and *B*. In both protoplast types we observed a sigmoid, time-dependent activation of the outward current (I_{K^+out}) after depolarizing voltage steps. Comparing the outward currents of PG and PT protoplasts, little differences concerning the current densities were detectable: sometimes a little higher conductance of

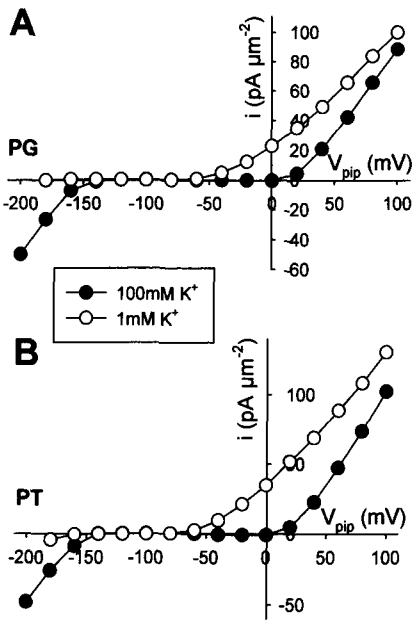

Fig. 2. Typical time-dependent current densities of pollen protoplasts at different K^+ concentrations of the bath solution. (*A*) PG protoplast. (*B*) PT protoplast. Standard pipette medium (PM-100K) and the bath media BS-100K (●) and BS-1K (○) were used, respectively.

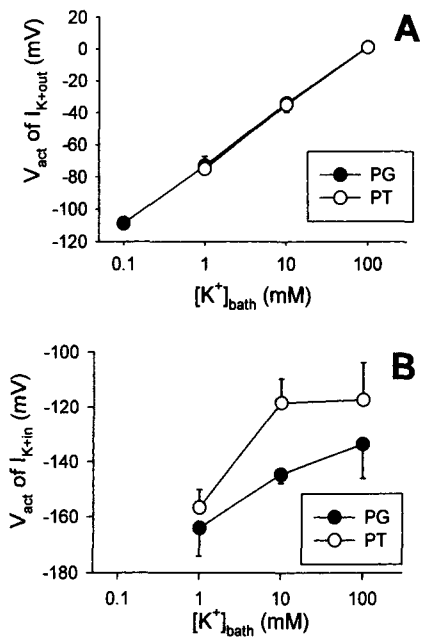

Fig. 3. Dependence on K^+ of the activation voltage (V_{act}) of the outward (*A*) and the inward (*B*) K^+ current. Filled circles and open circles represent mean values ± SD obtained from at least 5 whole-cell recordings from PG and PT protoplasts, respectively. Linear regression analysis gave slopes of 37 mV (PG) and 38 mV (PT) per decade bath K^+ concentration for I_{K^+out} (*A*). V_{act} of I_{K^+in} was less K^+-dependent: in PT protoplasts a slope of 38 mV/decade $[K^+]_o$ was only measured between 1 and 10 mM K^+ and in PG protoplasts the slope was 15 mV/decade. Pipette: PM-100K; bath: BS-100K, BS-10K, BS-1K, respectively.

I_{K^+out} was noticed in PT protoplasts (Fig. 1*C*). Notable was the very slow deactivation of the outward current in both protoplast types. After the activation of the outward current, the membrane voltage had to be clamped to negative voltages for a few seconds to deactivate I_{K^+out} completely. Voltages of 0 to −20 mV resulted in only a very slow closing of the channels in the range of minutes. Therefore, in order to measure the inward current, it was necessary to close all outward-conducting channels by preceding negative pulses (−120 mV). Otherwise the deactivation current of I_{K^+out} would cover the activation of I_{K^+in}. When applying this negative prepulse, an exponentially activating inward current was detected after hyperpolarization (Fig. 1*A* and *B*) in both protoplast types. Usually, in PG protoplasts the slope conductance of the inward current was much lower than the slope conductance of I_{K^+out} (90% of all experiments), whereas in 40% of the PT protoplasts I_{K^+in} was higher than I_{K^+out}. Additional to the higher conductance, the I_{K^+in} of PT protoplasts was already detectable at much less negative pipette voltages than in PG protoplasts (Fig. 1*C*, *see* Table 3).

Effect of the Bath K^+ Concentration

After changing the K^+ concentration of the bath solution from 100 to 1 mM, the conductance of I_{K^+out} was hardly affected at all, but the activation voltage (V_{act}) of the outward current shifted in both protoplast types to more negative voltages (Fig. 2). In contrast to the outward currents, I_{K^+in} of PG and PT protoplasts responded differently to a decrease in bath $[K^+]$. While a time-dependent inward current was detected in almost all PT protoplasts (Fig. 2*B*), in many PG protoplasts no inward current was detectable under these low K^+ conditions (Fig. 2*A*).

Regarding the voltage at which the currents could first be detected (V_{act}) in various bath media ranging from 0.1 mM to 100 mM K^+, no differences between PG and PT protoplasts concerning the outward currents were found (Fig. 3*A*). V_{act} of I_{K^+out} almost followed E_K (Fig. 3*A*: PG = 37 mV/decade $[K^+]_o$, PT = 38 mV/decade $[K^+]_o$). In contrast, V_{act} of the inward current of PGs was less voltage dependent (15 mV/decade $[K^+]_o$) and in PT protoplasts a voltage dependence of V_{act} could only be noticed between 1 and 10 mM KCl (38 mV/decade $[K^+]_o$, Fig. 3*B*), but I_{K^+in} already activated at less negative membrane voltages (Fig. 3*B*).

To further characterize the K^+ conductance of the currents, E_{rev} was determined, using a typical bipolar staircase voltage-pulse protocol. From the shift of the reversal potential of currents measured in 100 mM and 10 mM KCl in the bath, the relative permeability of K^+ to Cl^- was calculated, giving values for P_K/P_{Cl} of 23 (n = 3) and 18 (n = 4) for

Table 2. Block of inward- and outward-directed K^+ currents with external TEA^+ and Ba^{2+}

Current	TEA^+		Ba^{2+}	
	Tube protoplast	Grain protoplast	Tube protoplast	Grain protoplast
I_{K^+in}	68.39 ± 7.40	72.51 ± 7.31	98.89 ± 1.58	98.93 ± 0.97
I_{K^+out}	63.86 ± 3.09	63.94 ± 4.20	46.60 ± 2.21	39.59 ± 4.50

The inhibitors were added as chloride salts at a concentration of 10 mM. Data represent the mean inhibition in percent ± SD of at least 3 experiments.

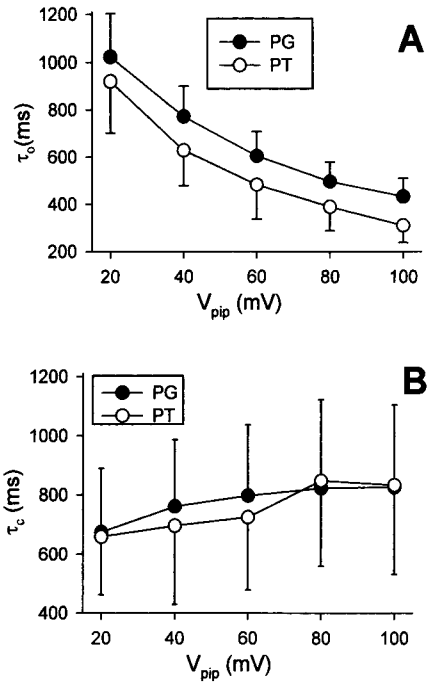

Fig. 4. The kinetics of the outward currents. The time-dependent currents of PG (●) and PT (○) protoplasts were fitted according to a Hodgkin-Huxley model and the time constants of the activation (τ_o) and the deactivation (τ_c) of the currents were plotted against the pipette voltage (V_{pip}). The data presented are mean values ± SD of at least 10 experiments. Pipette: PM-100K; bath: BS-100K.

I_{K^+out} of PG and PT protoplasts, respectively. The inward current was less selective, as reflected by P_K/P_{Cl} ratios of 9 (n = 3, PG) and 11 (n = 3, PT). However, no differences between the two protoplast types were detectable.

Inhibition by TEA^+ and Ba^+

To confirm that the measured currents are mainly carried by K^+ we treated the protoplasts with the K^+-channel blockers TEA^+ and Ba^{2+} and measured the percentage of inhibition (Table 2). The inhibitors were always applied at a concentration of 10 mM in the presence of 10 mM K^+ in the bath. TEA^+ blocked about 60% and 70% of I_{K^+out} and I_{K^+in}, respectively, and there were no significant differences between the currents of PT and PG protoplasts. Also, no differences between the two protoplast types were noticed when the currents were blocked with Ba^{2+}. Although the block of I_{K^+in} by Ba^{2+} was almost 100%, inhibition was reversible, as was the block by TEA^+. A minor effect of the Ba^{2+} block was a slower activation of the outward current (*data not shown*).

Analysis of the Opening and Closing Kinetics of I_{K^+out}

The sigmoid activating outward current was best described by a function according to the model of Hodgkin & Huxley (1952, Eq. 1), where I_0 is the leak current, I_t is the time-dependent current, τ_o is the opening time constant and p is the number of 'prerequisites' that must be fulfilled in order to open the channel:

$$I(t) = I_0 + I_t \cdot (1 - e^{-t/\tau_o})p \quad (1)$$

For the outward currents of PG and PT protoplasts best fits were obtained with p = 2. The time constants τ_o of PG and PT protoplasts after applying voltage steps from a holding potential of −40 mV to positive voltages between 20 mV and 100 mV are shown in Fig. 4*A*. In both protoplast types the activation of the outward channels was voltage dependent: the activation was faster with increasing voltage. In general, the outward current of PT protoplasts activated a little bit faster than the outward current in PG protoplasts.

The closing kinetics of I_{K^+out} was measured by means of voltage steps from positive voltages (20 mV to 100 mV) to −150 mV. In both protoplast types, channel closing followed an exponential function described by equation 2, where I_∞ is the steady state current when all channels are closed, I_{max} is the initial current after the voltage change and τ_c is the time constant of the closing kinetics:

$$I(t) = I_\infty + I_{max} \cdot e^{-t/\tau_c} \quad (2)$$

Fig. 4*B* shows that there is no significant difference in the closing behavior of I_{K^+out} between PT and PG protoplasts. Both protoplast types showed very little voltage dependence of τ_c.

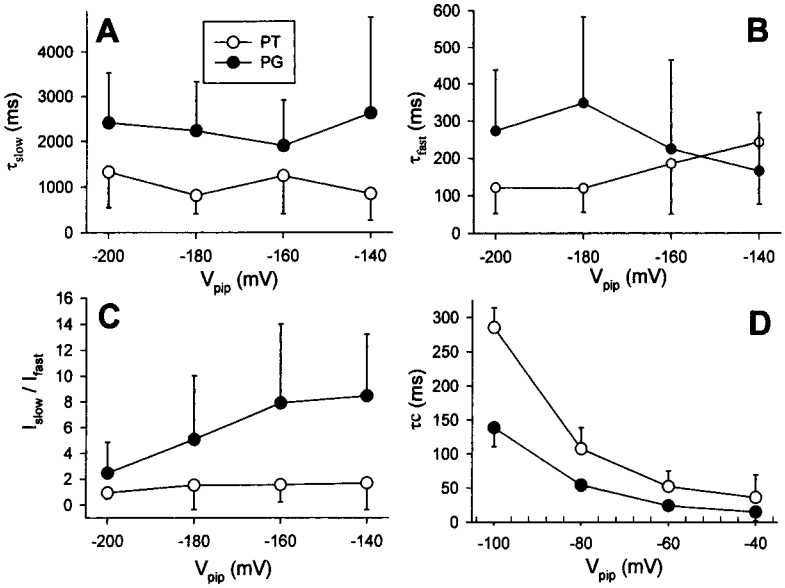

Fig. 5. Kinetics of the inward currents of PG (●) and PT (○) protoplasts. The activation kinetic of I_{K^+in} was described by the sum of two exponential functions including (*A*) a slow (I_s, τ_s) and (*B*) a fast (I_f, τ_f) component. (*C*) The ratio of the slow to the fast current amplitude (I_s/I_f) was plotted against V_{pip} showing the voltage-dependent contribution of the respective current component to the total I_{K^+in}. (*D*) The deactivation kinetics of the inward currents in PG and PT protoplasts could be fitted to a simple exponential function and the time constants of the deactivation kinetics (τ_c) were plotted against V_{pip}. The data shown are mean values ± SD of at least 6 measurements.

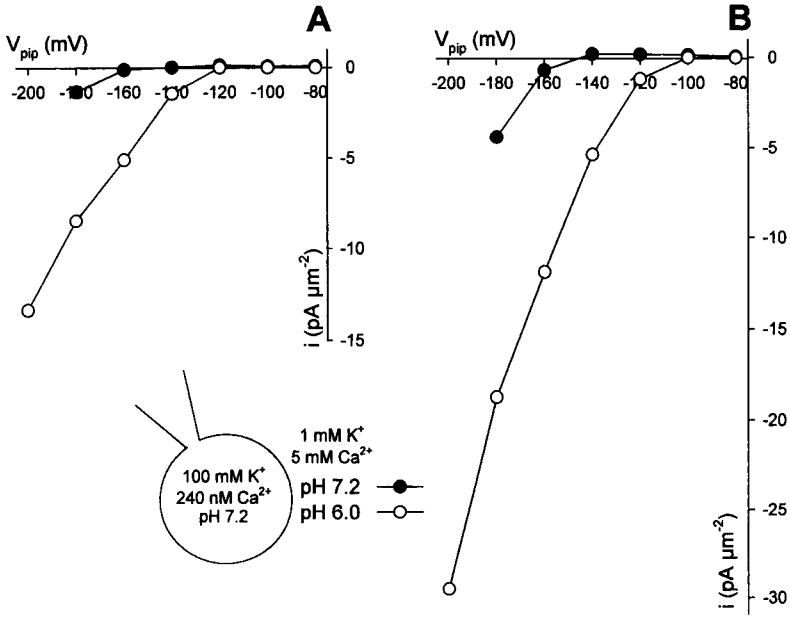

Fig. 6. Increase of I_{K^+in} by acidic external pH (pH_o). Inward current densities were measured at the same PG (*A*) or PT (*B*) protoplast at different pH_o values. Note the stronger pH_o effect on PT protoplasts. Pipette: PM-100K; bath: BS-1K with pH as indicated.

Analysis of the Opening and Closing Kinetics of I_{K^+in}

Unlike I_{K^+out}, the activation of I_{K^+in} of both protoplast types could be described by a sum of two exponential functions according to equation 3, where I_0 is the instantaneous current, I_s and τ_s are the current amplitude and the time constant of the slow component, respectively, and I_f and τ_f are the respective parameters of the fast component of I_{K^+in}.

$$I(t) = I_0 + I_s \cdot (1 - e^{-t/\tau_s}) + I_f \cdot (1 - e^{-t/\tau_f}) \quad (3)$$

The time constants of the inward currents of PG and PT showed no voltage dependence (Fig. 5*A* and *B*), but in PT protoplasts both components (slow and fast) of I_{K^+in} were activating a little bit faster than the corresponding current components of the PG protoplasts. A difference between PG and PT protoplast currents could be noticed in the ratio of the amplitudes of the fast and slow components (I_s/I_f), which reflects their contribution to the overall inward current (Fig. 5*C*). The contribution of the fast component relative to the slow current component is less for higher I_s/I_f ratios, which was observed for inward currents of PGs at less negative voltages (−140 to −160 mV). However, at more negative voltages the ratio decreased to a value of 2. In PT protoplasts, a ratio of about 1 was measured at all voltages, meaning an equal contribution of the fast and the slow current component even at less negative membrane voltages.

Table 3. Effect of the external pH on V_{act} and G_{ch} of the inward K^+ current of PG and PT protoplasts

	Pollen grain	*n*	Pollen tube	*n*	Significance
V_{act} pH 7.2; mV	−164.0 ± 10.1	14	−156.7 ± 6.5	4	Not significant
V_{act} pH 6.0; mV	−126.9 ± 6.2	9	−99.6 ± 11.4	13	$P = 1.74 \cdot 10^{-3}$
G_{ch} pH 7.2; fS μM^{-2}	7.9 ± 8.2	7	23.6 ± 30.8	3	Not significant
G_{ch} pH 6.0; fS μM^{-2}	116.4 ± 85.1	9	762.0 ± 404.5	11	$P = 3.18 \cdot 10^{-4}$

The chord conductance (G_{ch}) was determined at −160 mV. The K^+ concentration of the bath solution was always 1 mM. The significance of the difference was tested by Student's *t*-test at the 1% confidence level.

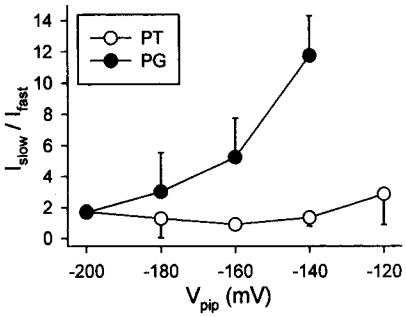

Fig. 7. Amplitude ratio of slow and fast component (I_s/I_f) of I_{K^+in} recorded at pH_o 6.0. The respective amplitude values were obtained by fitting the inward currents of PG (●) and PT (○) protoplasts to a sum of two exponential functions. The data shown are mean values ± SD from 8 (PG) and 11 (PT) measurements.

The closing kinetics of I_{K^+in} was measured by means of voltage steps from a negative voltage (−180 mV) to less negative voltages (−100 mV to −40 mV). In both protoplast types, channel closing followed an exponential function (Eq. 2). Fig. 5*D* shows that I_{K^+in} of PG protoplasts deactivated faster than I_{K^+in} of PT protoplasts. The closing time constant τ_c of both protoplast types was voltage dependent and increased with more negative membrane voltages.

pH Sensitivity of I_{K^+in}

Usually, pollen tubes are growing optimally in media of acidic pH. Therefore, we changed the pH of the bath solution from 7.2 to pH 6.0, and measured the K^+ currents of PG and PT protoplasts (Fig. 6). The decrease in external pH increased the inward conductance in both protoplast types. In PG protoplasts a 15-fold increase of the chord conductance from ca. 8 fS μM^{-2} to 116 fS μM^{-2} was measured when the pH was changed from 7.2 to 6.0 (Fig. 6*A*). A similar strong increase was observed in PT protoplasts: from 50 fS μM^{-2} to 750 fS μM^{-2} (Table 3, Fig. 6*B*). The outward currents of PT and PG protoplasts were almost not affected (*data not shown*). Additionally, a shift in V_{act} to more positive membrane potentials was observed. In PG protoplasts, V_{act} shifted to more positive membrane voltages by 29 mV and in PT protoplasts by 47 mV (Table 3). Note that under these ionic conditions (pipette: 100 K^+, bath: 1 K^+), an E_{rev} of ca. −120 mV is expected, but inward currents were observed only at voltages more negative than −140 mV, indicating a possible intrinsic voltage-gating mechanism of the involved K^+ channels. The origin of the observed outward currents at voltages between −140 and 0 mV is still unclear but may be caused by the activity of a plasma membrane H^+ATPase.

At an external pH of 6 the chord conductance and V_{act} of I_{K^+in} were significantly different in PT and PG protoplasts, already allowing inward K^+ currents at voltages slightly negative of the expected E_{rev}.

Finally, the opening kinetics of I_{K^+in} measured under these almost physiological conditions (1 mM K^+, pH 6.0), was fitted by equation 3. No significant differences in the time constants τ_s, τ_f, and τ_c could be noticed between PT and PG protoplasts (*data not shown*). However, the difference between PT and PG protoplasts in respect to the voltage dependence of the amplitude ratios (I_s/I_f) is larger at pH 6 (Fig. 7) than at pH 7.2 (Fig. 5*C*), indicating that the fast component of the inward current becomes much more important for the overall conductance at less negative voltages.

Discussion

The generation of cytoplasmic ion concentration gradients and of an external electrical field are main characteristics of tip-growing pollen tubes and are assumed to play a regulatory role in the initiation and maintenance of the polar growth pattern (Hepler et al., 2001). A major component of these endogenous currents are K^+ influxes along the growing pollen tube and K^+ channels are involved in mediating the uptake and release of K^+ across the plasma membrane. So far, K^+ channels and currents were characterized in pollen grains (Obermeyer & Kolb, 1993; Obermeyer & Blatt, 1995; Fan et al., 1999; 2001). In this study, the K^+ currents across the plasma membrane of pollen tube protoplasts were characterized for the first time and compared with the K^+ currents of pollen grain protoplasts.

K^+ Currents in Pollen Grain and Tube Protoplasts

Despite the large size of lily pollen grain protoplasts (diameter of ca. 80 μM), which implies a very low membrane input resistance (100 MΩ), reliable clamping of the voltage and thus reliable current recordings were achieved in the whole-cell configuration by reducing the access resistance of the pipette to 3–5 MΩ (Armstrong & Gilly, 1992). The detected outward and inward currents of lily pollen protoplasts are mainly carried by K^+ and were similar to those measured with the 2-electrode voltage-clamp technique in intact pollen grains (Obermeyer & Blatt, 1995). They are also comparable with currents recorded in other pollen species (Fan et al., 1999; 2001; Mouline et al., 2002). As in most plant cell and pollen grain protoplasts, I_{K^+out} was reversibly inhibited by the K^+ channel inhibitors TEA^+ and Ba^{2+} and exhibited sigmoid activation kinetics (Hodgkin-Huxley model with $n = 2$, Schroeder, 1989; Van Duijn, 1993). The conductance did not depend on the external K^+ concentration, whereas the activation voltage (V_{act}) of the outward current was strongly K^+ dependent (pollen grain protoplasts: 37 mV/decade $[K^+]_o$ in Fig. 3*A*, intact pollen grains: 38 mV/decade $[K^+]_o$, Obermeyer & Blatt, 1995). In general, no major differences were observed in PG protoplasts when compared with intact PGs except the sometimes observed exponential activation of the outward current in intact pollen grains. Therefore, the enzymatic removal of the cell wall did not affect the major ion currents in lily pollen.

The outward current of pollen tube protoplasts turned out to be more or less similar to I_{K^+out} of PG protoplasts, e.g., it showed the same V_{act}, similar inhibition by TEA^+ and Ba^{2+}, and there were no differences in the opening and closing kinetics. Also, the outward current density of PG and PT protoplasts was similar.

In contrast to I_{K^+out}, the inward current of PT protoplasts differed in all measured parameters from the PG inward currents. The current density was much higher, the current was already detectable at less negative membrane voltages, it activated faster, and the closing of I_{K^+in} in PT protoplasts was slower. In both protoplast types, I_{K^+in} was best fitted by a sum of two exponentials, which implies that either two different K^+ channel populations carry the current or that one K^+ channel type exhibits two open states with different activation kinetics. In lily pollen PT protoplasts, two current components could be distinguished based on their different opening kinetics. The contribution of the fast component of I_{K^+in}, was higher at less negative voltages, as revealed by the current amplitude ratio (I_s/I_f), which was 8 in PG and 1 in PT protoplasts at −140 mV (Fig. 5*C*). Therefore, in the case of pollen tubes, one may assume that a new type of K^+ channel is inserted into the plasma membrane, or the same K^+ channel type as in PGs is regulated differently during pollen tube growth.

At an acidic pH of the external medium (pH_o), the contribution of the fast component of I_{K^+in} was even higher (Fig. 7), indicating that the fast current component is more pH-dependent than the slow component. The pH-dependent increase in I_{K^+in} was also observed in *A. thaliana* PG protoplast, showing a maximal current at acidic pH (4.5–6.0, Fan et al., 2001). Such pH dependence was also reported for other plant cells, e.g., barley root cells (Amtmann, Jelitto & Sanders, 1999), maize bundle sheath cells (Keunecke & Hansen, 2000), and *Vicia* guard cells (Blatt, 1992). In guard cells not only did the conductance of I_{K^+in} increase with acidic pH_o but also a positive-going shift of the voltage gating of I_{K^+in}, as observed in lily pollen protoplasts, was detected. This high pH_o sensitivity may reflect a possible interaction between inward-rectifying K^+ channels and the plasma membrane H^+ ATPase in controlling K^+ fluxes during stomata movements as well as during pollen tube growth.

Pollen Tube Growth and Inward K^+ Currents

In contrast to pollen grains of *Brassica* and *Arabidopsis*, the previously measured K^+ currents of lily pollen grains did not show properties suited to drive a K^+ influx under conditions of pollen tube growth. The PG inward currents were only detected at membrane voltages more negative than the measured resting membrane potential (Obermeyer & Blatt, 1995; Weisenseel & Wenisch, 1980). Therefore, none of the measured K^+ inward currents of PG protoplasts could be responsible for the growth-related K^+ currents. On the other hand, the detected whole-cell inward currents in PT protoplasts show all parameters necessary to drive an influx of K^+ under physiological conditions, namely conditions of PT in-vitro cultures (1 mM KCl, pH 6).

As postulated before (Obermeyer et al., 1996; Pertl et al., 2001) an activation of the plasma membrane H^+ ATPase is one of the essential steps in initiating the germination and/or tube growth. An active proton pump will acidify the external medium (Southworth, 1983; Tupy & Rihova, 1984; Rodriguez-Rosales et al., 1989), especially in the vicinity of the pollen grain. Due to the acidic pH_o, K^+ channels open at membrane voltages that have been detected in PGs and growing PTs, and thus allow an uptake of K^+. The K^+ influx increases the osmotic value of the pollen tube cytoplasm, therefore enabling a water influx that drives the volume increase during tube growth. So far, the K^+ channels expressed in lily pollen grains and tubes were not yet identified by

molecular biology techniques. Only one type of K^+ channels important for pollen tube growth was identified in *Arabidopsis thaliana* pollen grains (Mouline et al., 2002). From the presented electrophysiological study, at least two types of inward-rectifying K^+ channels may be expected in lily pollen tubes.

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