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Voltage dependence of K^+ channels in guard-cell protoplasts

(patch clamp/K^+ current/ion channel/rectifier/stomata)

JULIAN I. SCHROEDER*, KLAUS RASCHKE†, AND ERWIN NEHER*

*Department of Membrane Biophysics, Max-Planck-Institut für biophysikalische Chemie, D-3400 Göttingen, Federal Republic of Germany; and †Pflanzenphysiologisches Institut und Botanischer Garten, Universität Göttingen, D-3400 Göttingen, Federal Republic of Germany

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ABSTRACT **Stomatal pores in leaves enable plants to regulate the exchange of gases with their environment. Variations of the pore aperture are mediated by controlled changes of potassium salt concentrations in the surrounding guard cells. The voltage-dependent gating of K^+-selective channels in the plasma membrane (plasmalemma) of cell-wall-free guard cells (protoplasts) was studied at the molecular level in order to investigate the regulation of K^+ fluxes during stomatal movements. Inward and outward K^+ currents across the plasmalemma of guard cells were identified by using the whole-cell configuration of the patch-clamp technique. Depolarizations of the membrane potential from a holding potential of −60 mV to values more positive than −40 mV produced outward currents that were shown to be carried by K^+. Hyperpolarizations elicited inward K^+ currents. Inward and outward currents were selective for K^+ over Na^+ and could be partially blocked by exposure to extracellular Ba^{2+}. In cell-attached and excised membrane patches, previously identified K^+-selective single channels in guard cells were studied. Averaging of single-channel currents during voltage pulses resulted in activation and deactivation kinetics that were similar to corresponding kinetics of inward and outward currents in whole cells, showing that K^+-selective channels were the molecular pathways for the K^+ currents recorded across the plasmalemma of single guard-cell protoplasts. Estimates demonstrate that K^+ currents through the voltage-gated K^+ channels recorded in whole guard cells can account for physiological K^+ fluxes reported to occur during stomatal movements in leaves.**

Elucidation of the molecular mechanisms involved in the transport of K^+ across membranes of higher plant cells is fundamental to the understanding of osmoregulation and cell-volume control. Our studies have focused on the specialized guard cells surrounding stomatal pores in the lower epidermis of leaves of *Vicia faba*. Two guard cells modulate the aperture of the pore they surround, allowing the exchange of CO_2 and O_2 for photosynthesis, while minimizing water loss to the atmosphere. Light, among other physiological factors, causes guard cells to accumulate K^+, leading to the opening of the pore (for reviews, see refs. 1–3). Darkening, high CO_2 concentrations, and the phytohormone abscisic acid (under a condition of drought) mediate pore closure by inducing the release of K^+ and counter ions (4, 5). Recently, ion channels have been identified in the plasmalemma of guard cells that are selectively permeable to K^+ and through which K^+ can be accumulated and released (6). In the present study, we have investigated the voltage-dependent activation of K^+-selective channels in guard-cell protoplasts to explore possible mechanisms involved in the regulation of stomatal apertures.

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METHODS

Guard-Cell Protoplasts. These were isolated from the lower epidermis of leaves from 2- to 3-week-old *Vicia faba* plants by 30- to 40-min incubation in 0.3 M D-mannitol/1 mM $CaCl_2$/2% cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan)/0.02% pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) as described (7). Experiments were conducted at 21–23°C.

Whole-Cell Recordings. The tight-seal whole-cell configuration of the patch-clamp technique was applied (8, 9). Briefly, the tip of the patch pipette was sealed against the plasmalemma of a protoplast. Subsequently, the underlying membrane patch was ruptured by a pulse of suction. The solution inside the pipette equilibrated with the cell so that cytoplasmic ion concentrations were well defined within minutes (9) (see Fig. 1 *Inset*). Whole-cell capacitive transients (9) revealed membrane capacitances of 5.5–7 pF per cell. The spherical protoplasts had a diameter of 15 ± 1 μm, leading to an estimated specific membrane capacitance of 0.9 $\mu F \cdot cm^{-2}$. An efficient whole-cell voltage clamp demands low values of the access resistance (R_a) to the cytoplasm at the tip of the recording pipette. R_a tended to rise to values greater than 20 MΩ within a few minutes. This behavior could be prevented by sufficiently buffering Ca^{2+} with EGTA (10) in the pipette solution by using wide-tipped pipettes, and by using solutions in the pipette that exceeded the osmolality of the bath solution by 10%. Seventy to 80% of the access resistance was compensated by analog circuitry (11). Residual errors in membrane potentials (<6 mV) were taken into account when calculating membrane potentials during voltage pulses. Instantaneous current-to-voltage relationships (12) with 105 mM K^+ in the cytoplasm and 11 mM K^+ in the bath indicated an ohmic membrane resistance of $R_m \approx 10$ GΩ (≈0.14 $S \cdot m^{-2}$) between potentials of −40 and −80 mV. Current-to-voltage relationships of voltage-dependent currents recorded in single guard cells were obtained by subtraction of the ohmic membrane resistance.

Single-Channel Currents. These were recorded by using the cell-attached and outside-out patch recording configurations (8) (see *Inset* in Fig. 3 *C* and *D*). Leakage and capacitive currents were subtracted by a P/4 voltage pulse procedure (13) before averaging of single-channel currents recorded in response to voltage pulses.

Control of membrane potential, measurement of currents, and compensation of cell capacitive transients were performed with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, F.R.G.). Data were recorded on video tape after pulse-code modulation (14). For off-line analysis, data were low-pass-filtered with eight-pole Bessel characteristics (15) and subsequently digitized and processed on a PDP-11/73 computer.

Solutions. Cells were bathed in either 10 mM potassium glutamate (solution I), 11 mM KCl (solution II), 100 mM sodium glutamate (solution III), or 100 mM NaCl (solution IV). Furthermore, all bathing solutions included 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KOH (or NaOH in solutions III

and IV), and 10 mM Mes [2-(*N*-morpholino)ethanesulfonic acid)], pH 5.5. The cytoplasm of whole cells was equilibrated with pipette solutions including either 100 mM potassium glutamate (solution V) or 100 mM KCl (solution VI). In addition, all intracellular solutions included 2 mM $MgCl_2$, 10 mM Hepes, 5 mM KOH, 2 mM EGTA, and 2 mM MgATP (pH 7.2). Solutions were adjusted to final osmolalities of 480 mmol·kg^{-1} (external solutions) or to 530 mmol·kg^{-1} (internal solutions) by addition of D-mannitol. All potentials were corrected for the liquid-junction potential that develops at the tip of the patch pipette when it is immersed into the bath solution (16). K^+ equilibrium potentials (V_{K+}) and relative permeabilities (P_{K+}/P_{Na+}) were computed from corrected ionic activities (17).

RESULTS

Voltage-Gated K^+ Currents Recorded in Single Guard Cells. Immediately after gaining access to the interior of a guard cell, resting potentials between −60 and −160 mV were recorded before the pipette solution equilibrated with the cytoplasmic contents. Fig. 1 shows typical voltage clamp recordings of ionic currents across the plasmalemma of a whole guard cell after cytoplasmic equilibration with 105 mM K^+ and with 11 mM K^+ in the bath (see Fig. 1*A Inset*). The membrane potential was held at −60 mV (V_H = −60 mV). Depolarizing voltage steps to potentials more positive than −40 mV produced large outward currents. When the membrane potential was stepped to hyperpolarized values, inward currents were elicited. Both inward and outward currents showed "slow" activation extending over seconds. When whole cells were clamped to potentials of +40 mV or −140 mV for prolonged durations of 15 min, the current inactivated by less than 25% (n = 5).

Addition of 1 mM Ba^{2+} to the external medium suppressed both inward and outward currents by 70–80% of the initial values (Fig. 1*B*). At 10 mM, external Ba^{2+} blocked 90 ± 3% (SD, n = 5) of inward and outward steady-state currents at potentials of +40 and −180 mV (Fig. 1 *C* and *D*). Upon removal of Ba^{2+} by bath perfusion, both inward and outward currents regained their initial values (not illustrated).

To determine which ions carried the inward currents across the plasmalemma of guard cells, the reversal potential was measured in asymmetric solutions (Fig. 2*A*). The membrane potential was stepped from a holding potential of −60 mV to a pulse potential of −191 mV, leading to a large inward current. From the pulse potential of −191 mV, the membrane potential was stepped back to more positive potentials (V_{Tail}) at which the decay of the inward currents (deactivation) could be monitored. The direction of the decaying "tail" currents reversed at −59 mV, corresponding closely to the Nernst potential for K^+, demonstrating that the inward currents were carried by K^+ ions. The Nernst potential for all other ions present in the pipette and bath solutions was more positive than −9 mV. In Fig. 2*A* for V_{Tail} = −18 mV, the deactivation of the inward current was followed by the slower activation of the outward current, suggesting the possibility that the gating mechanisms of inward and outward currents were independent from one another. The permeability ratio of K^+ to Na^+ was determined by measuring the reversal potential of the tail currents with 101 mM Na^+ in the bath (Fig. 2*B*). The reversal potential of −72 mV indicated that K^+ was permeating 17 times more readily than Na^+ as calculated from the Goldman equation.

Similarly the ionic selectivity of the outward currents was studied (Fig. 2*C*). The reversal potential of tail currents was near to the K^+ equilibrium potential. In symmetrical 105 mM K^+ solutions, the tail currents of inward and outward currents reversed at 0 mV (not illustrated). We concluded that the outward currents corresponded to an efflux of K^+.

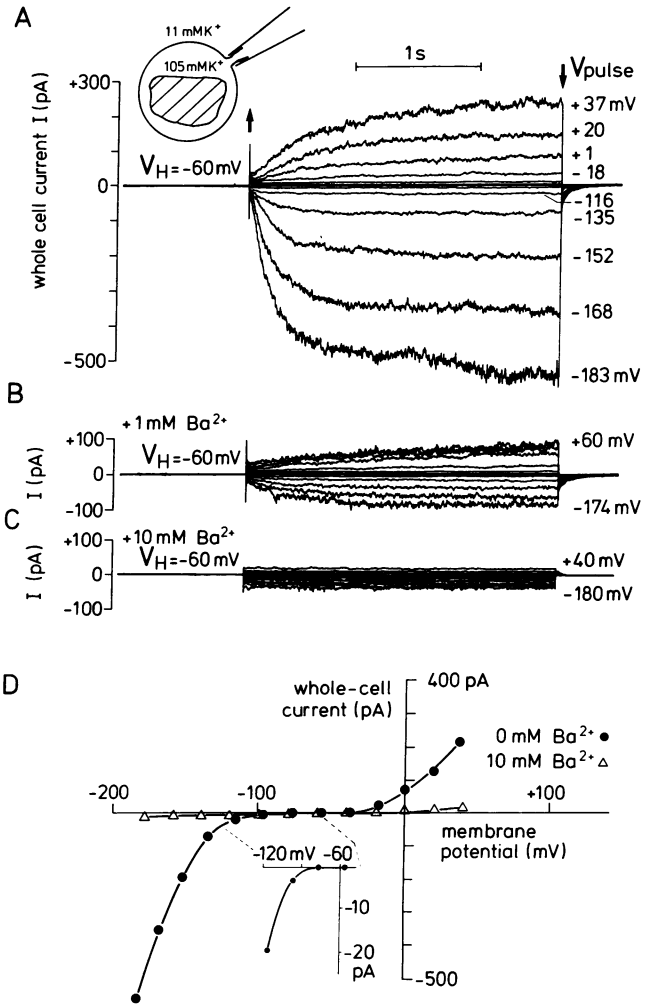

FIG. 1. (*A*) Recordings of K^+ currents in a whole guard-cell protoplast. (*B* and *C*) Partial block of K^+ currents by Ba^{2+} ions. (*D*) Current-to-voltage relationship of whole-cell currents. The cytoplasm of the protoplast was loaded with 105 mM K^+ (solution V) via the tightly sealed pipette, while the cell was bathed in 11 mM K^+ (solution I) (see *Inset*). (*A*) Superposition of consecutive recordings of ionic currents across the plasmalemma of a guard cell in response to individual voltage pulses of 2.5-s duration. Each voltage pulse started at the upward pointing arrow and stopped at the downward pointing arrow. The membrane potential was held at −60 mV (V_H) and stepped to potentials ranging from V_{Pulse} = −183 to +37 mV. Depolarizations caused outward currents (upward deflections), and hyperpolarizations produced inward currents seen as downward deflecting traces. (*B* and *C*) Both inward and outward currents were partially reduced by addition of 1 mM $BaCl_2$ (*B*) and 10 mM $BaCl_2$ (*C*) to the bathing solution. (*D*) Whole-cell current plotted before (•) and after (△) addition of 10 mM Ba^{2+} as a function of membrane potential during the voltage pulse. (*Inset*) Magnification of the plot between −60 and −120 mV in solutions without Ba^{2+}.

With 101 mM Na^+ in the bath, the permeability ratio of K^+ over Na^+ was 8 to 1 (Fig. 2*D*). The K^+/Na^+ permeability ratios obtained from whole-cell currents here were similar to the values for single K^+ channels described in a former report (6).

Currents Through Single K^+-Selective Channels: Molecular Correlates of Whole-Cell K^+ Currents. In an attempt to identify the unitary basis of the "macroscopic" outward and inward K^+ currents recorded in whole guard cells, currents through electrically isolated membrane patches were recorded at high resolution (8) (*Insets* in Fig. 3 *C* and *D*). Discrete steps in the current were recorded representing the opening and closing of single channels (Fig. 3*A Inset*). The single-

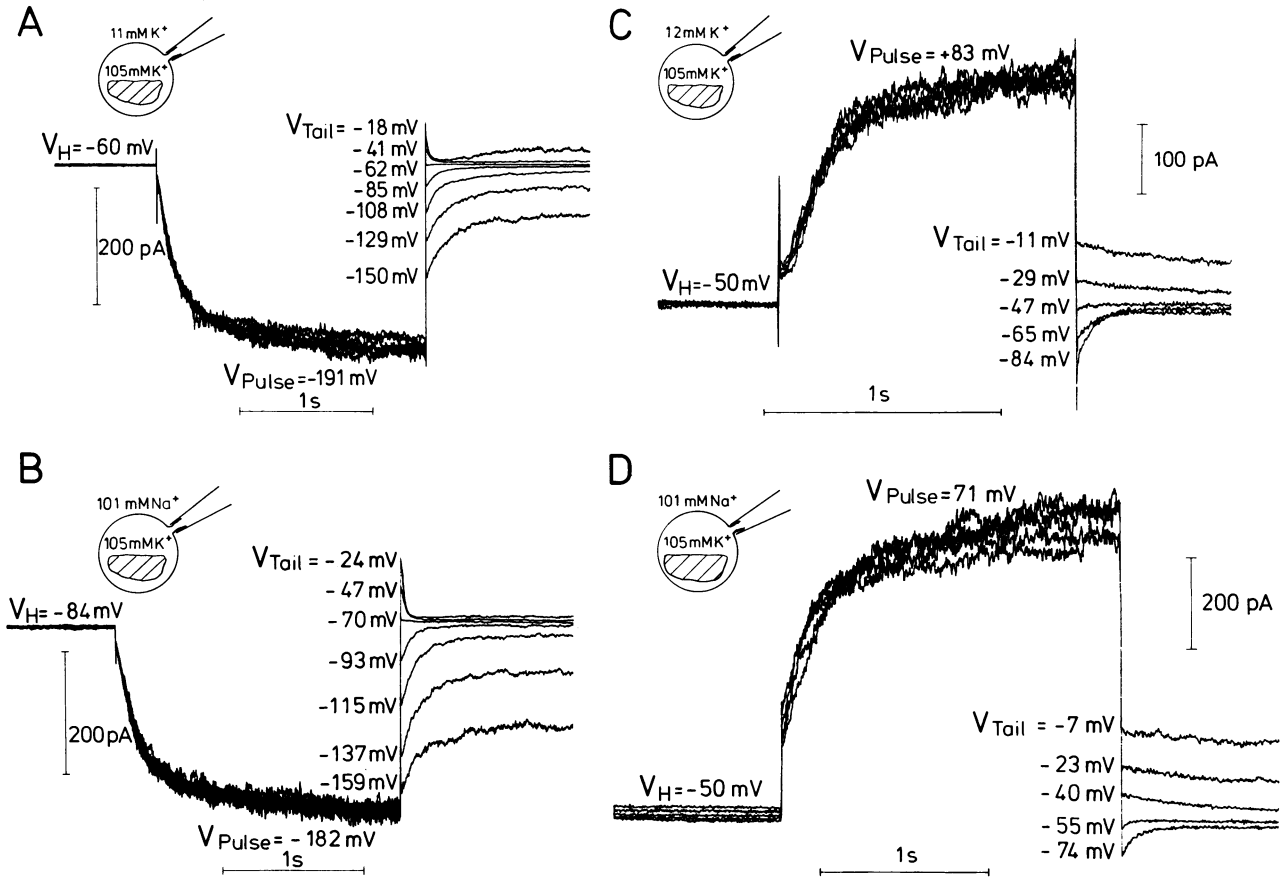

FIG. 2. Specificity of the inward (*A* and *B*) and outward (*C* and *D*) currents for K^+. (*A* and *B*) The selectivity of the inward currents to K^+ was studied by using a tail-current procedure (see text) with 105 mM K^+ in the cytoplasm (solution V) and 11 mM K^+ in the bath (solution I) in *A* and with 101 mM Na^+ in the bath (solution III) in *B* (see *Insets*). Both *A* and *B* show the superposition of seven consecutive recordings of currents across the plasmalemma of a whole guard-cell protoplast. In *A* the deactivating (tail) currents reversed close to the Nernst potential for K^+, demonstrating that inward currents were carried by K^+. In *B* the tail currents reversed at approximately −72 mV, showing that K^+ was 17 times more permeable than Na^+. (*C* and *D*) The selectivity of the outward currents to K^+ was investigated with 101 mM K^+ (solution VI) in the cytoplasm and 12 mM K^+ (solution II) in the bath in *C* and with 101 mM Na^+ in the bath in *D* (solution IV) (see *Insets*). The reversal potential of the deactivating currents was in the range of the Nernst potential for K^+ in *C*, indicating that outward currents corresponded to efflux of K^+ [$V_{K^+} = -51$ mV, after correction for ionic activities (17)]. In *D* deactivating currents reversed at −52 mV, indicating a permeability ratio of $P_{K^+}/P_{Na^+} \approx 8:1$.

channel currents had the same selectivity and conductance properties as the K^+-selective channels previously described in guard cells (6).

The distribution of the amplitudes of single-channel currents showed that K^+ channels had one main conductance state (Fig. 3*A*). In the presence of 11 mM K^+ on the extracellular and 175 mM K^+ on the cytoplasmic side of the membrane, inward K^+ channel currents could be fitted by a conductance of 10 pS ($10 \times 10^{-12}\ \Omega^{-1}$), and outward currents by a conductance of 25 pS (Fig. 3*B*). With symmetrical 105 mM K^+ solutions in outside-out patches, the conductance of the single channels for both inward and outward currents was 20 pS (Fig. 3*B*).

K^+ channels activated by hyperpolarization and by depolarization had very similar properties with respect to both single-channel conductance in symmetrical solutions (Fig. 3*B*) and blocking behavior by Ba^{2+} (Fig. 1 *B–D*). However, the observation that the deactivation of the inward current was followed by the slower activation of the outward current (e.g., Fig. 2*A*; $V_{Tail} = -18$ mV) suggests that two channel types may be involved. Moreover, in some preparations outward currents diminished with time of whole-cell recording, while inward currents remained stable for hours. Further evidence will be required to unambiguously determine whether inward and outward currents are passing by two different types of K^+ channels.

To investigate whether the single K^+ channels observed in small membrane patches were responsible for the whole-cell currents seen in Figs. 1 and 2, the mean time course of outward and inward single-channel currents was derived by averaging currents in response to voltage pulses (Fig. 3 *C* and *D*). Averaged single-channel currents showed a kinetic behavior that was similar to the kinetics observed for outward and inward whole-cell currents (Fig. 3 *C* and *D*). Half times for the deactivating tail currents agreed within 15% when whole-cell K^+ currents and averaged single-channel currents were recorded under equivalent conditions. However, activation time courses varied in different preparations in whole-cell recordings as well as in averaged single-channel recordings. In both types of recordings, half activation times were in the range of 150–400 ms. Analysis of the variance (18) of steady-state K^+ currents in whole cells ($n = 4$) predicted single-channel conductances that were ≈30% lower than those measured directly in outside-out patches. These results suggest that the K^+ channels described served as the permeation paths for the voltage- and time-dependent whole-cell K^+ currents.

DISCUSSION

K^+ Channels. Voltage-activated inward and outward rectifying K^+ channels have been described in the plasma membrane of animal cells (for review, see refs. 12 and 19).

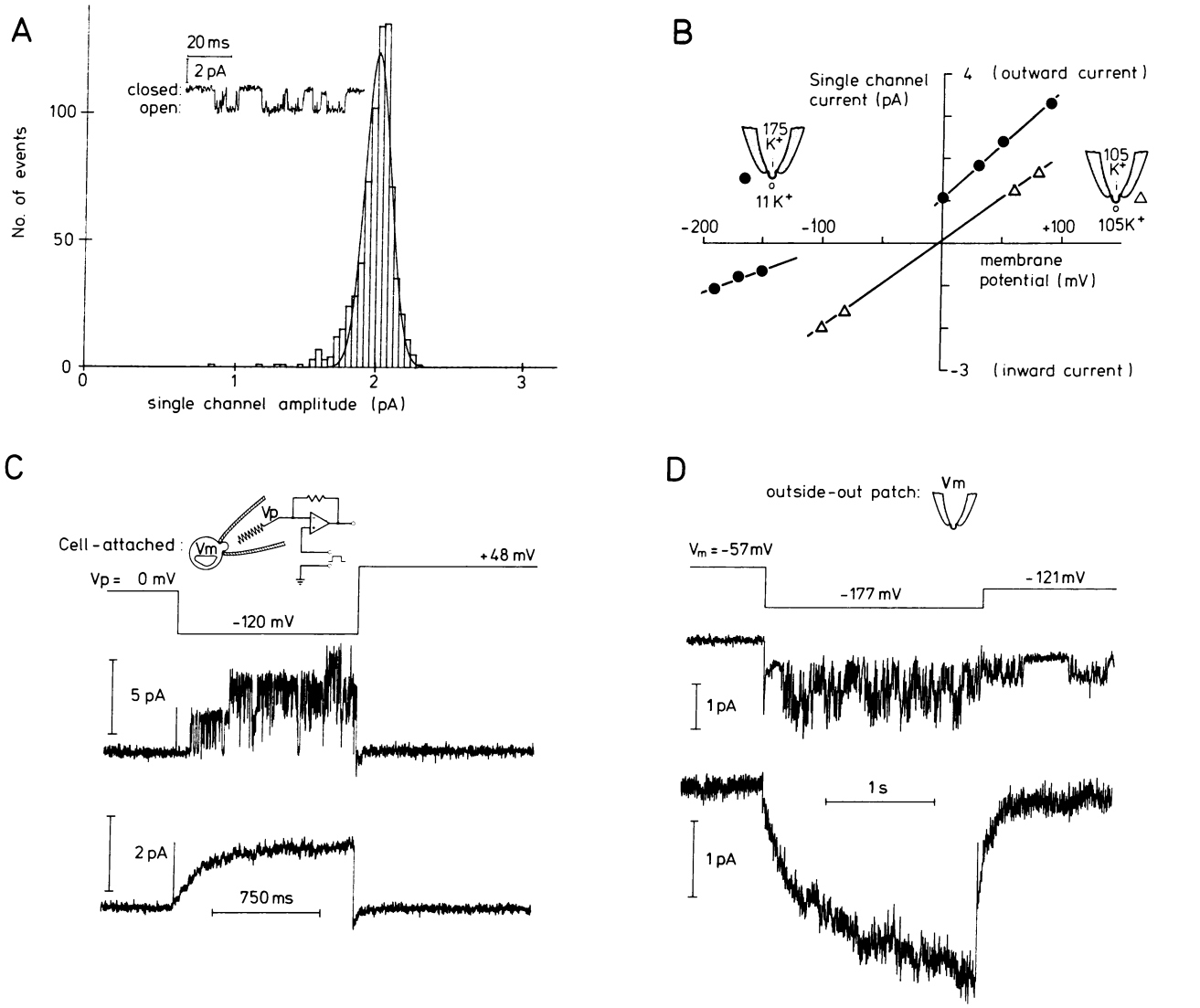

FIG. 3. Currents through individual K^+-selective channels: Amplitude distribution (*A*), current-to-voltage relationship (*B*), and averaged time courses of outward (*C*) and inward (*D*) single-channel currents in response to voltage pulses. (*A*) The distribution of single-channel current amplitudes (step sizes) at a membrane potential of −100 mV in an outside-out patch in symmetrical 105 mM K^+ solutions is shown. A Gaussian fit shows that the mean single-channel current was 2.0 ± 0.1 pA. (*Inset*) Sample recording of bursts of single-channel openings. (*B*) Amplitude of single K^+-channel currents as a function of membrane potential in two outside-out patches. Data from a patch with 175 mM K^+ (170 mM KCl/5 KOH) on the cytoplasmic membrane side and 11 mM K^+ (solution I) on the outside (see *Inset*) (●). The single-channel conductance of both inward and outward currents in symmetrical 105 mM K^+ solutions (solution VI) was fitted to 20.3 pS (△). Points were obtained as shown in *A*. (*C*) Recordings of outward currents through K^+ channels and averaged current in a cell-attached membrane patch with 12 mM K^+ (solution II) in the pipette and in the bath. The potential of the membrane patch underlying the pipette ($V_m - V_p$) was depolarized (inside positive) by imposing voltage steps ($V_p = -120$ mV) on the outer membrane face (see *Inset*). The upper current trace shows that upon depolarization ($V_p = -120$ mV), discrete outward current pulses appeared, which indicated the opening of single K^+ channels. After repolarization of the membrane to $V_p = +48$ mV, an inward channel current can be seen. The lower trace is an average of 80 consecutive single-channel recordings obtained as in the upper trace and shows the activation and deactivation time course of single channels. (*D*) Recording of inward currents through K^+-selective channels in an outside-out patch with 105 mM K^+ on the cytoplasmic membrane side and 11 mM K^+ on the outside (see *Inset*). The upper current trace shows that hyperpolarizing voltage steps from a membrane potential of −57 mV to a pulse potential of −177 mV elicited inward single-channel currents. When the potential was stepped back to −121 mV, channel activity decreased. The lower trace is an average of 58 consecutive current traces and shows a slowly developing inward current that deactivated upon repolarization to −121 mV.

Furthermore, voltage- and time-dependent outward K^+ currents have been reported in algae (20) and recently in yeast (21). The permeability ratios P_{K^+}/P_{Na^+} of inward and outward rectifying K^+ channels in animal cells are similar to the values reported here (19). Millimolar concentrations of Ba^{2+} have been found to block outward and inward rectifying K^+ currents in muscle, starfish eggs, and heart cells (22–24). The activation and deactivation kinetics of K^+ channels in animal cells are, however, on the order of 10^2 times faster than those observed here. The voltage dependence of K^+-selective channels in guard-cell protoplasts became apparent by using voltage pulses lasting 1.25 s or longer. When shorter pulses were used, the voltage dependence did not become evident (6), as voltage-gated K^+ channels in guard cells that activate at depolarized and hyperpolarized potentials have similar properties.

Whole-cell currents similar to the currents identified here were recorded in common epidermal cells (personal observations), and similar outward K^+-currents have been observed recently in extensor motor cells of pulvini (25), suggesting that the voltage-dependent K^+ channels as described here may provide a general mechanism for K^+ fluxes across plant membranes.

K^+ Fluxes in Guard-Cell Protoplasts Related to Stomatal

Movements. Guard cells of *Vicia faba* increase their intracellular K^+ concentration by an average of 400 mM when stomatal pores move from the closed to the open state (2). During accumulation, K^+ salts are taken up through the plasmalemma and then sequestered into the large intracellular organelle, the vacuole. In a recent patch-clamp study on isolated vacuoles, potential mechanisms for solute transport across the vacuolar membrane were identified (26). Stomatal pores close when K^+ and counter ions are released from the guard cells (4, 5).

Our experiments provide a way of quantitatively monitoring K^+ currents across the plasmalemma of single guard cells while simultaneously defining the composition of solutions on both the cytoplasmic and extracellular sides. The imposed K^+ concentrations in Figs. 1 and 2 were in the range of the physiologically expected free K^+ concentrations in the cell wall and the cytoplasm (2). The resting potential of nonstimulated guard cells has been postulated to be Nernstian with respect to K^+ (27). The voltage sensitivity of K^+ channels as described here implies that K^+ fluxes into and out of guard cells may be regulated by mechanisms that produce changes in the membrane potential. K^+ channels may be activated by displacements of the membrane potential from V_{K^+}, induced by physiological effectors of stomatal aperture such as light, CO_2, and abscisic acid (28–30).

Indeed, recent patch-clamp studies on guard cells have shown that blue light and the fungal toxin fusicoccin, both known stimulators of stomatal opening, activate electrogenic pumps that hyperpolarize the membrane potential and thus provide a driving force for K^+ influx (30, 31). Upon hyperpolarization, K^+ channels would open, providing a pathway for the accumulation of K^+. If the membrane potential is hyperpolarized to -120 mV, we predict a mean rate of K^+ accumulation of 0.2 fmol·s^{-1} per guard cell, as estimated from a mean inward K^+ current of 20 pA measured in single guard cells. This value is equivalent to a flux of 0.28 μmol·s^{-1} per m^2 of plasmalemma surface in a spherical guard cell of 15-μm diameter. The average of estimated fluxes of K^+ into guard cells of *Vicia faba* is 0.1 μmol·s^{-1}·m^{-2} (2). Taking our estimated values, a 400 mM K^+ concentration increase in guard cells during stomatal opening would require 1 hr, which is in the range of the physiologically observed time of 2 hr for *Vicia faba* at room temperature. The residual K^+ currents measured after reduction by 10 mM Ba^{2+} were estimated still to be sufficient to allow stomatal movements in the physiologically observed periods.

During stomatal closure, release of K^+ by guard cells would require a prolonged depolarization of the membrane potential. Depolarization could provide a driving force and activate opening of K^+ channels, allowing release of K^+. Taking the value for the mean outward K^+ current of 70 pA measured in response to depolarizations to 0 mV, calculations such as those above would predict a decline in the K^+ concentration by 400 mM within 16 min, which is in the range of the physiologically observed time for stomatal closure of 30 min in *Vicia faba* (1).

These estimates demonstrate that accumulation and release of K^+ during stomatal movements can be accounted for by the described K^+ channel currents recorded across the plasmalemma of single guard cells and that controlled changes of the plasmalemma potential are a conceivable mechanism for the modulation of K^+ fluxes during turgor and cell-volume regulation.

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