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Inward-rectifying K^+ channels in guard cells provide a mechanism for low-affinity K^+ uptake

(K^+ transport/voltage-dependent ion channel/stomata/anomalous rectification/patch clamp)

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ABSTRACT **The molecular mechanisms by which higher plant cells take up K^+ across the plasma membrane (plasmalemma) remain unknown. Physiological transport studies in a large number of higher plant cell types, including guard cells, have suggested that at least two distinct types of K^+-uptake mechanisms exist, permitting low-affinity and high-affinity K^+ accumulation, respectively. Recent patch clamp studies have revealed the presence of inward-conducting (inward-rectifying) K^+ channels in the plasma membrane of higher plant cells. Research on guard cells has suggested that these K^+ channels provide a major pathway for proton pump-driven K^+ uptake during stomatal opening. In the present study the contribution of inward-rectifying K^+ channels to higher plant cell K^+ uptake was investigated by examining kinetic properties of guard cell K^+ channels in *Vicia faba* in response to changes in the extracellular K^+ concentration. Increasing the extracellular K^+ concentration in the range from 0.3 mM to 11.25 mM led to enhancement of inward K^+ currents and changes in current–voltage characteristics of K^+ channels. The increase in K^+ conductance as a function of the extracellular K^+ concentration revealed a K^+-equilibrium dissociation constant (K_m) of ≈3.5 mM, which suggests that inward-rectifying K^+ channels can function as a molecular mechanism for low-affinity K^+ uptake. Lowering the extracellular K^+ concentration in the range from 11 mM to 1 mM induced negative shifts in the activation potential of K^+ channels, such that these channels function as a K^+ sensor, permitting only K^+ uptake. At low extracellular K^+ concentrations of 0.3 mM K^+, inward-rectifying K^+ channels induce hyperpolarization. Results from the present study suggest that inward-rectifying K^+ channels constitute an essential molecular mechanism for plant nutrition and growth control by providing a K^+-sensing and voltage-dependent pathway for low-affinity K^+ uptake into higher plant cells and additionally by contributing to plasma membrane potential regulation.**

Potassium plays a vital role as a macronutrient during plant growth, movements, enzyme homeostasis, and osmoregulation (1–3). Elucidation of molecular mechanisms by which higher plant cells transport and accumulate K^+ across the plasma membrane is essential to understanding plant nutrition and growth. Tracer flux studies of K^+ uptake have shown that at least two major mechanisms of K^+ uptake prevail, which are responsible for low-affinity and high-affinity K^+ uptake into plants as well as into various types of plant cells (ref. 4; for reviews see refs. 3 and 5). Additional K^+-uptake mechanisms may function at high extracellular K^+ concentrations (6). Deprivation of extracellular K^+ leads to induction of a high-affinity K^+ uptake system, which shows an equilibrium dissociation constant in the range of 5–40 μM (2–5, 7).

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Low-affinity K^+ uptake allows half-maximal K^+ influx to occur at extracellular K^+ concentrations in the range of approximately 2–8 mM (1, 4, 5, 8). Low-affinity K^+ uptake represents a major component of K^+ uptake into plants, as >80% of soils contain a soil solution concentration of 0.5 mM to >10 mM K^+ (9). Although many environmental stress factors are directly related to K^+ uptake into higher plant cells (1–3), the molecular mechanisms that allow high- or low-affinity K^+ accumulation remain unknown. Tracer flux studies of K^+ (Rb^+) uptake into guard cells suggest that these cells have low- and high-affinity K^+-uptake mechanisms analogous to K^+-uptake properties of other higher plant cells (for review, see ref. 10). These observations indicate that guard cells lend themselves to identification of the molecular mechanisms responsible for K^+ uptake.

Inward-rectifying K^+ channels ($I_{K^+,in}$ channels) were identified in guard cells (11, 12) and have been found in several higher plant cell types, including root cells (refs. 13–17; for a review, see ref. 18). These $I_{K^+,in}$ channels have been suggested to provide a major mechanism for K^+ uptake across the plasma membrane of guard cells during stomatal opening. $I_{K^+,in}$ channels are activated by membrane potentials more negative than approximately −90 mV and allow selective K^+ influx into higher plant cells (11, 13, 16, 19). Light-activated electrogenic proton-extruding pumps, which hyperpolarize the plasma membrane (19–23), provide the electrochemical driving force for $I_{K^+,in}$ channel-mediated K^+ uptake. Support for the hypothesis that $I_{K^+,in}$ channels constitute a major pathway for K^+ uptake during stomatal opening has been gained from detailed studies on *Vicia faba* guard cells, which resulted in the following findings. (*i*) Average physiological K^+-uptake currents during stomatal opening of ≈10 pA per guard cell (24) can be carried by $I_{K^+,in}$ channels (11, 12). (*ii*) The K^+ selectivity with respect to other alkali metal ions and steady-state activation properties of $I_{K^+,in}$ channels support physiological properties of the ionic specificity and long-term K^+ accumulation into guard cells (11, 12, 19). (*iii*) Aluminum ions, which inhibit stomatal opening (25), block $I_{K^+,in}$ channels at similar concentrations (19). (*iv*) Elevation of the cytosolic Ca^{2+} concentration to micromolar levels inhibits $I_{K^+,in}$ channels (26), which correlates to Ca^{2+} inhibition of stomatal opening and reduction of K^+ uptake (27, 28). In the present study the contribution of $I_{K^+,in}$ channels to physiological K^+ uptake fluxes was investigated by examining effects of naturally occurring changes in the extracellular K^+ concentration (9, 24) on K^+-channel properties and on electrical characteristics of the plasma membrane.

MATERIALS AND METHODS

Cell Isolation. *V. faba* plants were grown in a controlled environment growth chamber (Conviron E15) at 20°C (cham-

Abbreviation: $I_{K^+,in}$ channel, inward-rectifying K^+ channel.

ber humidity of 70–80%) with a 12-hr light/12-hr dark day/night cycle by using fluorescent and incandescent illumination at a photon fluence rate of 150 μmol·m^{-2}·s^{-1}. Plants were subirrigated with a nutrient solution containing Peters Professional 20-20-20 (W. R. Grace, Fogelsville, PA) at 2.6 g/liter. Guard cell protoplasts were isolated from 3- to 4-week-old plants following two different procedures, which gave identical results. One procedure has been described (19, 29), whereas the second isolation protocol was adapted by modification of a published procedure (30). In brief, two fully expanded leaves from *V. faba* were blended three times (Waring, 20,000 rpm), for 15 sec each, in deionized water (4°C) and passed and rinsed through a 200-μm mesh in order to purify epidermal tissue after each blending step. After a 10-min incubation in 0.5 M D-mannitol/0.1 mM $CaCl_2$, pH 5.5, pure epidermes were transferred to a digestion medium containing 1% Cellulase R10 and 0.5% Macerozym R10 (Yakult Honsha, Tokyo), 0.5 M mannitol, and 0.1 mM $CaCl_2$ and placed on a reciprocal shaker (ν = 0.5 Hz) for a 16-hr overnight incubation at 23°C. Isolated guard cell protoplasts were washed and purified in 0.5 M mannitol and 0.1 mM $CaCl_2$ as described (19).

Patch Clamp. Whole-cell patch clamp recordings (31) were performed on isolated guard cell protoplasts as described (12, 19). During whole-cell recordings the ionic contents of the cytosol of single guard cells can be controlled by perfusion with the patch clamp pipette solution (26, 31). This pipette solution included 75 mM potassium glutamate, 35 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA (potassium salt), 10 mM Hepes, 2 mM (or 0 mM) MgATP, 2 mM KOH at pH 7.2. Cells were bathed in solutions with the indicated concentrations of potassium glutamate, which included 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Mes at pH 5.5. Osmolalities were adjusted to 500 mmol·kg^{-1} with D-mannitol. K^+-equilibrium potentials were calculated after correction for ionic activities (32). Extracellular solutions were exchanged by perfusion (1 ml/min) of the recording chamber (volume $\approx$ 150 μl) with a peristaltic pump (Rainin). To ensure stable reference electrode potentials during solution changes, a 3 M KCl agar pipette was used as a bath electrode and was placed adjacent to the suction tube of the bath perfusion. Liquid junction potentials at the interface of the patch clamp pipette solution and the first bath solution are initially not accounted for and were corrected after whole-cell recordings were obtained (19, 33). All membrane potentials are specified as the potential on the cytoplasmic side of the membrane relative to that on the extracellular side of the membrane. Patch clamp recording and data analyses were performed with an Axopatch 1D patch clamp amplifier and stored on-line via an Axolab interface (Axon Instruments, Foster City, CA) on a 25-MHz, 386-based microcomputer as described (12). Current–voltage curves were obtained by subtracting the linear background conductance of cells, which showed whole-cell membrane resistances in the range of 7–25 GΩ (10^9 V/A). As in a previous study of Ca^{2+}-permeable channels (29), $I_{K^+,in}$ channels were analyzed after outward-rectifying K^+ currents had run down (12, 19), allowing detailed characterization of $I_{K^+,in}$.

RESULTS

When the membrane potential of single isolated guard cells was stepped to values more negative than −100 mV, an inward ion current was activated (Fig. 1 *A* and *C*), which has been shown to be carried by several hundred K^+-selective ion channels in the guard cell plasma membrane (11, 12). When guard cells were extracellularly perfused with a solution containing no K^+, voltage-dependent $I_{K^+,in}$ currents vanished, confirming the K^+ permeability of inward currents (Fig. 1 *B* and *C*).

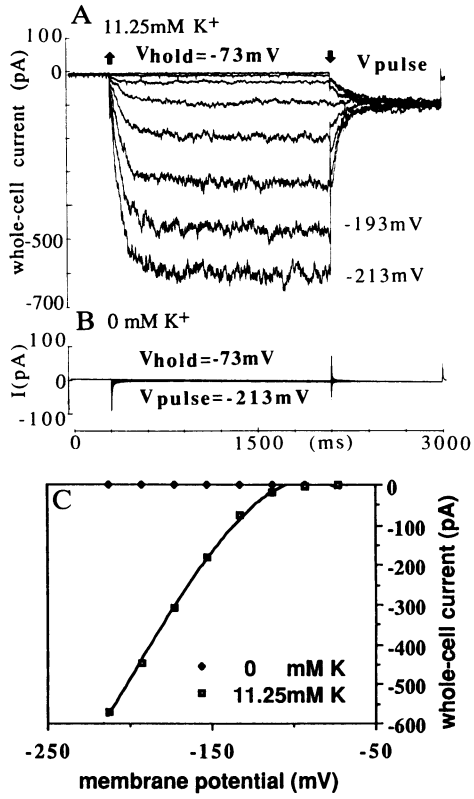

FIG. 1. $I_{K^+,in}$ channel currents in the plasma membrane of a guard cell protoplast. (*A*) K^+-channel currents measured in the presence of 11.25 mM external K^+. (*B*) Transmembrane currents measured in the absence of external K^+. (*C*) Transmembrane currents are plotted as a function of the applied voltage steps in *A* and *B*. *A* and *B* show the superposition of eight current recordings in response to hyperpolarizing voltage steps from a holding potential (V_{hold}) of −73 mV. $I_{K^+,in}$ channel currents (12) were activated by negative voltage steps (V_{pulse}) in the range of −73 mV to −213 mV. Pulse potentials (V_{pulse}) are indicated to the right of voltage pulse-activated current traces in Figs. 1*A*, 2, and 3. Pulse potentials were applied between the arrows. Downward current deflections represent influx of K^+ into cells in all figures. [The membrane was stepped to a final potential (after the downward pointing arrow) of −133 mV in Figs. 1 and 2 and −137 mV in Fig. 3.]

The whole-cell K^+ current allows comparison to measured K^+ fluxes in plant cells (5, 10). The sensitivity of $I_{K^+,in}$ channels to physiological changes in the extracellular K^+ concentration (24, 34) was examined (Fig. 2). Complete exchange of extracellular solutions during bath perfusions was verified by recording the reversal of $I_{K^+,in}$ channel currents using a relaxation ("tail") current procedure (12). Lowering the extracellular K^+ concentration from 11.25 mM K^+ to 1 mM K^+ led to reduction in the inward K^+ current (Fig. 2). Lowering the K^+ concentration also increased the proportion of the current that activated rapidly ("instantaneous current") with respect to the current contribution that activated slowly during the pulse (Fig. 2 *B* and *C*). This instantaneously rectifying current was also carried by K^+ influx, because perfusion with K^+-free solutions led to abolition of this current (Fig. 1*B*) and because reversal potential measurements confirmed the K^+ selectivity of the instantaneous current (data not shown).

Current–voltage analysis revealed that lowering the external K^+ concentration shifted the activation potential of $I_{K^+,in}$ channels to more negative values (Fig. 2*D*). This sensitivity of the activation potential to extracellular K^+ enables these K^+ channels to function as a voltage-dependent "valve", which allows steady-state K^+ influx when the membrane is

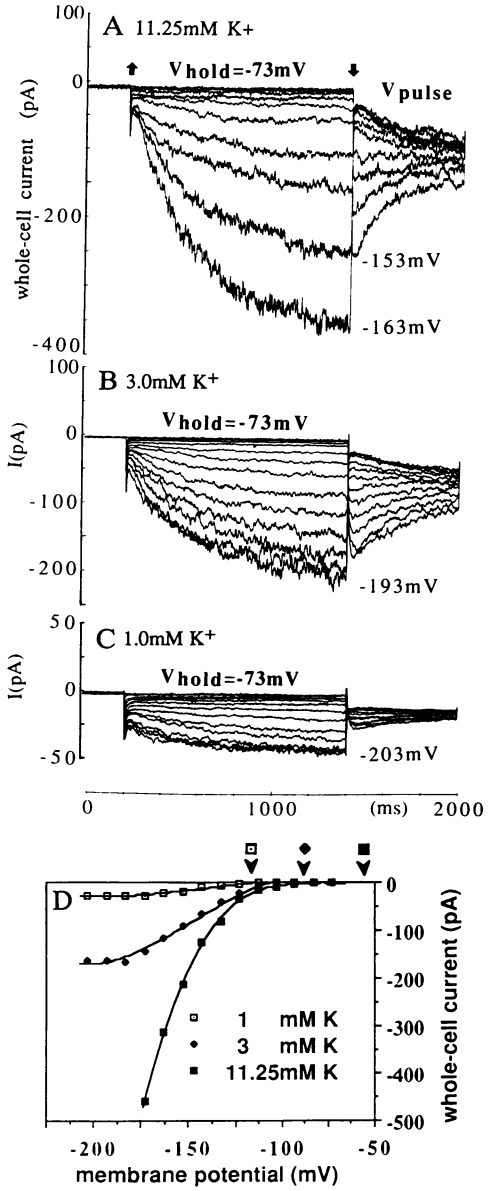

FIG. 2. Effect of changes in the extracellular K^+ concentration on $I_{K^+,in}$ channel currents in a *V. faba* guard cell. (*A*) $I_{K^+,in}$ currents recorded at an extracellular K^+ concentration of 11.25 mM. (*B* and *C*) $I_{K^+,in}$ currents recorded after perfusion with 3 mM K^+ (*B*) and 1 mM K^+ (*C*) in the bath. (*D*) The steady-state current–voltage curves of *A–C* are shown. Symbols and arrows at the top of *D* indicate K^+ equilibrium potentials for the respective extracellular K^+ concentrations. Extracellular K^+ concentrations for each symbol are indicated in *D*. V_{hold}, holding potential.

hyperpolarized to potentials more negative than the K^+ equilibrium potential.

At 0.3 mM extracellular K^+, recordings of inward K^+ currents at potentials negative to the K^+ equilibrium potential show that physiologically significant K^+ uptake fluxes (≥10 pA inward current per guard cell; refs. 10, 12 and 24) can be carried by $I_{K^+,in}$ channels at low extracellular K^+ (Fig. 3). Interestingly, at 0.3 mM external K^+, small voltage-dependent outward currents (+2.2 ± 1.2 pA; $n = 5$) could be detected at potentials slightly positive to the K^+ equilibrium potential (Fig. 3*B*). These small outward currents were also observed when the cytosol of cells was perfused with an ATP-free solution, suggesting that they were not carried by electrogenic plasma membrane proton pumps. The resting potential of cells recorded with patch pipettes containing

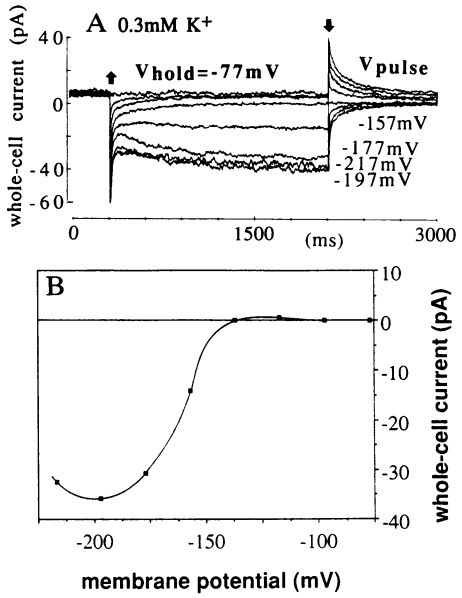

FIG. 3. (*A*) Recording of $I_{K^+,in}$ currents in the presence of 0.3 mM K^+ in the extracellular medium. (*B*) The steady-state current–voltage relationship of *A* is shown. V_{hold}, holding potential; V_{pulse}, pulse potential.

ATP-free solutions and bathed in 0.3 mM K^+ was in the range of the K^+ equilibrium potential (approximately −145 mV). Membrane potentials of guard cells extracellularly perfused with low K^+ (nominally K^+-free solutions) were as low as −250 mV. These findings as well as the voltage- and time-dependent activation of small outward currents (Fig. 3) indicate that the activation potential of $I_{K^+,in}$ currents was slightly positive to the K^+ equilibrium potential when cells were bathed in 0.3 mM K^+.

Previous findings with different recording solutions have indicated that the activation potential of *time-dependent* $I_{K^+,in}$ channel currents in guard cells did not clearly follow the K^+ equilibrium potential at very high extracellular K^+ concentrations (100 mM K^+) (19). This behavior differs markedly from $I_{K^+,in}$ channels in animal cells (35). Here, we have studied the activation potential of the complete $I_{K^+,in}$ channel currents as a function of the K^+ equilibrium potential in 16 guard cells (Fig. 4). Fig. 4 illustrates that $I_{K^+,in}$ channels allow

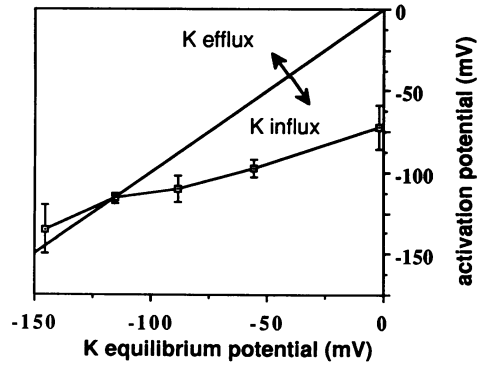

FIG. 4. The activation potential of $I_{K^+,in}$ channels is plotted as a function of the K^+-equilibrium potential. Data points below the diagonal line represent activation potentials that restrict steady-state K^+ currents to K^+ uptake, whereas data points above the diagonal line show activation potentials that allow small outward K^+ currents in addition to larger K^+ uptake currents (see text and Fig. 3). Extracellular K^+ concentrations for points shown from left to right were 0.3 mM K^+, 1 mM K^+, 3 mM K^+, 11.2 mM K^+, and 110 mM K^+ ($n = 16$ cells; error bars indicate the SD).

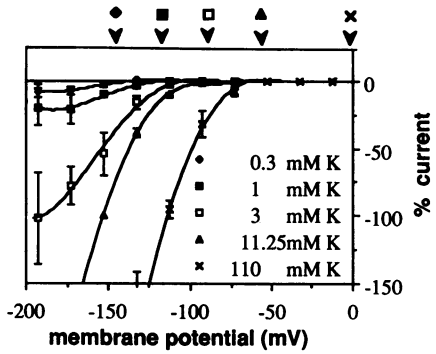

FIG. 5. Normalized current–voltage curves of $I_{K^+,in}$ currents recorded with extracellular K^+ concentrations in the range of 0.3 mM K^+ (smallest currents) to 110 mM K^+ (largest currents). K^+ currents were normalized with respect to current levels recorded at −153 mV and 11.25 mM external K^+ in each guard cell. Arrows on the top delineate the K^+-equilibrium potentials for the respective extracellular K^+ concentrations. (Extracellular K^+ concentrations for each symbol are defined in the figure; n = 11 cells; error bars indicate the SD.)

solely steady-state K^+ influx at extracellular K^+ concentrations ranging from >1 mM (K^+ equilibrium potential = −116 mV) to 110 mM (K^+ equilibrium potential = −1.3 mV). In this concentration range, the activation potential of instantaneous $I_{K^+,in}$ currents shows a very weak K^+ dependence, shifting ≈3.8 mV for every 10 mV change in the K^+ equilibrium potential (Fig. 4). At extracellular concentrations of 0.3 mM K^+, the mean activation potential of $I_{K^+,in}$ currents is slightly positive to the K^+ equilibrium potential (Fig. 4, leftmost point), thereby allowing small outward K^+ currents (Fig. 3), which were found to clamp the membrane potential of guard cells to the K^+-equilibrium potential.

To determine the K^+-concentration dependence of K^+ uptake through $I_{K^+,in}$ channels, current–voltage characteristics of K^+ channels were analyzed while exposing guard cells to various extracellular K^+ concentrations. Fig. 5 shows normalized and averaged current–voltage curves of $I_{K^+,in}$ channels from 11 guard cells. Saturation of current–voltage curves became apparent at potentials in the range of −185 mV at 0.3 mM K^+ and at 1 mM K^+ (Fig. 5). The whole-cell K^+ current is the product of the number of K^+ channels multiplied by the single-channel current and by the opening probability of K^+ channels. Instantaneous tail current analysis (36) shows that K^+-current saturation can be attributed to saturation of the single-channel conductance (J.I.S., unpublished results), which confirms results obtained from single K^+-channel recordings in guard cells (11, 37).

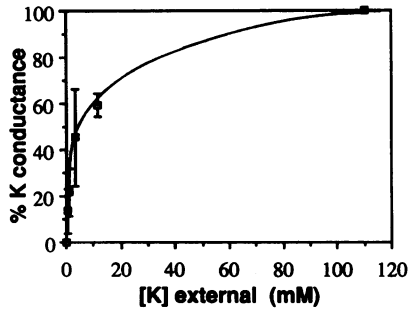

FIG. 6. Concentration dependence of the K^+ chord conductance (36) of $I_{K^+,in}$ channels. The fitted Michaelis–Menten relationship reflected an equilibrium dissociation constant (K_m) of ≈3.5 mM K^+. Chord conductances were determined at −173 mV and are plotted relative to the $I_{K^+,in}$ current conductance measured at 110 mM extracellular K^+ in each cell (n = 8 cells; error bars indicate the SD).

The K^+ conductance of $I_{K^+,in}$ currents was determined as a function of the extracellular K^+ concentration. The K^+ dependence was analyzed by fitting Michaelis–Menten and Hofstee equations (see ref. 5) to the data. The increase in K^+ conductance as a function of the extracellular K^+ concentration could be described by Michaelis–Menten kinetics with an equilibrium dissociation constant of 3.5 mM K^+ (Fig. 6). Ion channel conductances are known to follow Michaelis–Menten type saturation kinetics because of interaction of the permeating ion with the channel protein (38). The dependence of the K^+-channel conductance on extracellular K^+ resembles the kinetics of a low-affinity K^+-uptake transporter in higher plant cells (for reviews, see refs. 2, 3, and 5).

DISCUSSION

Low-Affinity K^+ Uptake by $I_{K^+,in}$ Channels. In spite of the central role of K^+ uptake for higher plant cell macronutrition, the molecular mechanisms of high- and low-affinity transmembrane uptake of K^+ remain unknown. Cell biological, biophysical, and pharmacological data have suggested that $I_{K^+,in}$ channels in guard cells provide a major pathway for proton pump-driven K^+ uptake during stomatal opening (11, 12, 19, 26). $I_{K^+,in}$ channels have been identified in numerous types of higher plant cells including aleurone, root, pulvinus, epidermal, and leaf cells (refs. 13–17; for a review see ref. 18), suggesting that they may provide a general pathway for higher plant cell K^+ uptake. Results from the present study and previous findings (11–19, 26) question the hypothesis that $I_{K^+,in}$ channels cannot contribute to K^+ uptake in higher plant cells (23).

In the present study, $I_{K^+,in}$ channels were shown to support K^+ uptake into guard cells at millimolar as well as at submillimolar (0.3 mM K^+) free K^+ concentrations. Measurements of physiological changes in the free K^+ activity in the cell wall space of guard cells have shown that extracellular K^+ concentrations of 1.8 mM occur during stomatal opening (2.8 ± 1.0 mM K^+; ref. 34). Similarly, apoplastic free K^+ concentrations in the range of 2–15 mM have recently been determined in leaf cell walls of *Pisum sativum* (39) and in the cell wall of expanding pulvinus cells of *Samanea* (5–15 mM K^+; ref. 40). Soil solution concentrations in the range of 0.5 mM to >10 mM K^+ were found to occur in >80% of 155 investigated soil types (9). These data suggest that a K^+ uptake mechanism with an affinity in the range of several millimolar K^+ contributes significantly to plant cell and higher plant K^+ accumulation (1, 3–5). The K^+-concentration dependence of $I_{K^+,in}$ channel conductances could be described by Michaelis–Menten kinetics with a K_m of ≈3.5 mM (Fig. 6), which is comparable to low-affinity K^+ uptake kinetics determined in various plant cells and plant tissues (1, 3–5). These findings lead us to suggest that inward-rectifying K^+ channels in higher plants provide an important mechanism for low-affinity K^+ uptake.

K^+ Sensing and Membrane Potential Control by K^+ Channels. Determination of the activation potential of $I_{K^+,in}$ channels showed that these K^+ channels can "sense" changes in the extracellular K^+ concentration (Figs. 2, 4, and 5), thereby furnishing a potassium- and voltage-dependent mechanism for proton pump-driven K^+ influx. At reduced extracellular concentrations of 0.3 mM K^+, small outward K^+ currents as well as large inward K^+ currents could be detected (Fig. 3). In the absence of H^+ pump activity, as well as the absence of depolarizing Ca^{2+}-permeable channels (29) and anion channels (26, 41), a minute K^+ efflux charge of 0.87 pA·s would be sufficient to shift the membrane potential of a guard cell with a 6-pF membrane capacitance (12) to −145 mV. Hence, small K^+ efflux currents recorded at low extracellular K^+ concentrations may be sufficient to contribute to hyperpolarization as was observed here. The finding that K^+

channels can hyperpolarize the membrane potential to large negative potentials is suggestive of a contribution of $I_{K^+,in}$ channels to the control of high-affinity K^+ uptake, because inducible high-affinity K^+-uptake transporters in higher plant cells and fungi may be stimulated by hyperpolarization (20, 42, 43) and because small H^+ pump currents of several pA per cell may be sufficient to drive low rates of K^+ uptake through $I_{K^+,in}$ channels, thus contributing to K^+ uptake at low extracellular K^+ concentrations.

It can be concluded that $I_{K^+,in}$ channels provide an important K^+- and voltage-sensing pathway for low-affinity K^+ uptake into higher plant cells and additionally that these K^+ channels may contribute to membrane potential control at low extracellular K^+ concentrations.

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