

# K<sup>+</sup> Transport Properties of K<sup>+</sup> Channels in the Plasma Membrane of *Vicia faba* Guard Cells

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**ABSTRACT** Electrical properties of the plasma membrane of guard cell protoplasts isolated from stomates of *Vicia faba* leaves were studied by application of the whole-cell configuration of the patch-clamp technique. The two types of K<sup>+</sup> currents that have recently been identified in guard cells may allow efflux of K<sup>+</sup> during stomatal closing, and uptake of K<sup>+</sup> during stomatal opening (Schroeder et al., 1987). A detailed characterization of ion transport properties of the inward-rectifying ( $I_{K^+,in}$ ) and the outward-rectifying ( $I_{K^+,out}$ ) K<sup>+</sup> conductance is presented here. The permeability ratios of  $I_{K^+,in}$  and  $I_{K^+,out}$  currents for K<sup>+</sup> over monovalent alkali metal ions were determined. The resulting permeability sequences ( $P_{K^+} > P_{Rb^+} > P_{Na^+} > P_{Li^+} \gg P_{Cs^+}$ ) corresponded closely to the ion specificity of guard cell movements in *V. faba*. Neither K<sup>+</sup> currents exhibited significant inactivation when K<sup>+</sup> channels were activated for prolonged periods (>10 min). The absence of inactivation may permit long durations of K<sup>+</sup> fluxes, which occur during guard cell movements. Activation potentials of inward K<sup>+</sup> currents were not shifted when external K<sup>+</sup> concentrations were changed. This differs strongly from the behavior of inward-rectifying K<sup>+</sup> channels in animal tissue. Blue light and fusicoccin induce hyperpolarization by stimulation of an electrogenic pump. From slow-whole-cell recordings it was concluded that electrogenic pumps require cytoplasmic substrates for full activation and that the magnitude of the pump current is sufficient to drive K<sup>+</sup> uptake through  $I_{K^+,in}$  channels. First, direct evidence was gained for the hypothesis that  $I_{K^+,in}$  channels are a molecular pathway for K<sup>+</sup> accumulation by the finding that  $I_{K^+,in}$  was blocked by Al<sup>3+</sup> ions, which are known to inhibit stomatal opening but not closing. The results presented in this study strongly support a prominent role for  $I_{K^+,in}$  and  $I_{K^+,out}$  channels in K<sup>+</sup> transport across the plasma membrane of guard cells.

## INTRODUCTION

Vital processes in higher plant cells such as solute transport, plant movements, osmoregulation, and growth require the transport and accumulation of K<sup>+</sup> salts in

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specialized cells of various tissues (Satter et al., 1974; Lüttge and Pitman, 1976). This study focuses on guard cells that surround stomatal pores in the epidermis of leaves. Movements of two guard cells lead to variations in the aperture of their central pore (stomatal pore). Changes in the pore aperture regulate the gas exchange for photosynthesis while controlling water loss of the plant to the atmosphere. Stomatal pores open when the two surrounding guard cells augment their osmotic potential by increasing their concentration of  $K^+$  and counter ions (Imamura, 1943; Humble and Raschke, 1971; Raschke, 1979). Release of  $K^+$  and counter ions by guard cells leads to closure of the stomatal pore. Environmental signals such as blue light, red light,  $CO_2$ , temperature, and drought regulate the gas exchange of plants by controlling the  $K^+$  salt content of guard cells (for review see: Raschke, 1979; Zeiger, 1983).

Guard cells provide an ideal model system for the purpose of studying processes that underlie  $K^+$  transport across higher plant membranes. Patch-clamp studies have revealed the existence of inward- and outward-rectifying  $K^+$  channels in the plasma membrane of guard cells. It has been suggested that these  $K^+$  channels allow uptake and release of  $K^+$  during the stomatal opening and closing (Schroeder et al., 1984; Schroeder et al., 1987). In recent voltage-clamp studies time-dependent outward-rectifying  $K^+$  channels have been found in the plasma membrane of algae, yeast, and various other higher plant cells, and have been suggested to play a role in  $K^+$  release during osmoregulation (Findlay and Coleman, 1983; Beilby, 1985; Gustin et al., 1986; Satter et al., 1986; Sokolik and Yurin, 1986; Bertl and Gradmann, 1987; Iijima and Hagiwara, 1987). The voltage-dependent inwardly rectifying  $K^+$  channels, which activate in a time-dependent manner ( $I_{K^+,in}$ ) were originally described in guard cells (Schroeder et al., 1987). Electrogenic  $H^+$  pumps, which are activated by blue and red light, have been suggested to provide the driving force for  $K^+$  uptake through  $K^+$  channels (Assmann et al., 1985; Shimazaki et al., 1986; Serano et al., 1988).

The object of this study is to examine critically whether voltage-dependent  $K^+$  channels represent a significant pathway for the uptake and release of  $K^+$  in guard cells during the regulation of gas exchange in leaves. For this purpose biophysical properties of the plasma membrane have been investigated in detail that can be compared to previous studies of guard cell movements. The following questions were addressed: Are the alkali metal ion selectivities of  $I_{K^+,in}$  and  $I_{K^+,out}$  comparable to selectivities found in tracer flux studies of stomatal movements (Raschke, 1975; MacRobbie, 1983; Zeiger, 1983)? As  $K^+$  fluxes in guard cells occur on a slow time scale, how do the kinetics of  $I_{K^+,in}$  and  $I_{K^+,out}$  behave in response to sustained ( $\geq 10$  min) depolarizations and hyperpolarizations? Do activation potentials of  $I_{K^+,in}$  and  $I_{K^+,out}$  channels shift when  $K^+$  gradients are varied? Can blockers of  $K^+$  channels that also block stomatal movements be found? Slow-whole-cell recordings (Lindau and Fernandez, 1986) were established to examine whether blue light stimulation of electrogenic pumps could provide sufficient driving force to activate  $I_{K^+,in}$  channels and to propel  $K^+$  uptake through these channels. In this report, direct investigation of these questions with the patch-clamp technique allows evaluation of the importance of  $K^+$  channels for  $K^+$  transport during guard cell movements. Some aspects of this study have been published in abstract form (Schroeder, 1988).

## METHODS

*Solutions*

Solutions were developed that permitted good yields in sealing and stable recordings from single protoplasts for periods of up to several hours. The composition of the bath solution was 10 mM K<sup>+</sup>-glutamate, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1 mM KOH, 10 mM MES ([*N*-morpholino]ethanesulfonic acid), pH 5.5, osmolality adjusted to 480 mmol · kg<sup>-1</sup> with D-mannitol. The composition of the intracellular medium was 100 mM K<sup>+</sup>-glutamate, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 5 mM KOH, 10 mM HEPES, 2 mM MgATP, pH 7.2, osmolality adjusted to 530 mmol · kg<sup>-1</sup> with D-mannitol. Osmolalities were measured with a vapor pressure osmometer (5100 c; Wescor Inc., Logan, UT). The 10% higher osmolality of the pipette solution with respect to the bath made it possible to obtain stable and low access resistances between the patch pipette and the cytoplasm, thus enabling well defined voltage-clamp conditions (effective  $R_{\text{access}} < 10 \text{ M}\Omega$ ). This behavior may be explained by an osmotic shrinking of the vacuole, which can take up over 90% of the cell volume.

Permeability ratios of  $I_{\text{K}^+, \text{in}}$  and  $I_{\text{K}^+, \text{out}}$  for K<sup>+</sup> over other alkali metal ions were determined by replacing 10 mM K<sup>+</sup>-glutamate and 1 mM KOH in the bathing medium with 100 mM of various alkali metal ion glutamate salts. Relative permeabilities and K<sup>+</sup> equilibrium potentials were calculated from ionic activities. Ionic activity coefficients for 10 mM K<sup>+</sup>-glutamate, 100 mM K<sup>+</sup>-glutamate, 100 mM Li<sup>+</sup>-glutamate, 100 mM Na<sup>+</sup>-glutamate, 100 mM Rb<sup>+</sup>-glutamate, and 100 mM Cs<sup>+</sup>-glutamate solutions were 0.89, 0.77, 0.79, 0.78, 0.76, and 0.755, respectively. These values were approximated from activity coefficients of various salt solutions as determined by Robinson and Stokes (1955). Membrane potential values were corrected for the liquid junction potential that develops at the opening of the patch pipette when it is immersed into the bath solution. Liquid junction potentials were measured with a 3-M-KCl agar bridge as described elsewhere (Fenwick et al., 1982). The liquid junction potential between the 100-mM K<sup>+</sup>-glutamate internal solution and the 10-mM K<sup>+</sup>-glutamate external solution was -17 mV. With 100 mM K<sup>+</sup>-glutamate internal and 100 mM of the alkali metal ions Li<sup>+</sup>, Na<sup>+</sup>, Rb<sup>+</sup>, and Cs<sup>+</sup> in the bath, corrections of liquid junction potentials were -6, -4, +1, and +2 mV, respectively.

*Protoplast Isolation*

Protoplasts were isolated from abaxial epidermal strips of 2-3-wk-old *Vicia faba* (Gruenker-nige Hangdown (Göttingen, F.R.G.) leaves by modification of a published procedure (Gotow et al., 1984). Epidermal strips were incubated in 15 ml of 1.6% Cellulase Onozuka RS (Yakult Honsha Co., Japan), 0.016% Pectolyase Y-23 (Seishin Pharmaceutical Co., Japan), 23.2 g liter<sup>-1</sup> Gamborg's culture medium B-5 (no hormones added; Gibco, Grand Island, NY), 1 mM CaCl<sub>2</sub>, 1 mM Spermidine, 0.2% BSA (Sigma Chemical Co., St. Louis, MO), and 185 mM D-Mannitol, pH 5.57, osmolality: ~350 mmol · kg<sup>-1</sup>. The incubation medium was centrifuged at 2,000 *g* for 5 min to remove starch grains and other enzyme debris. Epidermal strips were exposed to the incubation medium in a shaking water bath at 29°C (*f* = 0.5 Hz) for 35-50 min. Released protoplasts were purified by passage through a 30- $\mu\text{m}$  mesh and centrifuged at 125 *g* and 5°C for 12 min. The pellet consisting of guard cell protoplasts was washed twice (at 125 *g* and 5°C for 10 min) in Gamborg's culture medium B-5 (no hormones added; Gibco), with 380 mM D-mannitol, 1 mM CaCl<sub>2</sub>, osmolality 500 mmol · kg<sup>-1</sup>. The ability of protoplasts to swell in the light was verified as an indication of intactness of guard cells.

*Patch Clamp and Seal Formation*

The mechanical work station for patch clamping was as described by Hamill et al. (1981) (see also Schroeder, 1987). The whole-cell configuration of the patch-clamp

technique (Hamill et al., 1981; Marty and Neher, 1983) was applied to isolated guard cell protoplasts as described previously (Schroeder et al., 1987). In general, obtaining gigaseals ( $R_{\text{seal}} \geq 10 \text{ G}\Omega$ ) between recording pipettes and plant protoplasts is more difficult than with animal cells or plant vacuoles. Therefore, procedures that may facilitate seal formation on guard cell protoplasts were developed for this study and are described in the following.

The addition of membrane-stabilizing agents and culture media to the isolation and washing solutions as well as to the  $\text{K}^+$ -glutamate solutions described above were optimized for improved yields in seal formation. In the case of very difficult sealing, preliminary experiments can be performed with up to 40% lower osmolality in the pipette solution with respect to the bathing medium. However, osmolalities needed to be adjusted as specified for reliable whole-cell voltage-clamp recordings (see *Solutions* above). Good seals were obtained when patch pipettes were fabricated from a thin-walled borosilicate glass (Kimax glass, Kimble 34500; Kimble Div., Owens-IL, Inc., Toledo, OH). Seal formation can be very slow. An adequate procedure for reproducibly forming seals was developed in which the suction is slightly increased stepwise every minute by  $\sim -1$  to  $-10 \text{ cm H}_2\text{O}$ . As soon as the seal starts to form ( $R_{\text{seal}} > 100 \text{ M}\Omega$ ) negative voltage is applied to the pipette ( $-40$  to  $-80 \text{ mV}$ ). Frequently, the gigaseal forms quickly after following this procedure for several minutes. In this case, the suction should be released immediately before breaking into the cell. The seal can be improved ( $R_{\text{seal}} \geq 20 \text{ G}\Omega$ ) by applying slight suction ( $5 \text{ cm H}_2\text{O}$ ) for 1–5 s and releasing it again several times. When all procedures described here were followed closely, high resistance seals ( $\geq 20 \text{ G}\Omega$ ) could be obtained in up to  $\sim 90\%$  of the trials. When these suitable procedures for patch clamping guard cells were applied to protoplasts from other types of higher plant tissue, gigaseals could be obtained.

For slow-whole-cell recordings the pipette solution was backfilled (100 nl behind the tip) with a solution including 50 hemolytic units of the purified bacterial porin Haemolysin from *Escherichia coli* (Menestrina, G., N. Mackman, I. B. Holland, and S. Bhakdi, manuscript in preparation). After establishment of the cell-attached recording configuration, capacitive transients were monitored. Usually, after 10–20 min slow-whole-cell capacitive transients appeared (time constant  $\sim 5 \text{ ms}$ ). Capacitances and series resistances of  $\leq 1 \text{ G}\Omega$  could be compensated by modification of an integration circuit in the EPC-7 patch-clamp amplifier.

#### *Data Recording and Acquisition*

Voltage-clamp measurements of whole-cell currents were performed at  $21\text{--}23^\circ\text{C}$  with an EPC-7 patch-clamp amplifier (List Elektronik, Darmstadt, FRG). Errors in membrane potential ( $< 6 \text{ mV}$ ) due to the voltage drop across the effective access resistance, were compensated as described previously (Schroeder et al., 1987). Data were low-pass-filtered with eight-pole Bessel characteristics and digitized at a sample rate of five times the filter cutoff frequency. Data were stored on a PDP-11/73 computer (INDEC, Sunnyvale CA) operating on-line. All data values and error bars are mean  $\pm$  SD.

#### RESULTS

In a previous study it was shown that both outward-rectifying and inward-rectifying  $\text{K}^+$  currents are present in the plasma membrane of guard cell protoplasts from *V. faba*. These  $\text{K}^+$  currents were shown to be carried by several hundred single  $\text{K}^+$

channels that are located in the plasma membrane of a guard cell, and which activate in a voltage- and time-dependent manner (Schroeder et al., 1987).

#### *Alkali Metal Ion Selectivity*

Various monovalent cations have been used in former whole tissue studies of guard cell movements (MacRobbie, 1983; see: Raschke, 1975; Zeiger, 1983). To compare

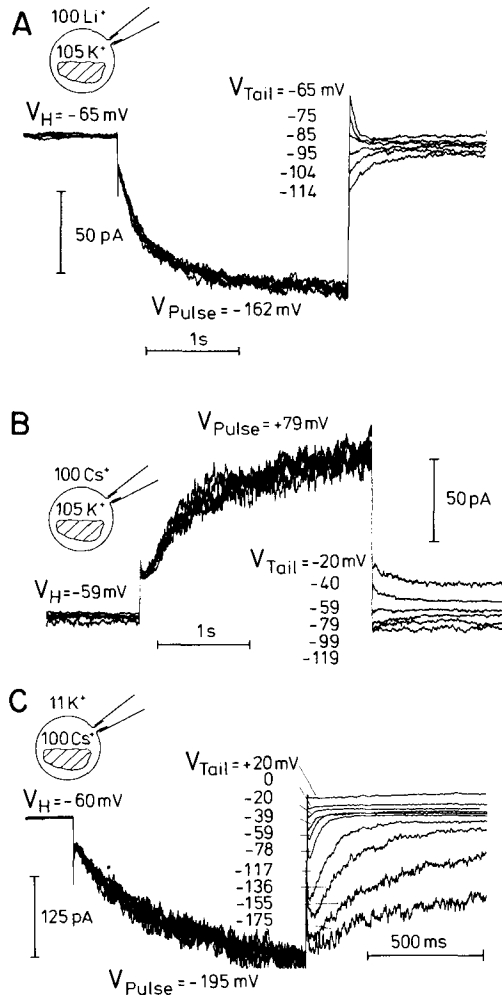


FIGURE 1. Tail current recordings, which were used to determine the reversal potential of  $K^+$  currents with 100 mM  $Li^+$  in the bath (A). With 100 mM  $Cs^+$  in the bath (B) and with 100 mM  $Cs^+$  in the cytoplasm (C), no typical tail current reversals could be found. A and B show the superposition of 6, and C the superposition of 10 current recordings at different tail potentials ( $V_{Tail}$ ).  $K^+$  currents were activated by stepping the membrane to values indicated by  $V_{pulse}$ . Subsequently, the potential was stepped to values ( $V_{Tail}$ ) at which deactivating currents were recorded.

patch-clamp recordings of  $K^+$  permeation through  $K^+$  channels with previous studies of guard cell movements it is of interest to study the complete alkali metal ion selectivity of these channels. Permeability ratios for  $K^+$  ions over the alkali metal ions  $Li^+$ ,  $Na^+$ ,  $Rb^+$ , and  $Cs^+$  were measured by determining the reversal potential of tail currents with 105 mM  $K^+$  in the cytoplasm and 100 mM of an alkali metal ion in the bath (for the method see: Schroeder et al., 1987). Fig. 1 A shows a typical recording of tail currents of  $I_{K^+,in}$  with 100 mM  $Li^+$  in the bath. It should be noted

that with 100 mM Cs<sup>+</sup> in the bath no inward currents nor characteristic inward directed tail currents could be measured (Fig. 1 B). The same was found for outward currents when cells were loaded with 100 mM Cs<sup>+</sup>-glutamate and bathed in 10 mM K<sup>+</sup>-glutamate solution (Fig. 1 C). These results suggest that both  $I_{K^+,in}$  and  $I_{K^+,out}$  channels have a low permeability for Cs<sup>+</sup> ions. The permeability ratios for K<sup>+</sup> over other alkali metal ions X<sup>+</sup> were calculated by the Goldman equation:

$$\frac{P_{K^+}}{P_{X^+}} = \frac{[X^+]_i \exp(V_{rev}F/RT) - [X^+]_o}{[K^+]_o - [K^+]_i \exp(V_{rev}F/RT)}, \quad (1)$$

where  $V_{rev}$  is the reversal potential of tail currents,  $R$  is the gas constant,  $T$  the absolute temperature, and  $F$  is Faraday's constant. The ionic concentrations in brackets were corrected for their activities in solution (i = internal, o = external). Table I summarizes the permeability ratios derived from reversal potentials of tail currents. Minimum values for  $P_{K^+}:P_{Cs^+}$  in Table I result from potentials up to which no clear tail reversal was measured. The permeability ratios in Table I show that inward K<sup>+</sup>

TABLE I  
Permeability Ratios ( $P_{K^+}:P_{X^+}$ ) of  $I_{K^+,in}$  and  $I_{K^+,out}$  Channels

|                               | Li <sup>+</sup> | Na <sup>+</sup> | K <sup>+</sup> | Rb <sup>+</sup> | Cs <sup>+</sup> |
|-------------------------------|-----------------|-----------------|----------------|-----------------|-----------------|
| $I_{K^+,in} P_{K^+}:P_{X^+}$  | 32 (±10)        | 17 (±5)         | 1 (±0.1)       | 5 (±1)          | >40             |
| $V_{rev}$ (mV)                | -89 ± 10        | -72 ± 7         | -54 ± 3        | -42 ± 6         | > +40           |
| <i>n</i>                      | 6               | 5               | 10             | 3               | 3               |
| $I_{K^+,out} P_{K^+}:P_{X^+}$ | 27 (±5)         | 8 (±3)          | 1 (±0.1)       | 3 (±1)          | >100            |
| $V_{rev}$ (mV)                | -85 ± 5         | -52 ± 10        | -53 ± 4        | -27 ± 10        | < -119          |
| <i>n</i>                      | 3               | 4               | 10             | 3               | 3               |

Permeability ratios were derived from reversal potentials ( $V_{rev}$ ) of tail currents measured with 105 mM K<sup>+</sup> in the cytoplasm and 100 mM of other alkali metal ions (X<sup>+</sup>) in the bath ( $P_{K^+}:P_{X^+}$  was measured with 11 mM K<sup>+</sup> in the bath, and  $P_{K^+}:P_{Cs^+}$  for  $I_{K^+,in}$  as shown in Fig. 1 C) (± SD; *n* is the number of cells). Data for  $P_{K^+}:P_{Na^+}$  are taken from Schroeder et al., 1987.

currents and outward K<sup>+</sup> currents had the same permeability sequence:  $P_{K^+} > P_{Rb^+} > P_{Na^+} > P_{Li^+} \gg P_{Cs^+}$ .

#### Special Gating Properties of $I_{K^+,out}$ and $I_{K^+,in}$ Channels

In previous studies (Schroeder et al., 1984, 1987) estimates of the magnitude of K<sup>+</sup> fluxes through  $I_{K^+,in}$  and  $I_{K^+,out}$  channels were based on the assumption that  $I_{K^+,in}$  and  $I_{K^+,out}$  channels do not inactivate upon prolonged stimulation. When whole cells were clamped for prolonged durations (≥10 min) to potentials at which  $I_{K^+,out}$  and  $I_{K^+,in}$  were activated, little or no inactivation could be found (Fig. 2). For outward-rectifying K<sup>+</sup> currents an average decrease of the current by 12 ± 5% (*n* = 10) was found after a 10-min stimulation at +40 mV. Inward currents showed a mean reduction of 9 ± 6% (*n* = 15) when stimulated for 10 min at -130 to -140 mV. These experiments indicate that K<sup>+</sup> channels can remain activated for physiological durations in which K<sup>+</sup> fluxes occur in guard cells. In *Chara* a sufficient number of K<sup>+</sup> channels can remain activated, which continuously polarize the membrane potential to the equilibrium potential for K<sup>+</sup> (Beilby, 1985).

In some protoplast preparations the magnitude of  $I_{K^+,out}$  has been reported to diminish ("run down") with the duration of whole-cell recording (Schroeder et al., 1987). Only preparations with stable outward  $K^+$  currents were studied in experiments as shown in Fig. 2 A. The reduction of  $I_{K^+,out}$  with time, which occurred in some preparations, could not be attributed to inactivation as this irreversible current reduction was also observed after cells were constantly clamped to  $-75$  and  $-100$  mV. Similar phenomena have been observed for different types of  $K^+$  channels in animal cells and have been attributed to washing out of cytoplasmic factors during whole-cell recordings (Levitan, 1985).

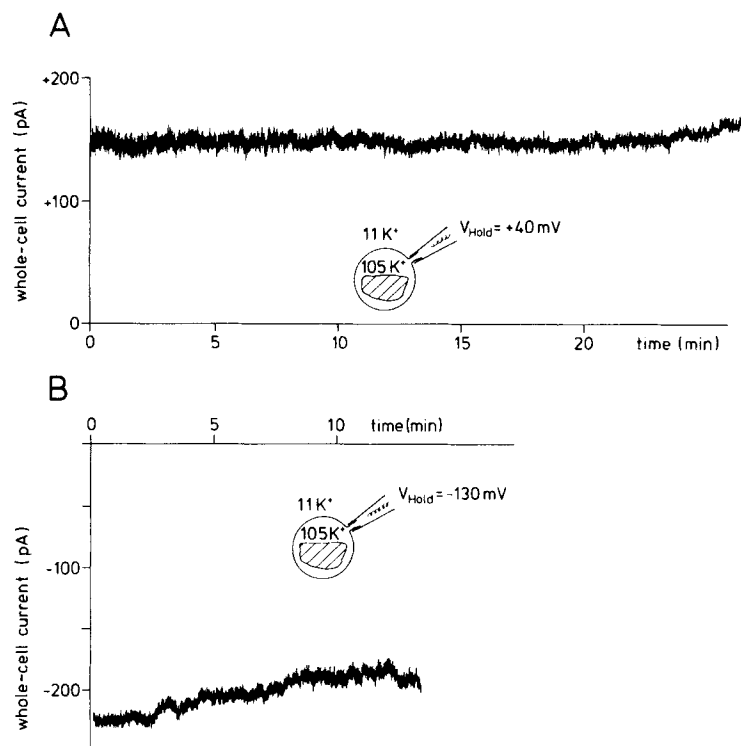


FIGURE 2. Outward  $K^+$  currents (A) and inward  $K^+$  currents (B) were recorded during continuous stimulation by holding the membrane potential at +40 mV (A) and  $-130$  mV (B). Little or no inactivation was observed.

As  $I_{K^+,in}$  channels have properties similar to inward-rectifying  $K^+$  channels in animal tissues, experiments were designed to test whether the activation potential of  $I_{K^+,in}$  shifts with changes in the equilibrium potential for  $K^+$  as has been shown in animal cells (Hagiwara and Takahashi, 1974). In Fig. 3 A  $I_{K^+,in}$  and  $I_{K^+,out}$  were recorded with 100 mM  $K^+$ -glutamate in the bath. Reversal potentials of tail currents were measured after each bath perfusion to test whether  $K^+$  concentrations were effectively changed in the vicinity of the plasma membrane. Reversal potentials shifted from  $-53$  mV with 11 mM  $K^+$  in the bath to  $-2$  mV with 100 mM  $K^+$  in the

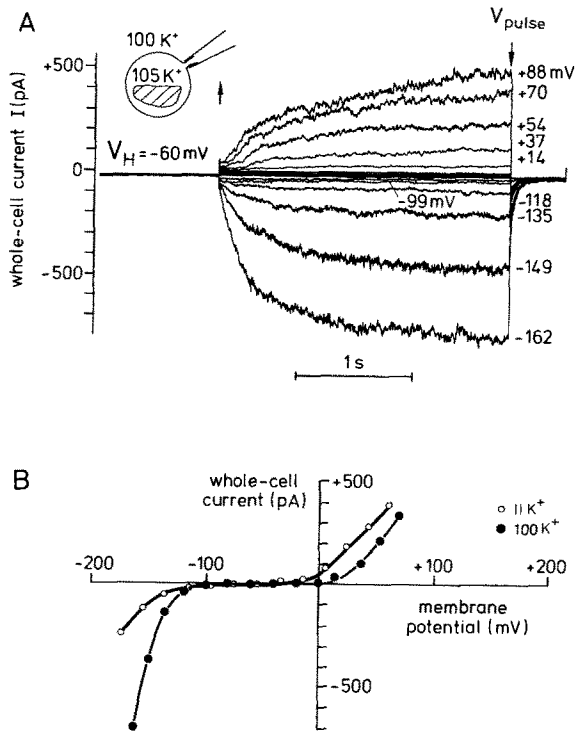


FIGURE 3. (A) Recordings of  $I_{K^+,out}$  and  $I_{K^+,in}$  in a guard cell bathed in 100 mM  $K^+$ -glutamate external solution. (B) The superposition of current-voltage relationships of the cell in A with 100 mM  $K^+$ -glutamate in the bath (filled circles) and after bath perfusion with the 10-mM  $K^+$ -glutamate solution (open circles). The activation potential of  $I_{K^+,in}$  was not shifted significantly with respect to the  $K^+$  gradient.

bath (not shown). In Fig. 3 B it becomes apparent that the activation potential of  $I_{K^+,in}$  was not shifted significantly by the change in the  $K^+$  gradient. This property of  $I_{K^+,in}$  channels reflects a pronounced difference from inward rectifiers in animal cells. Interestingly, the activation potential of  $I_{K^+,out}$  shifted by  $\sim +35$  mV with respect to recordings with 11 mM  $K^+$  in the bath. The activation potential dependence of  $I_{K^+,out}$  and  $I_{K^+,in}$  on extracellular  $K^+$  concentrations are summarized in Table II.

#### Driving Force for $K^+$ Uptake

The assumption that  $I_{K^+,in}$  channels represent a permeation path for  $K^+$  uptake during stomatal opening requires that an active transport system be capable of hyperpolarizing the membrane potential to values of  $\sim -120$  mV (Schroeder et al., 1987).

TABLE II  
Activation Potentials ( $V_{act}$ ) of  $I_{K^+,in}$  and  $I_{K^+,out}$  Measured  
with 11 mM  $K^+$  and 100 mM  $K^+$  in the Bath

|                            | 11 mM $K^+$  | 100 mM $K^+$ |
|----------------------------|--------------|--------------|
| $I_{K^+,in}: V_{act,in}$   | $-83 \pm 15$ | $-87 \pm 15$ |
| $I_{K^+,out}: V_{act,out}$ | $-39 \pm 15$ | $-2 \pm 5$   |

Activation potentials were defined as the potential at which a measurable time-dependent whole-cell current ( $\geq 1$  pA) was resolved. Values are  $\pm$  SD and  $n = 5$ .



In Fig. 4 the effect on membrane currents by the stimulators of stomatal opening blue light (A) and the fungal toxin fusicoccin (Marré, 1979; Clint and MacRobbie, 1984) (B) were investigated. Exposure to blue light ( $n = 57$ ) or bath application of fusicoccin ( $n = 17$ ) lead to the activation of an outward current. When the membrane potential was held at  $-60$  mV in symmetrical solutions, blue light and fusicoccin induced currents that surpassed the zero-current level. The blue light-activated outward current has been shown to induce membrane hyperpolarization and could only be measured when MgATP was added to the pipette solution (Assmann et al., 1985). No significant changes of  $I_{K^+,in}$  and  $I_{K^+,out}$  channels in response to blue light were found (not shown). Furthermore, outward currents could be measured in symmetrical  $K^+$ -free solutions (Fig. 4, A and B). These data imply that currents activated by blue light and fusicoccin are mediated by an electrogenic ATPase. When guard cells are exposed to light and fusicoccin, acidification of the extracellular medium has been measured (Raschke and Humble, 1973; Shimazaki et al., 1986). These data are in accord with the hypothesis that the electrogenic current is mediated by a plasma membrane  $H^+$ -ATPase (Gradmann et al., 1978; Serrano, 1985; Shimazaki et al., 1986).

Previous experiments were performed with 9.2 mM ATP in the pipette solution (Assmann et al., 1985). In order to address the question of whether physiological cytoplasmic ATP levels of  $\sim 1$  mM (Roberts, 1984; Blatt, 1987b; Michalke and Schnabl, 1987) are sufficient to activate electrogenic pumps, experiments were performed with various concentrations of MgATP in the pipette solution that dialyzes the cytoplasm. With 10 mM MgATP and 2 mM MgATP in the pipette solution, peak blue light-stimulated outward currents were  $3.4 \pm 1$  pA ( $n = 10$ ) and  $3.2 \pm 1$  pA ( $n = 10$ ), respectively. With 1 mM MgATP the peak current was reduced by 35% ( $2.1 \pm 0.6$  pA,  $n = 10$ ), and with 0.1 mM MgATP clear electrogenic currents ( $>0.2$  pA) were seldom resolved.

To provide the full driving force for  $K^+$  uptake during stomatal opening in *V. faba* leaves the magnitude of the electrogenic current would need to exceed  $+12$  pA as judged from the activation potential of  $I_{K^+,in}$  (Fig. 3, Table II), the membrane input resistance ( $\sim 10$  G $\Omega$ ) and the estimated mean rates of physiological  $K^+$  uptake for which a membrane potential of  $-120$  mV would be sufficient (Outlaw, 1983; Schroeder et al., 1987). However, in whole-cell recordings blue light-induced outward currents were always smaller than  $+12$  pA (see above). Possibly cytoplasmic substrates, which are washed out during whole-cell recordings, are required during physiological stimulation of electrogenic pumps. To test this suggestion, the recently developed slow-whole-cell recording technique, which leaves the biochemistry of the cell largely unperturbed (Lindau and Fernandez, 1986), was applied to guard cells. The magnitude of electrogenic currents activated by blue light in slow-whole-cell recordings was  $18 \pm 6$  pA ( $n = 5$ ) (Fig. 4 C). This finding suggests that diffusible cytoplasmic substrates other than MgATP, which are washed out in whole-cell recordings, may be required to fully activate blue light-stimulated pumps. At the peak of the current response in Fig. 4 C a resting potential of  $-140$  mV was measured by clamping the membrane to zero current. Resting potentials after blue light exposure were more negative than the required potential of  $-120$  mV. Similar resting potentials were recently reported in guard cells (Blatt, 1987a, b). Thus light-

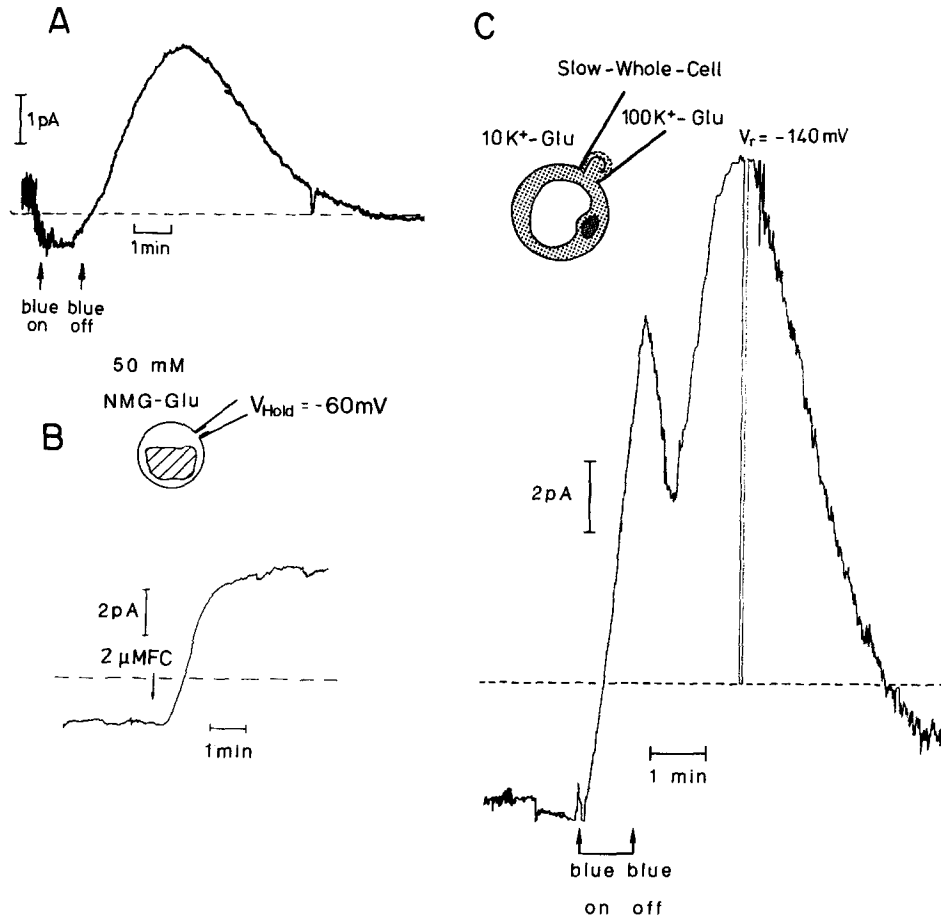


FIGURE 4. Activation of electrogenic pumps in whole-cell recordings by blue light (A) and by bath perfusion of  $2 \mu\text{M}$  of the fungal toxin fusicoccin (B) (Marré, 1979) at a holding potential of  $-60 \text{ mV}$ . C shows the activation of electrogenic pumps by blue light in a nondialyzed cell (slow-whole-cell) at  $-60 \text{ mV}$ . The protoplasts were kept in the dark for 2 min prior to exposure to fusicoccin or blue light at an irradiance of  $100 \mu\text{mol s}^{-1} \text{ m}^{-2}$ . Experiments in A and B were performed in  $\text{K}^+$ -free solutions;  $100 \text{ mM}$  and  $10 \text{ mM}$   $\text{K}^+$ -glutamate in the standard solutions were replaced by  $50 \text{ mM}$  concentrations of *n*-methylglucamine-glutamate (NMG-Glu) in both the pipette and the bath solution, and KOH was replaced by *n*-methylglucamine. In A, the relaxation of an outward current can be seen before blue light application, which can be attributed to perfusion of the cell with symmetrical solutions. In C, two peaks can be seen in the blue light-induced current. This type of behavior was observed in three out of five slow-whole-cells. The underlying mechanism for the response is not yet understood. Slow-whole-cell parameters in C were:  $R_{\text{access}} = 500 \text{ M}\Omega$ ,  $R_{\text{membrane}} = \sim 8 \text{ G}\Omega$ , and membrane capacitance =  $5.9 \text{ pF}$ .

induced hyperpolarization by electrogenic pumps appears adequate to drive K<sup>+</sup> uptake through  $I_{K^+,in}$  channels.

#### *Al<sup>3+</sup> Blocks $I_{K^+,in}$ Channels*

A direct test of the hypothesis that K<sup>+</sup> channels may be responsible for K<sup>+</sup> transport in guard cells can be accomplished by finding specific externally applicable blockers of K<sup>+</sup> channels. In a previous study it was found that externally applied Ba<sup>2+</sup> ions

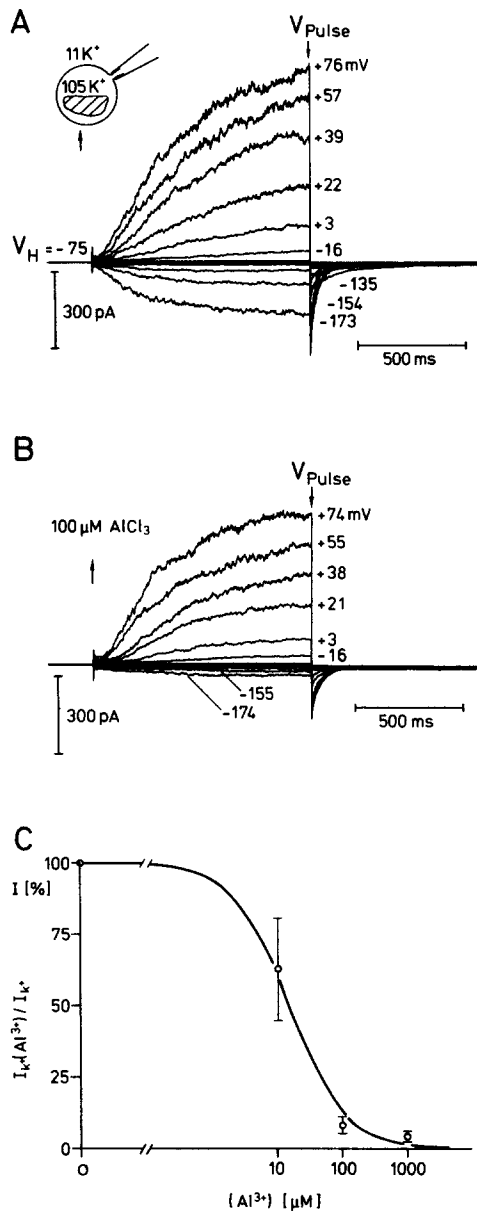


FIGURE 5. Block of  $I_{K^+,in}$  by Al<sup>3+</sup> ions. (A) K<sup>+</sup> currents measured in standard K<sup>+</sup>-glutamate solutions. (B) K<sup>+</sup> currents in the same cell after the addition of 100 μM AlCl<sub>3</sub> to the bath. (C) Normalized steady state  $I_{K^+,in}$  currents measured at -170 to -175 mV as a function of the Al<sup>3+</sup> concentration. Currents were normalized with respect to currents measured before Al<sup>3+</sup> application. The fitted dose-response curve had a  $K_D$  of 15 μM Al<sup>3+</sup> and a Hill coefficient of 1 (error bars, SD; n = 7).

block both  $I_{K^+,in}$  and  $I_{K^+,out}$  rather nonspecifically ( $K_D \sim 0.8$  mM) (Schroeder et al., 1987). It has been shown by Schnabl and Ziegler (1975) that  $Al^{3+}$  ions at a concentration of 1 mM inhibit stomatal opening but not closing in *V. faba* epidermal strips. In search of a specific  $K^+$  channel blocker, the effect of  $Al^{3+}$  ions on whole-cell currents was tested (Fig. 5). It was found that bath application of 100  $\mu$ M  $AlCl_3$  reduced the time-dependent  $I_{K^+,in}$  by >80% (Fig. 5 B). In Fig. 5 B, a reduction of  $I_{K^+,out}$  by 25% can also be detected (compare Fig. 5, A and B). This reduction of  $I_{K^+,out}$  may be attributed to "run down" (see above), as in other whole-cells, the addition of 100  $\mu$ M  $Al^{3+}$  had no significant blocking effect on  $I_{K^+,out}$  (<10% current reduction), while  $I_{K^+,in}$  was abolished (>98% current reduction). Fig. 5 C shows the dose-response curve of the  $Al^{3+}$  block of  $I_{K^+,in}$  measured at  $\sim -170$  mV. The data were fitted by a Hill equation with a  $K_D$  of 15  $\mu$ M  $Al^{3+}$  and a Hill coefficient of 1. These data represent first direct evidence for the hypothesis that  $I_{K^+,in}$  channels constitute a prominent molecular pathway for  $K^+$  uptake in guard cells.

## DISCUSSION

### *K<sup>+</sup> Channels and Guard Cell K<sup>+</sup> Transport*

The whole-cell recording technique (Hamill et al., 1981; Marty and Neher, 1983), as applied to guard cell protoplasts in this study, provides a powerful tool to investigate molecular mechanisms of transmembrane ion transport and cell biological events coupled to these processes in higher plant cells. It has been suggested in previous studies that  $I_{K^+,out}$  and  $I_{K^+,in}$  channels provide a pathway for the release and uptake of  $K^+$  ions by guard cells during the regulation of the gas exchange in leaves (Schroeder et al., 1984, 1987). The suggested significance of  $K^+$  channels was tested in this report by examination of ion transport properties of  $I_{K^+,in}$  and  $I_{K^+,out}$  and  $H^+$  pumps. The investigated transport properties can be brought in relation to investigations of the physiology of guard cell movements.

The permeability sequence among alkali metal ions of  $I_{K^+,out}$  and  $I_{K^+,in}$  was:  $P_{K^+} > P_{Rb^+} > P_{Na^+} > P_{Li^+} \gg P_{Cs^+}$ . This sequence is similar to the specificity of stomatal movements to various salts in leaves and epidermal strips of *V. faba* as studied by many researchers (for review see: Raschke, 1975; Zeiger, 1983). Both  $I_{K^+,out}$  and  $I_{K^+,in}$  did not show significant inactivation when stimulated for prolonged durations (Fig. 2), during which  $K^+$  fluxes occur in guard cells. The effects of external  $K^+$  ions on the activation potentials of  $I_{K^+,out}$  and  $I_{K^+,in}$  (Fig. 3, Table II) suggest that future studies of guard cell movements in symmetrical solutions may be of interest.

Blue light and fusicoccin stimulate electrogenic pumps (Fig. 4). In addition to activation of a plasma membrane pump, tracer flux studies suggest that fusicoccin may also inhibit efflux of cations by yet unknown mechanisms (Clint and MacRobbie, 1984). The magnitude of blue light-dependent electrogenic pumps in whole-cell recordings was not sufficiently large (< +12 pA) to drive  $K^+$  uptake through  $I_{K^+,in}$  channels. This was found with either physiological or with excess concentrations of MgATP (1, 2, and 10 mM) in the cytoplasmic solution. Therefore, slow-whole-cell recordings of electrogenic pump currents in response to blue light in nondialyzed guard cells were established. Blue light-induced  $H^+$  pump currents in slow-whole-cell recordings were approximately six times larger than in whole-cell

recordings. Furthermore, from microelectrode recordings calculated pump currents of ~200 pA have been inferred for guard cells at a membrane potential of -140 mV (Blatt, 1987b). These results imply that soluble cytoplasmic substances other than MgATP play an important role in the amplification of H<sup>+</sup> pumps. In voltage-clamp studies of electrogenic pumps in guard cells it was reported that these pumps are voltage dependent and have a stoichiometry of one charge transported per hydrolyzed ATP molecule (Blatt, 1987b). The voltage dependence indicates a decrease in pump current for potentials negative to -100 mV. This decrease in pump current suggests that blue light-stimulated currents may be smaller when measured at a holding potential of -120 mV than when measured at -60 mV as shown in Fig. 4 C. Slow-whole-cell recordings showed that sufficient hyperpolarization can be achieved by blue light-stimulated pumps (e.g., -140 mV in Fig. 4 C) to activate  $I_{K^+,in}$  channels and drive physiological rates of K<sup>+</sup> uptake through these channels. These data are supportive of the hypothesis that K<sup>+</sup> uptake can be mediated via passive diffusion of K<sup>+</sup> through  $I_{K^+,in}$  channels.

This hypothesis was strongly supported by the finding that Al<sup>3+</sup> ions at a concentration of 1 mM block both stomatal openings (Schnabl and Ziegler, 1975) and  $I_{K^+,in}$  channels ( $K_D = 15 \mu\text{M}$ , Fig. 5) in *V. faba* guard cells. Al<sup>3+</sup> ions have been shown to interfere with cytoplasmic processes in guard cells (Schnabl, 1976) and other plant cells (Haug, 1986). The selective Al<sup>3+</sup> block of  $I_{K^+,in}$  channels may be of interest for future studies as it allows pharmacological separation of functions of  $I_{K^+,in}$  and  $I_{K^+,out}$  channels. All results found in this report affirm the hypothesis that  $I_{K^+,in}$  and  $I_{K^+,out}$  channels may represent prominent molecular pathways for K<sup>+</sup> accumulation and release, respectively.

#### *Comparison of Guard Cell K<sup>+</sup> Channels with K<sup>+</sup> Channels in Other Cells*

The existence of voltage-dependent inward-rectifying K<sup>+</sup> channels that activate in a time-dependent manner ( $I_{K^+,in}$ ) similar to those identified in guard cells (Schroeder et al., 1987) has to my knowledge not been clearly shown hitherto in previous voltage-clamp studies of higher plant cells. It should be noted however that *instantaneous* inward-rectifying K<sup>+</sup> currents have been recorded in *Nitella*, which consist of two components (Sokolik and Yurin, 1986). One component resembles a deactivating tail current seen after activation of an outward-directed K<sup>+</sup> current. The other component may represent a small instantaneous activation of an  $I_{K^+,in}$  type current, as the current-voltage relationship is similar to that of the time-dependent  $I_{K^+,in}$  currents reported here.

The voltage- and time-dependent activation of outwardly directed K<sup>+</sup> currents have previously been measured in voltage-clamp recordings in preparations from algae, yeast, and higher plant cells (Findlay and Coleman, 1983; Gustin et al., 1986; Satter et al., 1986; Sokolik and Yurin, 1986; Bertl and Gradmann, 1987; Iijima & Hagiwara, 1987). Indirect measurements by current-clamp recordings in *Nitella* and *Chara* have suggested the existence of a K<sup>+</sup> conductance activated by depolarization for many years (Cole and Curtis, 1938; see: Findlay and Hope, 1976; Tazawa et al., 1987). Interestingly, the permeability sequence of  $I_{K^+,out}$  channels in guard cells is identical to the selectivity sequence recently found for K<sup>+</sup> currents in *Nitella* (Sokolik and Yurin, 1986).

Voltage-dependent  $K^+$  channels have been characterized in many tissues from animal cells (for review see: Latorre and Miller, 1983; Hille, 1984). The permeability sequence of  $K^+$  channels in guard cells as determined here is similar to that found by Hille in the myelinated nerve of frogs (1973). Although the activation time courses of voltage-dependent  $K^+$  channels in animal cells are generally on the order of 10–100 times faster than those studied here (Bezanilla, 1985), outward-rectifying  $K^+$  channels with similar activation time courses have recently been described in frog heart (Hume et al., 1986; Simmons et al., 1986). Inwardly rectifying  $K^+$  channels in guard cells, on the other hand, differ in a basic characteristic from those studied in animal tissue; the activation potential of guard cell  $I_{K^+,in}$  currents did not follow the equilibrium potential for  $K^+$  ions in symmetrical solutions (Fig. 3, Table II).

#### *The Physiological Role of $I_{K^+,out}$*

Two major physiological roles for outwardly directed  $K^+$  currents have been proposed in plant cells. (a) It has been suggested that voltage-dependent  $K^+$  conductances play a role in the repolarization of plant action potentials in both algae and higher plant cells (Sibaoka, 1966; Findlay and Hope, 1976; Simons, 1981). (b) In guard cells,  $I_{K^+,out}$  channels have also been implicated as a pathway for  $K^+$  release.

In both cases, mechanisms that lead either to transient or prolonged depolarization of the membrane potential are required. The magnitude of outward  $K^+$  currents has been estimated to account for  $K^+$  fluxes during stomatal closing, when prolonged depolarizations are assumed. Prolonged depolarizations have been observed in guard cells under current clamp (Schroeder, unpublished observation), however, the underlying mechanisms remain unresolved. It has been suggested previously that the phytohormone abscisic acid stimulates stomatal closure by inhibition of  $H^+$  pumps (Shimazaki et al., 1986) and by increasing the open probability of  $K^+$  channels (Schauf and Wilson, 1987). It becomes apparent from the electrical properties of the guard cell plasma membrane studied here that pump inhibition and increased  $K^+$  channel open time alone would not be sufficient to depolarize the membrane beyond the activation potential for  $I_{K^+,out}$ . Therefore, other mechanisms need to be postulated that induce depolarization for prolonged periods. Preliminary evidence exists for one specialized mechanism of depolarization. Ion channels have been found that are activated by stretching of the plasma membrane and have a moderate selectivity for anions over cations (Edwards and Pickard, 1987; Schroeder, 1987). Opening of stretch-activated channels would depolarize the membrane, which would lead to activation of  $I_{K^+,out}$  and to simultaneous release of  $K^+$  and anions through  $I_{K^+,out}$  and stretch channels. It is well established that a turgor sensor and regulator must exist in higher plant cells (Le Rudulier et al., 1984). Stretch-activated channels may fulfill both functions.

#### *Conclusions*

The finding that  $Al^{3+}$  ions block both  $I_{K^+,in}$  channels and stomatal opening, and the increased magnitude of blue light-stimulated pumps in slow-whole-cell recordings, taken together with the finding that  $I_{K^+,in}$  currents have been shown to be carried by passive transport through single  $K^+$  channels (Schroeder et al., 1987) strongly sug-

gest that  $I_{K^+,in}$  channels may represent a predominant pathway for  $K^+$  uptake in guard cells. The experimental results reported here, taken together with the characterization of  $I_{K^+,out}$  type channels in other plant tissue (Findlay and Coleman, 1983; Beilby, 1985; Gustin et al., 1986; Satter et al., 1986; Sokolik and Yurin, 1986; Bertl and Gradmann, 1987; Iijima & Hagiwara, 1987) and observations of  $I_{K^+,in}$  and  $I_{K^+,out}$  channels in protoplasts from various higher plant tissues (Schroeder, unpublished observation) leads to the postulate that  $I_{K^+,in}$  and  $I_{K^+,out}$  channels may constitute general mechanisms of  $K^+$  transport across the plasma membrane of plant cells.

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