Plant Physiol. (1989) 89, 1184–1192
0032-0889/89/89/1184/09/$01.00/0

Received for publication July 6, 1988
and in revised form October 14, 1988

Characterization of Potassium-Dependent Currents in Protoplasts of Corn Suspension Cells[1]

Karen A. Ketchum*, Alvin Shrier, and Ronald J. Poole

Centre for Plant Molecular Biology, Department of Biology (K.A.K., R.J.P.) and Department of Physiology (A.S.), McGill University, Montréal, Québec, Canada H3A 1B1

ABSTRACT

Protoplasts obtained from corn (*Zea mays*) suspension cells were studied using the whole cell patch-clamp technique. One time-independent current, as well as two time-dependent currents were identified. All three currents were reduced by tetraethylammonium (9 millimolar), a K^+ channel blocker. The time-independent current had a nearly linear current-voltage relationship and its reversal potential, defined as the voltage at which there is zero current, was highly dependent on the extracellular potassium concentration. One of the two time-dependent currents was activated, with rapid kinetics, by membrane hyperpolarization to potentials more negative than -100 millivolts. The second time-dependent current was activated with a sigmoidal time course by membrane depolarization to potentials more positive than -60 millivolts. It exhibited no inactivation and was carried primarily by potassium ions. These characteristics suggest that this latter current is caused by the voltage-dependent opening of delayed-rectifier K^+ channels. These three currents, which are not generated by the plasmalemma H^+-ATPase, are likely to assist in the regulation of the cellular K^+ fluxes and membrane potential.

Ion channels play a central role in the regulation of ionic and electrical gradients that energize nutrient uptake. There is also increasing evidence of their involvement in polar growth (23), turgor regulation (22), and responses to hormones (24, 25), light (11), and environmental stress (19). Channel proteins are able to serve these many functions by virtue of their ability to switch, or oscillate, between open and closed states in response to changes in membrane potential or to the binding of various ligands.

A variety of ion channels have been identified in plant cell membranes including Cl^- channels (6, 25), Ca^{2+} channels (27), and K^+ channels (2, 3, 9, 16, 20, 21, 24–26, 28). In particular, K^+ channels are thought to modify the membrane potential under specific environmental conditions. One example, that is well documented in plants of the Characeae family, is the shift in membrane potential from the hyperpolarized, 'pump state' to the depolarized, 'K^+-state' (15). This depolarization, promoted by the absence of Ca^{2+} or concentrations of external K^+ that exceed 1.0 mM, is caused by an increase in the K^+ permeability (*i.e.* opening of K^+ channels) in the plasma membrane (2–4, 16).

[1] Supported by Natural Sciences and Engineering Research Council of Canada and Department of Education of Québec. K. A. K. is supported by a McGill Major Fellowship from The Friends of McGill.

Higher plant cells do not show such conspicuous shifts to and from a K^+-permeable state. Nevertheless, there is evidence (5, 18) that K^+ influx from low external concentrations requires active transport against an electrochemical gradient (mechanism I) (7), whereas K^+ influx from external concentrations above 1 mM (mechanism II) occurs by diffusion down an electrochemical gradient. This arrangement can only work efficiently if there are K^+ channels which are closed in low K^+ and open in high K^+ solutions, and there are indications from measurements of resistance (1, 13) and of potentials (1, 5, 12) that this is the case.

Another plausible function for K^+ channels in the plant plasma membrane is the restoration of the membrane potential after a depolarization event such as a calcium influx or the presentation of solutes that are electrogenically transported with protons. In animal cells, this function is served by a class of K^+ channels (delayed rectifier channels) (14) which open in response to membrane depolarization to allow a transient K^+ efflux. Kinraide *et al.* (17) demonstrated that the cell membrane potential in oat coleoptiles first depolarized, following the addition of amino acids to the extracellular medium, and then spontaneously repolarized. This repolarization could not be attributed entirely to increased proton efflux from the plasma membrane ATPase. Hence, they hypothesized that the repolarization current was partly due to passive K^+ efflux from the cytoplasm.

Although K^+ channels have been identified in higher plant membranes (20, 24–26) little is known about the characteristics of different types of K^+ channels in plants, the distribution of these proteins between species, their occurrence in various cell types, or the factors which regulate their activity. In this paper we identify one time-independent current and two time-dependent currents present in protoplasts obtained from corn suspension cells. These currents reflect the activity of three classes of ion channels which are all K^+-dependent, but differ in their voltage sensitivity. We speculate that these currents have distinct and independent roles in controlling K^+ fluxes and modulating the membrane potential during physiological and/or environmental challenges.

MATERIALS AND METHODS

Cell Cultures

Corn suspension cells (*Zea mays*, Black Mexican sweet corn University of Illinois #78-002) derived from root tissue were grown in culture medium composed of Murashige and Skoog salt mixture (Gibco Laboratories) supplemented with

0.5 mM myoinositol, 1 μM thiamine HCl, 4 μM nicotinic acid, 2.5 μM pyridoxine HCl, 25 μM glycine, 18 μM 2,4-D, and 300 mM sucrose, (pH 5.6). Cells were maintained in continuous light and agitated on a gyratory shaker at 150 rpm. Typically, 5-d-old cells (mid log phase) were used for patch-clamp experiments.

Protoplast Isolation

Cells used for protoplast isolation were plasmolyzed in two sequential 30 min periods. This was done by allowing the cells to settle, aspirating off the supernatant and then replacing it with a new medium of progressively higher osmotic potential. The two plasmolysis solutions consisted of culture medium adjusted to 350 and 700 mOsm with mannitol. Protoplasts were prepared by incubating 1.0 g cells (wet weight) in 10 mL culture medium supplemented with the following reagents: 1.5% Cellulase Y-C, 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), 0.5% Macerozyme R-10 (Yakult Biochemical Co., Nishinomiya, Japan), 0.5% BSA, 20 mM citric acid, 1 mM DTT, and 300 mM mannitol. The cells were maintained in the dark at 25°C and gently agitated for 3.5 to 4 h until the majority of the cells had released protoplasts. The protoplasts in digestion buffer were diluted twofold with rinse medium (digestion buffer minus the enzymes and BSA), and allowed to settle to the bottom of the flask. The solution was then aspirated, leaving approximately 5 mL containing the protoplasts, and replaced by new rinse medium. This step was repeated four times. After the final rinse protoplasts were stored on ice, kept in the dark and utilized within 12 h.

Tight-Seal Whole-Cell Recording

The whole-cell patch-clamp technique (10) was used to record potential or currents from protoplasts at 22°C with a List EPC-7 patch-clamp amplifier (Medical Systems Corp., Greenvale, NY). In the whole-cell configuration the pipette interior is continuous with the cytoplasm and the currents recorded are a summation of all the open channels in the plasmalemma. Pipettes were made from borosilicate glass capillaries (Kimax-51, Kimble Products) with an average resistance of 6 MΩ. The current signal was balanced to zero with the pipette immersed in the bathing solution. Electrical access to the cytoplasm was confirmed by monitoring the resting potential of the protoplasts and by the change in capacitance. In these experiments, only those cells having a resting potential more negative than −30 mV were used. The total series resistance, or access resistance, was approximately 28 MΩ (averaged from 12 cells). This value represents the pipette resistance plus the resistance of the broken patch of membrane at the pipette tip. The resistance of the cell averaged 19 ± 11 GΩ (n = 7) with 1 mM external K-Glu.[2] This corresponded to a specific membrane resistance of 93 $\Omega \cdot m^2$. Cell capacitance varied in proportion to cell size ranging from 19 pF to 95 pF (average protoplast surface area = 4.9×10^{-9} m^2, n = 14). The average specific capacitance was 1.0 ± 0.26 $\mu F/cm^2$ (n = 14). The values for series resistance and cell capacitance corresponded with a time constant for voltage clamping of approximately 1.0 ms.

During the experiment membrane current and voltage were filtered at 10 kHz, digitized at 44 kHz by a pulse code modulation unit (Sony Digital Audio Processor PCM-501ES) and recorded on a Beta cassette recorder (Sony SL-HF900). Current amplitudes were later analyzed on a digital oscilloscope (Nicolet Explorer, model 206) and traces were plotted on an X-Y recorder (Hewlett Packard 7015B). Currents that were acquired at a high amplifier gain (i.e. those with a low signal to noise ratio) occasionally required additional filtering (as noted in the text). This was done with an RC filter (Krohn-Hite, model 3323).

Solutions

The pipette filling solution consisted of 100 mM K-Glu, 2 mM $MgCl_2$, 4 mM EGTA-Tris, 5 mM BTP-ATP, 400 mM mannitol, and 10 mM Hepes adjusted to pH 7.0 with concentrated Tris. This same solution supplemented with 2.5% agar was used to form the reference electrode. Extracellular media consisted of 1 mM K-Glu (replaced where indicated with 10 mM K-Glu), 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 575 mM mannitol buffered with 10 mM Mes to a pH of 6.25. In experiments where the external pH was 8.0, 10 mM Hepes was substituted for 10 mM Mes. Variations in K^+ concentration were osmotically compensated by the addition of NMG-Glu. TEA-Gluc, when present, was used at a concentration of 9 mM.

RESULTS

General Electrophysiological Characteristics

In the voltage clamp mode, step changes in membrane potential elicited a time-independent current and two prominent time-dependent currents. Typical examples are shown in Figure 1. Membrane depolarization induced an instantaneous change in current and, at potentials more positive than −60 mV, activated an outward current which developed with a sigmoidal time course (Fig. 1A). This current which was present in approximately 90% of the cells tested (n = 40), showed little inactivation and was similar to delayed rectifier currents described previously (14). Membrane hyperpolarization to −140 mV, in bathing medium containing 1 mM K-Glu, induced only instantaneous changes in current (Fig. 1B).

The second time-dependent current was most evident during hyperpolarization of cells bathed in media with extracellular K^+ concentrations greater than 1 mM (Fig. 1C). The development of this current was rapid, as compared to the current elicited with membrane depolarization, and the current was inwardly directed at the negative potentials required for its activation. Five of the eight cells tested in the 10 mM extracellular K^+ medium exhibited an inward current with hyperpolarizing voltage clamp steps. As well, this current was displayed in 6 of the cells bathed in 1 mM extracellular K^+ when examined at potentials more negative than −150 mV (data not shown).

[2] Abbreviations: K-Glu, potassium-glutamate; BTP, Bis-Tris-propane; NMG-Glu, *N*-methyl D-glucamine-glutamate; TEA-Gluc, tetraethylammonium-gluconate; I_i, instantaneous current; I_{ss}, steady state current; I_t, time-dependent current; S, Siemens.

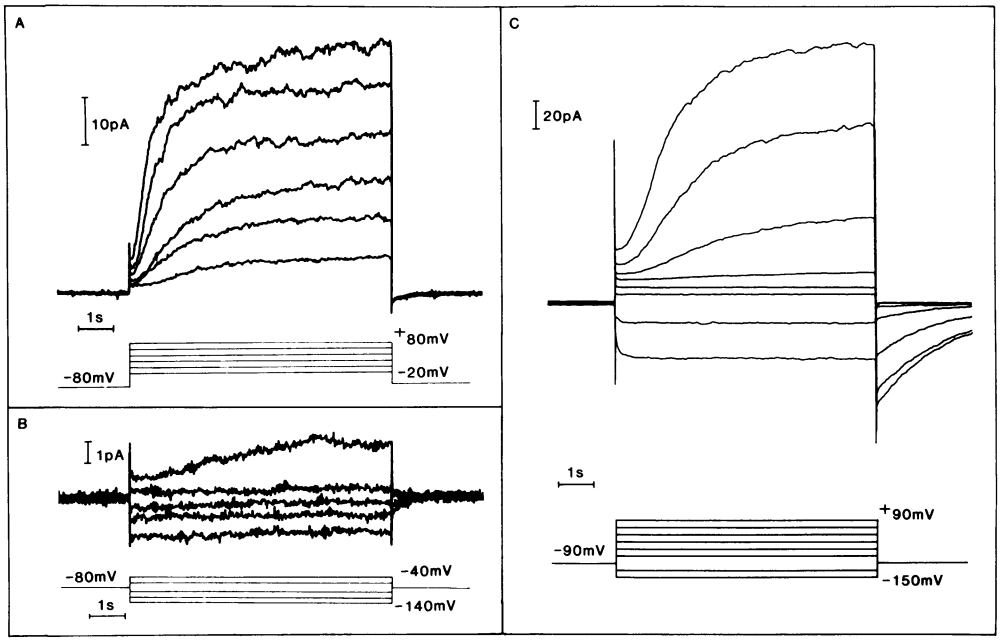

Figure 1. Voltage-dependent whole cell currents recorded from corn protoplasts. (A) Time-independent and time-dependent currents initiated by depolarizing voltage-clamp steps (−20 mV to +80 mV at 20 mV intervals) from a holding potential of −80 mV. Each current recording (upper trace) corresponds to one voltage step (lower trace) with outward currents represented by upward deflections. Extracellular K-Glu was 1 mM. (B) Currents recorded from the same cell (filtered at 1500 Hz, note increase in gain) with voltage steps between −140 mV and −40 mV. The threshold voltage for the time-dependent outward current was between −60 mV and −40 mV. (C) A different cell with 10 mM K-Glu in the extracellular medium. The holding potential was −90 mV, voltage-clamp steps ranged from −150 mV to +90 mV (30 mV intervals). Hyperpolarizing steps elicited a second time-dependent current, which was inwardly directed, in addition to the time-independent current and time-dependent outward current.

The general current-voltage characteristics of corn protoplasts with 1 mM and 10 mM K-Glu are shown in Figure 2. The *I-V* relationships are of three types: (a) instantaneous *I-V* relationships (I_i-V) express the initial, time-independent current observed at the beginning of the voltage step, (b) steady state *I-V* relationships (I_{ss}-V) express the current measured at the end of the voltage step as a function of the step potential, and (c) time-dependent *I-V* relationships (I_t-V), the I_i-V curve subtracted from the I_{ss}-V curve, which reflect the activation of time-dependent currents during the voltage step.

In Figure 2, A and B, it can be seen that the I_i-V was largely linear, although in the higher K^+ medium there was a slight increase in slope at the most positive and negative potentials. The reversal potential of this current (which was also the membrane potential of the protoplast) appeared to be quite potassium-selective, shifting in this case from −120 mV in a cell in 1 mM K-Glu, to −40 mV in a cell in 10 mM K-Glu. The average reversal potential, recorded after the presumed equilibration of the pipette contents with the cytoplasm, was consistently more negative for cells bathed in 1 mM as opposed to 10 mM K-Glu (Table I). In addition, the instantaneous conductance of the protoplasts increased in response to the increase in external potassium (Table I). The conductance was determined by normalizing the slope of the I_i-V relationship to the surface area of the cell as calculated from measurements of the cell diameter. Differences in conductance within treatments were also reflected in the membrane potential where, in general, a lower conductance correlated with a more negative potential.

In both potassium concentrations there was a distinct increase in the slope of the I_{ss}-V and I_t-V relationships at potentials more positive than −60 mV (Fig. 2, A and B). This time-dependent conductance change corresponded to the outward currents of Figure 1. Cells tested in 10 mM K-Glu exhibited a second region below −100 mV, in which the membrane conductance increased during the voltage step, which corresponded to the rapid inward currents of Figure 1C. The I_t-V relationship in this region (Fig. 2B) shows a marked inward rectification. In a few cases in which it was possible to record at more negative potentials than in Figure 2, an inward current was activated even in 1 mM K-Glu. Hence the activation potential of this current appears to be shifted by an increase in external potassium. These features are consistent with the properties of the inward rectifier current observed in other cell types (14).

Sensitivity to Inhibitors

TEA^+, a cation commonly employed to block potassium channels, inhibited the time-independent and the two time-dependent currents which were observed in corn protoplasts. Outward currents activated by membrane depolarization were reversibly blocked by the presence of 9 mM external TEA-Gluc (Fig. 3). With the removal of TEA^+ 90% of the original current could be recovered as illustrated by the I_t-V relationships in Fig. 4. As well, the addition of TEA-Gluc caused a decrease in the instantaneous conductance and a concomitant hyperpolarization of the membrane potential to −115 mV

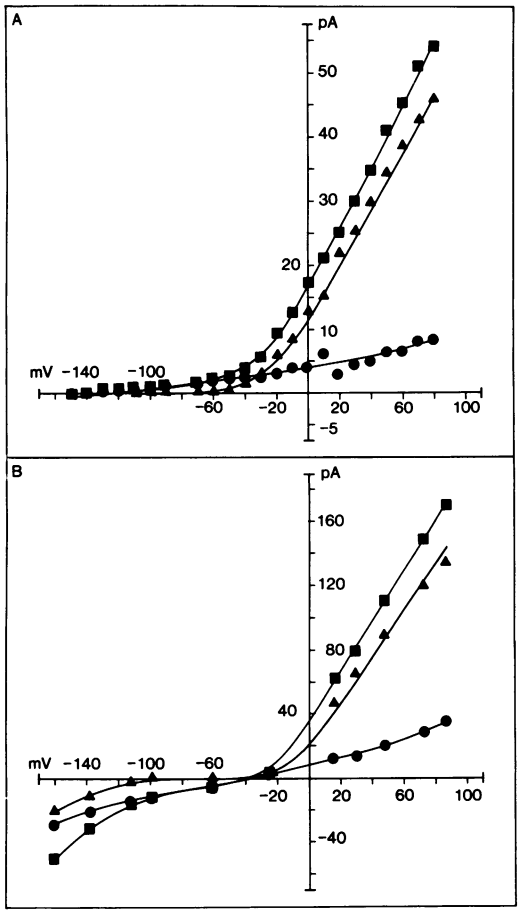

Figure 2. Current-voltage relationships for two different cells in (A) 1 mM and (B) 10 mM K-Glu. Cell A, surface area = 4.6×10^{-9} m^2; cell B, surface area = 5.3×10^{-9} m^2 (note difference in scale). (●) Instantaneous I-V curve (I_i-V); (■) steady state I-V curve (I_{ss}-V); and (▲) time-dependent I-V curve (I_t-V)

from −75 mV (data not shown). The inward currents observed with membrane hyperpolarization were also partially blocked by TEA^+ (data not shown).

We were also interested in assessing whether the time-dependent outward and inward currents were caused by changes in plasma membrane ATPase activity. To address this question currents were monitored in the presence of sodium-orthovanadate. When 1 mM vanadate was added to the extracellular medium no inhibition of these currents was observed. As well, the inclusion of 0.1 mM vanadate in the pipette filling solution did not prevent their measurement (data not shown). Our observations indicated that under these experimental conditions the two voltage- and time-dependent currents were not significantly affected by changes in activity of the proton pump.

Characteristics of the Delayed Outward Current

Ion Selectivity

A 'fully activated' I-V relationship was determined from a two-step protocol (Fig. 5). In this experiment the voltage was first stepped from the holding potential of −90 mV to a positive potential (*e.g.* +64 mV) to fully activate all the channels. The potential was then stepped to a second level (*e.g.* +10 mV) and the current was measured immediately. This value represents the current that flows through the channels that were open at +64 mV *and* the current which is contributed by the time-independent membrane conductance (see I_i-V, above). After re-establishing the holding potential of −90 mV, the membrane potential is stepped directly to the potential of the second step, +10 mV, to ascertain the magnitude of the time-independent current. This time-independent current is subtracted from the initial measurement to obtain the current due only to the channels opened during the first step. We plotted this current as a function of the voltage of the second step to obtain the fully activated I-V relationships shown in Figure 6. These experiments allowed us to measure the conductance and reversal potential of the time-dependent outward current.

Table I. *Average Values of the Conductance and Reversal Potential for the Time-Independent Current and the Delayed-Rectifier Current*[a]

The instantaneous membrane conductance was determined from the slope of the I_i-V relationship (Fig. 2) and the corresponding membrane potential was obtained when the current was clamped at zero. The delayed-rectifier conductance was determined by dividing the time-dependent current, observed when the membrane voltage was clamped at +50 mV, by the driving force, taken as the membrane potential minus the reversal potential of the current. The delayed-rectifier reversal potential was assessed from a "fully activated" I-V relationship (Fig. 5, see text).

K-Glu	Instantaneous Conductance	Membrane Potential	Delayed-Rectifier Conductance	Delayed-Rectifier Reversal Potential
mM	*S/m² × 10²*	*mV*	*S/m² × 10²*	*mV*
1	1.90 ± 1.3 [7]	−96 ± 34 [7]	4.48 ± 2.1 [7]	−70 ± 8.3 [12]
10	3.87 ± 1.8 [7]	−64 ± 23 [7]	11.14 ± 7.5 [7]	−40 ± 8.0 [8]

[a] Values represent the average and standard deviation with the number of cells indicated in brackets. Measurements at different K^+ concentrations were made on different cells.

The fully activated I-V relationship was nearly linear with a slight increase in slope at more depolarized membrane voltages (Fig. 6). This suggested that the channels have a slightly larger conductance at more positive potentials. Figure 6 illustrates that a 100-fold K^+ gradient, with $[K^+]_i > [K^+]_o$, produced a reversal potential of approximately −75 mV. The equilibrium potentials for the other ions, Cl^-, Mg^{2+}, H^+, glutamate$^-$, and Ca^{2+} were approximately −10, 0, +58, +116, and > +150 mV, respectively. Ions included in the buffers, Mes^-, $Tris^+$, BTP^+, and $Hepes^-$, had equilibrium potentials < −116 or > +116. When the chamber was perfused with medium containing 10 mM K-Glu, there was a corresponding shift in the reversal potential to a more positive value. The change in voltage was approximately 35 mV/decade change in the K^+ gradient. In addition, in the cell used for Figure 6, the conductance for the delayed outward current was approximately four fold greater in 10 mM external K^+ than in 1 mM K^+. The average conductance of the delayed rectifier-like current with 10 mM K^+ was 2.5-fold greater than at 1 mM K^+ (measured in different cells, Table I). The average reversal potential was 30 mV more negative in protoplasts which had a 100-fold K^+ gradient as compared to those with a 10-fold

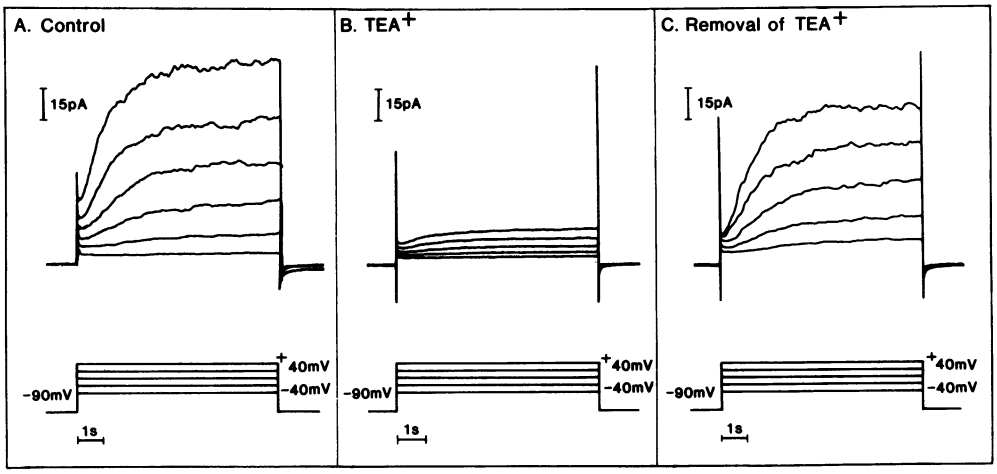

Figure 3. Inhibition of the time-dependent and time-independent outward currents by TEA^+. External K^+ = 1 mM. (A) Control currents obtained by single, voltage-clamp steps from the holding potential of −90 mV, step potentials between −40 mV and +40 mV at 20 mV intervals. Lowest current trace is for step to −60 mV. (B) Currents reduced by the addition of TEA-Gluc, final concentration was 9 mM. (C) Currents recorded after TEA^+ was perfused from the chamber.

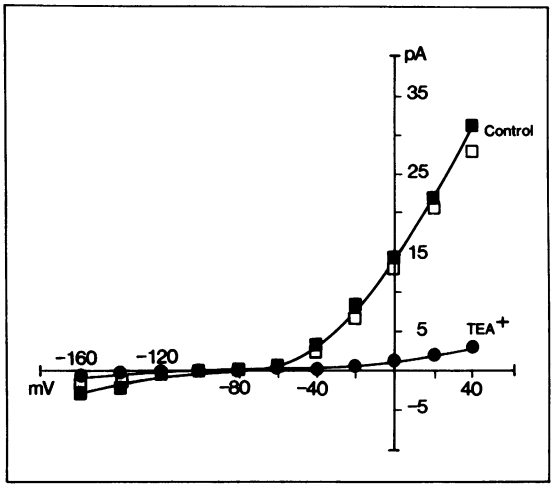

Figure 4. Time-dependent I-V curves (I_t-V), illustrated that 90% of the outward rectifying current was inhibited by TEA^+. (■) control; (●) with TEA-gluconate; (□) current after the removal of TEA^+.

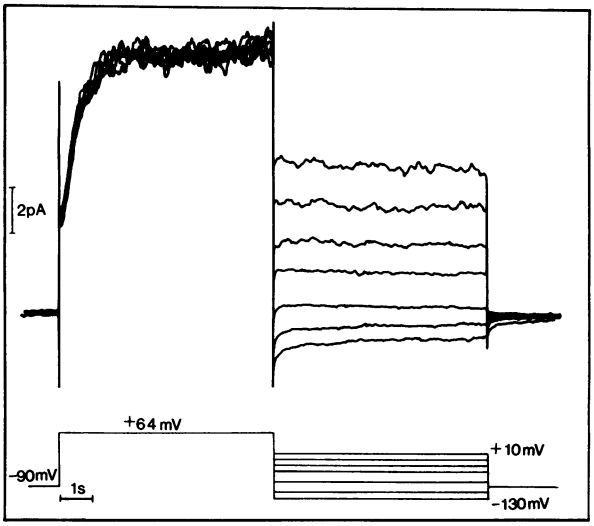

Figure 5. Reversal potential of the time-dependent outward current, determined from a two-step experiment, was approximately −80 mV with an extracellular K^+ concentration of 1 mM (intracellular K^+ = 100 mM). The membrane voltage was stepped to +64 mV, from a holding potential of −90 mV, to activate the outward current. Next, the voltage was stepped to a second potential, here steps to +10 mV, −10 mV, −30 mV, −50 mV, −80 mV, −110 mV, and −130 mV are shown, and the current was measured at the beginning of the second step.

K^+ gradient (Table I). Hence one of the major ions contributing to this current was potassium.

Preliminary tests were done to assess whether protons also contributed to this current. To address this question we elevated the external pH so that E_{H^+} (the proton equilibrium potential) was equal to E_{K^+} (the K^+ equilibrium potential, *i.e.* $E_{H^+} = -58$ mV and $E_{K^+} = -58$ mV). If proton fluxes comprised part of this current the change in the proton gradient should cause a corresponding shift in the reversal potential from −40 mV to −58 mV. We found that alterations of the H^+ gradient had no effect on the reversal potential (Fig. 7). Thus it is unlikely that H^+ fluxes contribute significantly to these currents.

Voltage Activation Range

The conductance change underlying this time-dependent K^+ current expressed as a function of membrane potential is depicted in Figure 8. These values were obtained by dividing the current by its driving force, which is defined as the membrane potential minus the reversal potential of the current. The current was activated by membrane potentials between −60 mV and +50 mV. The activation curve is well fit by the Boltzmann distribution (14), Equation 1, with V' = −1 mV and k = 25 (Fig. 8, solid line).

$$G = \frac{G_{max}}{1 + exp\dfrac{(V' - V_m)}{k}} \quad (1)$$

G in Equation 1 is the conductance change, V_m is the clamped membrane potential, V' corresponds to the potential giving half maximal activation and k is defined as KT/ze, where K is the Boltzmann constant, T is the absolute temperature, e is the electronic charge and z is the valency of the gating charge. The valency of the gating charge for this current was found to be 1.0.

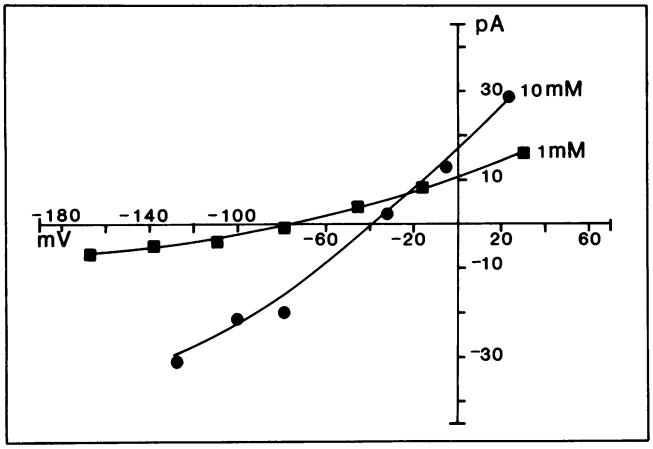

Figure 6. Reversal potential of the time-dependent outward current was influenced by the K^+ gradient. I-V relationship obtained from a two-step procedure similar to that shown in Figure 5. One cell, first bathed in 1 mM K-Glu (■), with a reversal potential of −75 mV (100/1 K^+ gradient), and then incubated in 10 mM K-Glu (●), with a reversal potential of −40 mV (10/1 K^+ gradient).

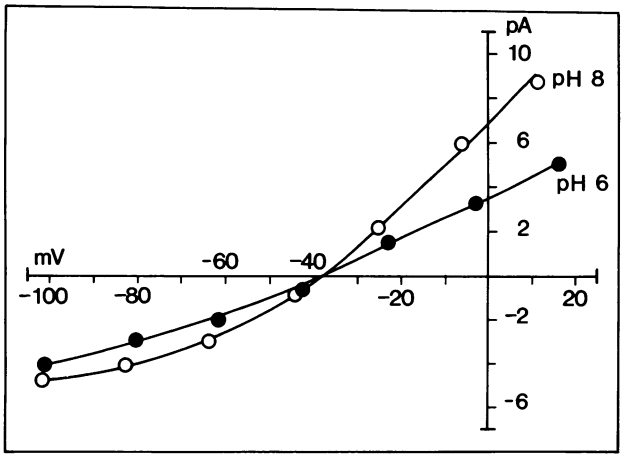

Figure 7. External pH does not affect the reversal potential of the outward current. I-V relationships obtained as in Figure 5 with the extracellular pH = 6.0 (●) or pH = 8.0 (○). The external and internal K^+ concentrations were 10 mM and 100 mM, respectively.

Kinetics of Activation and Inactivation

The time course of current development during depolarizing steps was markedly sigmoidal (Fig. 1). This implies that activation is dependent upon either a series of ordered steps that lead to channel opening or positive cooperativity between channels or their subunits (14). The rate of current development was strongly voltage-dependent. As seen in Figure 9B, the half activation time was inversely related to the membrane potential and ranged from 3.3 s to 1.0 s at potentials of −40 mV and +80 mV, respectively.

We also determined the rate at which currents decayed when the membrane potential was returned to the holding level. To investigate this we utilized a pair of voltage steps that were separated by varying lengths of time (Fig. 10). When the interval between pulses was too short to allow any of the channels to close, the current observed at the beginning of the second pulse was equal to that seen at the end of the first step. However, as the length between steps was increased, allowing some channels to close, the current monitored at the beginning of the second step was reduced. As seen in the inset, the decay of the current followed a simple exponential time course. The rate of this decay was markedly voltage-dependent as illustrated by the plot of half time for closing, *versus* membrane voltage (Fig. 9A). The more negative closing potentials caused a more rapid loss of current with values extending from 5.7 s at −60 mV, (note that this is the threshold voltage for activation), to 0.7 s at −140 mV. Pulses to more negative potentials frequently resulted in the breakdown of the cellular membrane thus preventing an accurate determination of closing rates beyond this point.

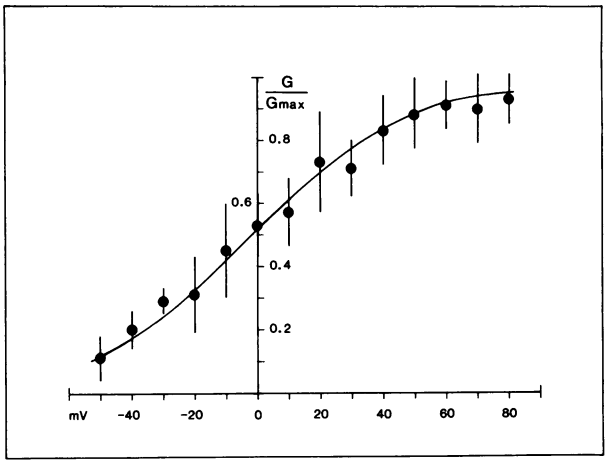

Figure 8. Conductance change (G) underlying the time-dependent outward current expressed as a function of the step potential. Points represent the average from five cells, in 1 mM external K^+, with the vertical lines denoting the standard deviation. Solid line indicates the Boltzmann distribution (Equation 1) which fits these data (see text).

DISCUSSION

Characteristics of the Outward K^+ Current

The outward rectifying current, which was the main focus of this study, had properties which reflect the activity of a voltage-gated K^+ channel. Its characteristics are similar to those which denote a class of K^+ channels termed delayed rectifiers (14). These channels are found in many types of animal cells and have also been described in specialized plant cells, such as guard cells in *Vicia faba* (24, 26) and cells derived from the pulvini of *Samanea* (20). As well, voltage-gated K^+ currents have been identified in some algal species (9, 28) and in suspension cultures of *Asclepias tuberosa* (25), although the activation range for these currents is not identical to the threshold voltage observed in corn. The features which distinguish these currents are the activation by membrane depolarization, the marked sigmoidal time course of current development and the dependence of the current's reversal potential on the K^+ gradient.

It is apparent from measurements of the reversal potential that potassium ions are the primary ionic species composing the outward current. It is also evident, however, that these channels are not ideally selective for K^+ but have some low

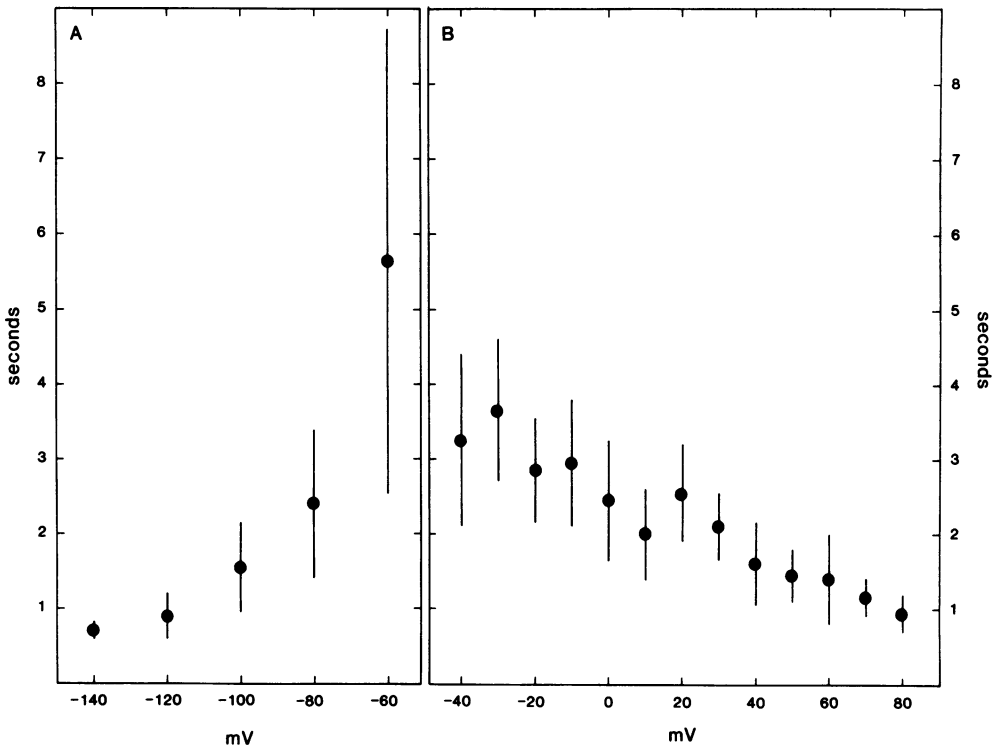

Figure 9. Rate at which the time-dependent outward current inactivates (A) and activates (B). External K^+ = 1 mM. Points represent the sample average and the vertical lines define the standard deviation. (A) The half time of inactivation decreased as the membrane potential became more negative, average from six cells. (B) The half activation time was inversely related to the step potential, average from five cells.

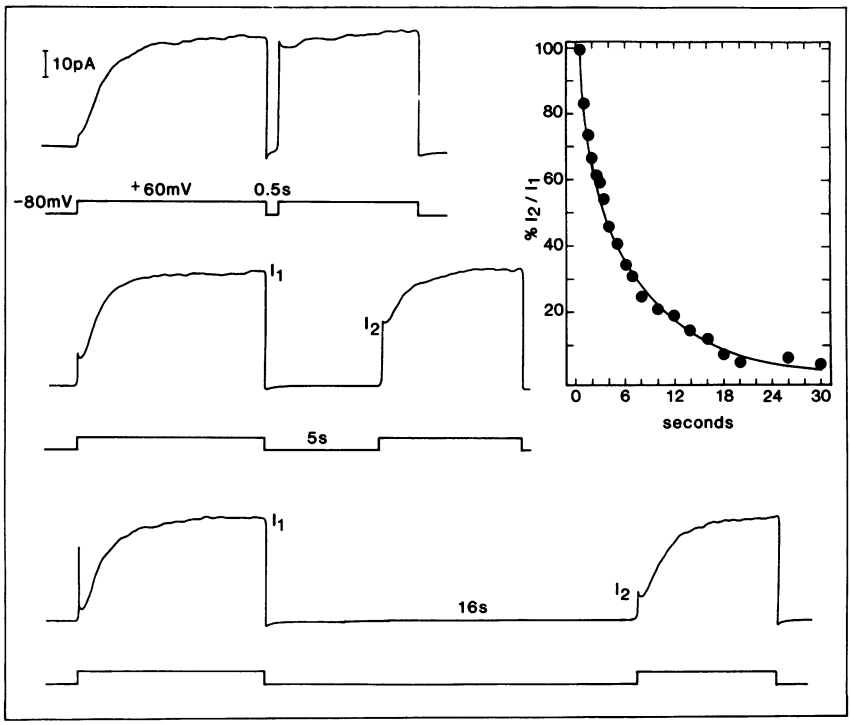

Figure 10. Time-dependent outward current decayed in a simple exponential fashion when the membrane voltage was returned to the holding potential (inset). Each point was obtained from a pair of voltage steps that were separated by varying lengths of time. In this experiment the holding potential was −80 mV and the potential of the voltage step was +60 mV. Representative current traces are shown from pulses separated by 0.5, 5, and 16 s. I_1 = the magnitude of the original current, I_2 = the current which has not decayed during the time between the voltage steps.

permeability to other, as yet undetermined, ions. Of particular interest is the observation that protons do not contribute to these currents (Fig. 7). This suggests that channel activation does not, in itself, dissipate the pH gradient and thus would not hinder electroneutral cotransport events at the plasma membrane.

The ability of TEA^+ to inhibit the outward rectifying current (Fig. 4) lends further support to the concept that this current is effected by the activation of K^+ channels. In plant systems TEA^+ has been shown to inhibit K^+ currents in cells of *A. tuberosa* (25) and *Hydrodictyon africanum* (9). In addition TEA^+ has been utilized to block K^+-dependent ion fluxes in intact cells (3, 18). However, TEA^+ is an inhibitor which affects a variety of K^+ channels. Thus additional pharmaco-

logical studies must be done to help differentiate this channel from other types of K^+ channels that may be present in the plasma membrane.

There are several additional properties of the outward K^+ current which should be noted. It was seen in Figure 6 that the conductance of the *I-V* curve, obtained when presumably all of the channels had been opened, was approximately four fold greater in 10 mM external K^+ than in 1 mM K^+. A similar effect of external K^+ on the K^+ conductance was seen in *Chara* (3) during the transition to the K^+-state described in the Introduction. Since the internal K^+ remains constant, one explanation for these observations is that the increase in current reflects an increase in the number of open channels. If so, the effect of K^+ on channel opening seems quite independent of the effect of the membrane potential on channel opening. Alternatively, if the number of open channels remains constant, external K^+ must in some manner enhance channel conductance. Single channel recording is required to differentiate between these two possibilities.

A plot of time-dependent conductance versus potential (Fig. 8) showed the potential for half-maximal channel opening to be −1 mV. The voltage sensitivity of the conductance is low compared with that for the delayed rectifier channels of nerve axons, and corresponds to an equivalent gating charge of 1.0, *versus* 4.5 for the delayed rectifier of the squid axon (14). The half-times for channel opening and closing (around one second or more, Fig. 9) are also very slow compared with nerve axons. In other plants half activation times in the range of 100 to 400 ms have been recorded (24–26).

A question remains concerning the variation in delayed rectifier conductance among the cells tested (Table I). This may be related to the variable state of differentiation of these cultured cells.

Characteristics of the Inward Current

This current has not been characterized in any detail in this study, but it is interesting to note that our observations, as far as they go, are consistent with the properties of inward rectifier K^+ channels (14). Thus the inward current is activated on hyperpolarization, and is partially inhibited by TEA^+, while the activation potential is strongly dependent on K^+ concentration. Similar channels have been described in *Nitella* (28) and in guard cells of *Vicia faba* (26).

The Time-Independent (Instantaneous) Current

The instantaneous *I-V* curve, unlike the steady state or time-dependent *I-V* curves, was fairly linear over a wide range of potentials (Fig. 2). Thus the instantaneous conductance is largely insensitive to potential. The conductance of individual cells, observed prior to eliciting the voltage- and time-dependent currents, but after the presumed equilibration of cytoplasmic ions with the micropipette contents, showed considerable variation from cell to cell. Nevertheless, the magnitude of I_i, as well as its reversal potential, was sensitive to the external K^+ concentration (Table I, Fig. 2) and to TEA^+ inhibition. This suggests that K^+ channels make a major contribution to the conductance, and argues against nonspecific leaks resulting from protoplast formation or poor sealing at the membrane pipette junction.

Sensitivity to K^+ and TEA^+ raises the question whether the instantaneous current is mediated by the same types of channels as the two time-dependent currents. A degree of rectification of I_i-V (Fig. 2B) toward the negative end of the voltage spectrum suggests a contribution of the 'inward rectifier' channels to the instantaneous current in 10 mM external K^+. This is unlikely at 1 mM K^+ since the activation range of the inward current is shifted outside the normal range of measurements and far from the holding potential of −80 or −90 mV. The holding potential is also quite far from the range of potentials at which the delayed-rectifier-like current is activated. The Boltzmann curve (Fig. 8) would predict a finite number of open channels at the holding potential, but their conductance would be only a small percent of G_{max}. In fact, the instantaneous conductance was on average 40% of the G_{max} for the delayed rectifier, and in the most extreme case was equal to G_{max}. The time-independent conductance and the delayed rectifier conductance both varied from cell to cell by as much as five to 10-fold, yet they showed no correlation. It is concluded that the instantaneous current is probably mediated by a class of K^+ channels different from those carrying the time-dependent currents.

The values for membrane conductance reported here (around 0.04 S/m^2 in 10 mM K^+) are comparable with the value of 0.14 S/m^2 reported by Schroeder *et al.* (26) for protoplasts of guard cells. The results of these patch-clamp studies are however two orders of magnitude lower than conductance measurements made by traditional methods in higher plant cells (8). This may be partly due to the effect of plasmodesmata in the intact tissue or to better sealing of the patch-clamp micropipette. Conductance values in the Characeae vary widely but the specific conductance of *Nitella* can approach the low values recorded here (29). Moreover, the measurements by Schroeder *et al.* (26) suggested that the K^+ fluxes which are known to accompany stomatal movements, could be accounted for by the conductance of the K^+ channels found in guard cell plasma membrane. Although it remains an open question whether the overall conductance values of protoplasts are representative of intact cells, this does not invalidate the use of patch-clamp data to characterize the various classes of ion channels which may contribute to the normal cell conductance.

Physiological Roles of the K^+ Currents

We speculate that the main function of the delayed rectifier current is to restore the membrane potential after a depolarizing event, as suggested in the Introduction. In contrast the time-dependent inward current would mediate the uptake of K^+ under appropriate conditions (*i.e.* membrane hyperpolarization and high extracellular K^+). The characteristic shift in activation range, with increasing levels of extracellular K^+, lead us to question whether this current may be involved in the low affinity, mechanism II, K^+ uptake noted in tissues exposed to high salts (18).

The role of the time-independent current is more difficult to infer, but it seems that this K^+ current may take part in regulating the membrane potential under 'resting' conditions.

We observed that the membrane potential of the corn protoplasts, like the conductance, varied considerably between cells. The range of variation, approximately −60 to −130 mV at 1 mM K^+, and −40 to −90 mV at 10 mM K^+, may be limited in each case by the threshold of activation of the delayed rectifier and inward rectifier channels. Within this range there is a correlation between the membrane potential and I_i, such that the smallest conductances give the most negative potentials. Furthermore, treatment with TEA^+, which reduced the conductance, resulted in hyperpolarization of the cell (results not shown). Thus the membrane potential appears to be largely controlled by I_i. The nature of the initial membrane conductance and the cause of its variability are currently under further investigation.

ACKNOWLEDGMENT

The authors would like to thank Dr. Ellis Cooper for his many helpful suggestions on experimental techniques and data interpretation. We also thank Dr. Stephen K Randall, Vahé Sarafian and Heike Pöpperl for critically reading the manuscript.

LITERATURE CITED

1. **Bates GW, Goldsmith MHM, Goldsmith TH** (1982) Separation of tonoplast and plasma membrane potential and resistance in cells of oat coleoptiles. J Membr Biol **66:** 15–23
2. **Beilby MJ** (1985) Potassium channels at *Chara* plasmalemma. J Exp Bot **36:** 228–239
3. **Beilby MJ** (1986) Factors controlling the K^+ conductance in *Chara.* J Membr Biol **93:** 187–193
4. **Bisson MA** (1984) Calcium effects on electrogenic pump and passive permeability of the plasma membrane of *Chara corallina.* J Membr Biol **81:** 59–67
5. **Cheeseman JM, Hanson JB** (1979) Energy-linked potassium influx as related to cell potential in corn roots. Plant Physiol **64:** 842–845
6. **Coleman HA** (1986) Chloride currents in *Chara*-A patch-clamp study. J Membr Biol **93:** 55–61
7. **Epstein E, Rains DW, Elzam OE** (1963) Resolution of dual mechanisms of potassium absorption by barley roots. Proc Natl Acad Sci USA **49:** 684–692
8. **Etherton B, Keifer DW, Spanswick RM** (1977) Comparison of three methods for measuring electrical resistances of plant cell membranes. Plant Physiol **60:** 684–688
9. **Findlay GP, Coleman HA** (1983) Potassium channels in the membrane of *Hydrodictyon africanum.* J Membr Biol **75:** 241–251
10. **Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ** (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch **391:** 85–100
11. **Hepler PK, Wayne RO** (1985) Calcium and plant development. Annu Rev Plant Physiol **36:** 397–439
12. **Higinbotham N, Etherton B, Foster RJ** (1964) Effect of external K, NH_4, Na, Ca, Mg and H ions on the cell transmembrane electropotential of *Avena* coleoptile. Plant Physiol **39:** 196–203
13. **Higinbotham N, Hope AB, Findlay GP** (1964) Electrical resistance of cell membranes of *Avena* coleoptile. Science **143:** 1448–1449
14. **Hille B** (1984) Ionic Channels of Excitable Membranes. Sinauer, Sunderland, MA
15. **Hope AB, Walker NA** (1975) The Physiology of Giant Algal Cells. Cambridge University Press, Aberdeen
16. **Keifer DW, Lucas WJ** (1982) Potassium channels in *Chara corallina.* Control and interaction with the electrogenic H^+ pump. Plant Physiol **69:** 781–788
17. **Kinraide TB, Newman IA, Etherton B** (1984) A quantitative simulation model for H^+-amino acid cotransport to interpret the effects of amino acids on membrane potential and extracellular pH. Plant Physiol **76:** 806–813
18. **Kochian LV, Xin-Zhi J, Lucas WJ** (1985) Potassium transport in corn roots. IV. Characterization of the linear component. Plant Physiol **79:** 771–776
19. **Minorsky PV** (1985) An heuristic hypothesis of chilling injury in plants: a role for calcium as the primary physiological transducer of injury. Plant Cell Environ **8:** 75–94
20. **Moran N, Iwasa K, Ehrenstein G, Mischke C, Bare C, Satter R** (1987) Effects of external K^+ on K^+ channels in *Samanea* protoplasts (abstract 678). Plant Physiol **83:** S-112
21. **Ohkawa T, Tsutsui I, Kishimoto U** (1986) K^+ channel in the *Chara* plasmalemma: estimations of K^+ channel density and single K^+ channel conductance. Plant Cell Physiol **27:** 1429–1438
22. **Okazaki Y, Tazawa M** (1986) Involvement of calcium ion in turgor regulation upon hypotonic treatment in *Lamprothamnium succinctum.* Plant Cell Environ **9:** 185–190
23. **Reiss HD, Herth W** (1985) Nifedipine-sensitive calcium channels are involved in polar growth of lily pollen tubes. J Cell Sci **76:** 247–254
24. **Schauf CL, Wilson KJ** (1987) Effects of abscisic acid on K^+ channels in *Vicia faba* guard cell protoplasts. Biochem Biophys Res Commun **145:** 284–290
25. **Schauf CL, Wilson KJ** (1987) Properties of single K^+ and Cl^- channels in *Asclepias tuberosa* protoplasts. Plant Physiol **85:** 413–418
26. **Schroeder JI, Raschke K, Neher E** (1987) Voltage dependence of K^+ channels in guard-cell protoplasts. Proc Natl Acad Sci USA **84:** 4108–4112
27. **Shiina T, Tazawa M** (1987) Demonstration and characterization of Ca^{2+} channel in tonoplast-free cells of *Nitellopsis obtusa.* J Membr Biol **96:** 263–276
28. **Sokolik AI, Yurin VM** (1986) Potassium channels in plasmalemma of *Nitella* cells at rest. J Membr Biol **89:** 9–22
29. **Spanswick RM** (1970) The effects of bicarbonate ions and external pH on the membrane potential and resistance of *Nitella translucens.* J Membr Biol **2:** 59–70