Plant Physiol. (1993) 101: 81-88

Whole-Cell K^+ Currents across the Plasma Membrane of Tobacco Protoplasts from Cell-Suspension Cultures[1]

Bert Van Duijn*, Dirk L. Ypey, and Kees R. Libbenga

Institute of Molecular Plant Biology, Department of Botany, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands (B.V.D., K.R.L.); and Department of Physiology, Leiden University, P.O. Box 9604, 2300 RC Leiden, The Netherlands (B.V.D., D.L.Y.)

The whole-cell configuration of the patch clamp technique was used to study both outward and inward ion currents across the plasma membrane of tobacco (*Nicotiana tabacum*) protoplasts from cell-suspension cultures. The ion currents across the plasma membrane were analyzed by the application of stepwise potential changes from a holding potential or voltage ramps. In all protoplasts, a voltage- and time-dependent outward rectifying current was present. The conductance increased upon depolarization of the membrane potential (to >0 mV) with a sigmoidal time course. The reversal potential of the outward current shifted in the direction of the K^+ equilibrium potential upon changing the external K^+ concentration. The outward current did not show inactivation. In addition to the outward rectifying current, in about 30% of the protoplasts, a time- and voltage-dependent inward rectifying current was present as well. The inward rectifying current activated upon hyperpolarization of the membrane potential (<−100 mV) with an exponential time course. The reversal potential of the inward conductance under different ionic conditions was close to the K^+ equilibrium potential.

The patch-clamp technique (Hamill et al., 1981) has been shown to be a technique of major importance for plant physiological and biochemical research. Measurements of single and whole-cell ion channel activities provide the possibility to directly measure the existence and regulation of ion channels and their role in different cell physiological processes and responses. In guard cells and pulvinar cells, patch-clamp experiments provided a large contribution toward the understanding of the regulation of the function of these cells (e.g. Schroeder et al., 1987; Moran et al., 1988; Schroeder, 1988; Blatt, 1991; Marten et al., 1991; Schroeder and Fang, 1991). Besides an important role in osmo- and turgor regulation and selective uptake of ions in root cells, ion fluxes are likely involved in many other processes, such as hormone signal transduction. Both outward (Iijima and Hagiwara, 1987; Moran et al., 1988; Schroeder, 1988; Ketchum et al., 1989; Schroeder, 1989; Fairley et al., 1991; Fairley-Grenot and Assmann, 1992) and inward (Schroeder et al., 1987; Bush et al., 1988; Schroeder and Hagiwara, 1989; Colombo and Cerana, 1991; Fairley-Grenot and Assmann, 1991; Schroeder and Fang, 1991; Blatt, 1992; Fairley-Grenot and Assmann, 1992; Kourie and Goldsmith, 1992) rectifying voltage-dependent K^+ channels can be found in different plant cells. Analysis of the outward currents shows both similarities and differences among the conductances underlying these currents in different species and tissues. In guard cells and pulvinar cells, the outward rectifying K^+conductance functions as a K^+ release channel during cell shrinking (Schroeder et al., 1987; Moran et al., 1988; Blatt, 1991). The inward rectifying conductance provides a mechanism for low-affinity K^+ uptake, e.g. in guard cells during stomatal opening (Schroeder et al., 1987; Blatt, 1991; Schroeder and Fang, 1991).

Here we report on the presence of both outward- and inward-directed K^+ currents across the plasma membrane of tobacco (*Nicotiana tabacum*) protoplasts from tobacco cell-suspension cultures. The currents through these channels were measured in the whole-cell configuration of the patch-clamp technique. Properties of the outward rectifying K^+ current and the inward rectifying K^+ current from tobacco protoplasts are compared with the properties of similar currents in other plant cells. We used protoplasts from tobacco because this plant is among the most widely used in molecular biological and biochemical studies. The use of the tobacco protoplasts in patch-clamp studies provides the possibility to investigate ion channel function and regulation in combination with molecular biological approaches. Some preliminary results of this study have been published in a conference proceeding (Van Duijn et al., 1992).

MATERIALS AND METHODS

Cell Cultures and Protoplast Isolation

Tobacco suspension cells (*Nicotiana tabacum* L., cv Bright Yellow) were grown in Linsmaier and Skoog medium (Lins-

[1] This work was partly supported by European Economic Community Bridge contract BIOT CT90-0158-C. B.V.D. was financially supported by the Dutch Organization for Pure Research through Dutch Society for Biological Research/Biophysical Society thema I (Intracellular conduction of signals) project 811-416-112.

* Corresponding author; Department of Physiology, Leiden University, P.O. Box 9604, 2300 RC Leiden, The Netherlands; fax 31-71-276782.

Abbreviations: 4-AP, 4-amino-pyridine; E_{Cl}, equilibrium potential for chloride; E_H, holding potential; E_K, equilibrium potential for potassium; E_m, membrane potential; E_{Na}, equilibrium potential for sodium; E_{rev}, reversal potential; ECS, extracellular solution; ICS, intracellular solution; K-ECS, high potassium extracellular solution; G_K, chord conductance; $G_{K,max}$, maximal chord conductance; *I-V* relationship, current-voltage relationship; nS, nanosiemens; TEA, tetraethylammonium; V_m, applied membrane potential.

maier and Skoog, 1965) supplemented with aneurine-HCl (0.6 $mg \cdot L^{-1}$) and 180 $mg \cdot L^{-1}$ KH_2PO_4 in the presence of 8.8 $\times 10^{-7}$ M 2,4-D at 25°C in the dark according to An (1985). Cells in the stationary phase, i.e. 7 d after transfer to fresh medium, were used to prepare protoplasts. Protoplasts were prepared in analogy with a method described for the preparation of parsley protoplasts (Dangle et al., 1987), because good giga-seal formation could be obtained on protoplasts prepared with this method (in contrast to tobacco suspension protoplasts obtained with other methods). Cells were washed three times by centrifugation (about 200*g*) with a 0.24 M $CaCl_2$ solution. Subsequently, cells (about 10 g fresh weight) were incubated for 10 to 12 h at 25°C in the dark in a 2-L Erlenmeyer flask in 150 mL of 0.24 M $CaCl_2$ solution supplemented with 0.05 g of cellulase (Onozaku RS, Yakult Honsha, Japan) and 0.005 g of macerozyme (Macerozyme R-10, Yakult Honsha, Japan). Protoplasts were collected after washing at least three times with the 0.24 M $CaCl_2$ solution by centrifugation (3 min, 100*g*). Just before starting the experiments, 50 μL of cell suspension was added to 2 mL of extracellular solution (see "Solutions") in a Teflon culture dish. The glass coverslip mounted in the Teflon culture dish was carefully cleaned with 96% ethanol before use. Protoplasts were allowed to settle on the glass coverslip for about 15 min. Only protoplasts that were firmly attached to the coverslip were chosen for experiments. Protoplasts used for experiments had an average surface area of $2.9 \times 10^{-9} \pm 1.8 \times 10^{-9}$ m^2 ($n = 30$).

Solutions

The standard ECS was composed of 10 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KOH, and 10 mM Mes (pH 5.5). Addition of cell suspension increased the bath concentration of Ca^{2+} by 5.9 mM and of Cl^- by 11.7 mM. The osmolarity of ECS was adjusted to about 1010 or 570 mOsm with mannitol. Both the high- and the low-osmolarity ECS were used for experiments. No differences in seal formation or in the outward currents and inward currents were observed between experiments using high- and low-osmolarity ECS. In addition, protoplasts in ECS showed normal expression of auxin-inducible genes (K. Boot, personal communication; Van der Zaal et al., 1987). A K-ECS was used that consisted of ECS supplemented with 45 mM KCl (total K^+ concentration 55 mM). The ICS (i.e. pipette fillings) consisted of 100 mM K-gluconate, 2 mM $MgCl_2$, 1.1 mM EGTA, 0.45 mM $CaCl_2$, 4 mM MgATP, 6 mM KOH, and 10 mM Hepes (pH 7.0). The osmolarity of ICS was adjusted to about 708 mOsm with mannitol. The MgATP (Sigma Chemical Co.) was added to the solutions just before starting the experiments. Ion channel blocker-containing solutions were freshly made before use. Quinidine and 4-AP were from Sigma. TEA was from BDH (Poole, UK).

Patch-Clamp Experiments

Whole-cell currents from tobacco protoplasts were recorded at room temperature (about 20°C) with an L/M EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, F.R.G.) using conventional patch-clamp technique (Hamill et al., 1981). Electrodes were pulled from borosilicate glass (GC150TF-15, Clark Electromedical Instruments, Reading, U.K.) and had, after fire polishing, resistances ranging from 5 to 10 MΩ (measured in ECS). The seal-success ratio was about 30% (ranging from 0 to 80% from culture to culture). The seal resistance usually ranged between 2 and 10 GΩ. After formation of a giga seal, the whole-cell configuration was obtained by application of a strong suction pulse to the pipette interior.

Before analog to digital conversion, the measured currents were low-pass filtered at 3 kHz. The generation of sequences of test voltage potentials, data recording, and data storage was controlled by the software package pClamp (version 5.5.1., Axon Instruments, Burlingame, CA). The data were analyzed with the help of the software packages pClamp and FigP (version 6.0, Biosoft, Cambridge, UK).

Values are expressed as means ± SD, with n = number of cells measured, unless stated otherwise. Significance of differences in mean values was tested with Student's t test ($P < 0.05$).

RESULTS

Resting E_m

The resting E_m of the tobacco protoplasts was in many cases measured by switching to the current-clamp mode of the patch-clamp amplifier directly after gaining access to the protoplast interior (whole-cell configuration). This allowed E_m measurement before perfusion of the protoplasts with the patch-pipette content occurred. The mean E_m measured in protoplasts bathed in ECS was −38.7 ± 24.9 mV ($n = 11$). This mean E_m value is in agreement with values found by intracellular microelectrode measurements (Briskin and Leonard, 1979; unpublished observations).

Whole-Cell Currents

After obtaining the whole-cell configuration, the membrane potential was clamped at −50 mV. Unless stated otherwise, the applied voltage-step and ramp protocols were started from this holding potential (E_H). In 36 whole-cells from about 120 experiments, two different responding cell types could be distinguished upon the application of step-wise voltage changes to the pipette interior; 24 cells that showed only outward currents (Fig. 1, A and B) and 12 cells showing both outward and inward currents (Fig. 1, C and D). The ohmic membrane resistance (measured with ECS in the bath and ICS in the pipette) could be measured between −10 and −80 mV. The mean ohmic plasma membrane resistance was 5.2 ± 3.4 GΩ ($n = 27$). All currents displayed and values given are, where appropriate, corrected for the leak conductance.

Identification of Outward Current

Step-wise depolarization of the protoplast membrane from a holding potential of −50 mV activated a time- and voltage-dependent outward current (Fig. 1). The maximal outward current amplitude was very stable in time, and did not show run down as long as the whole-cell configuration could be

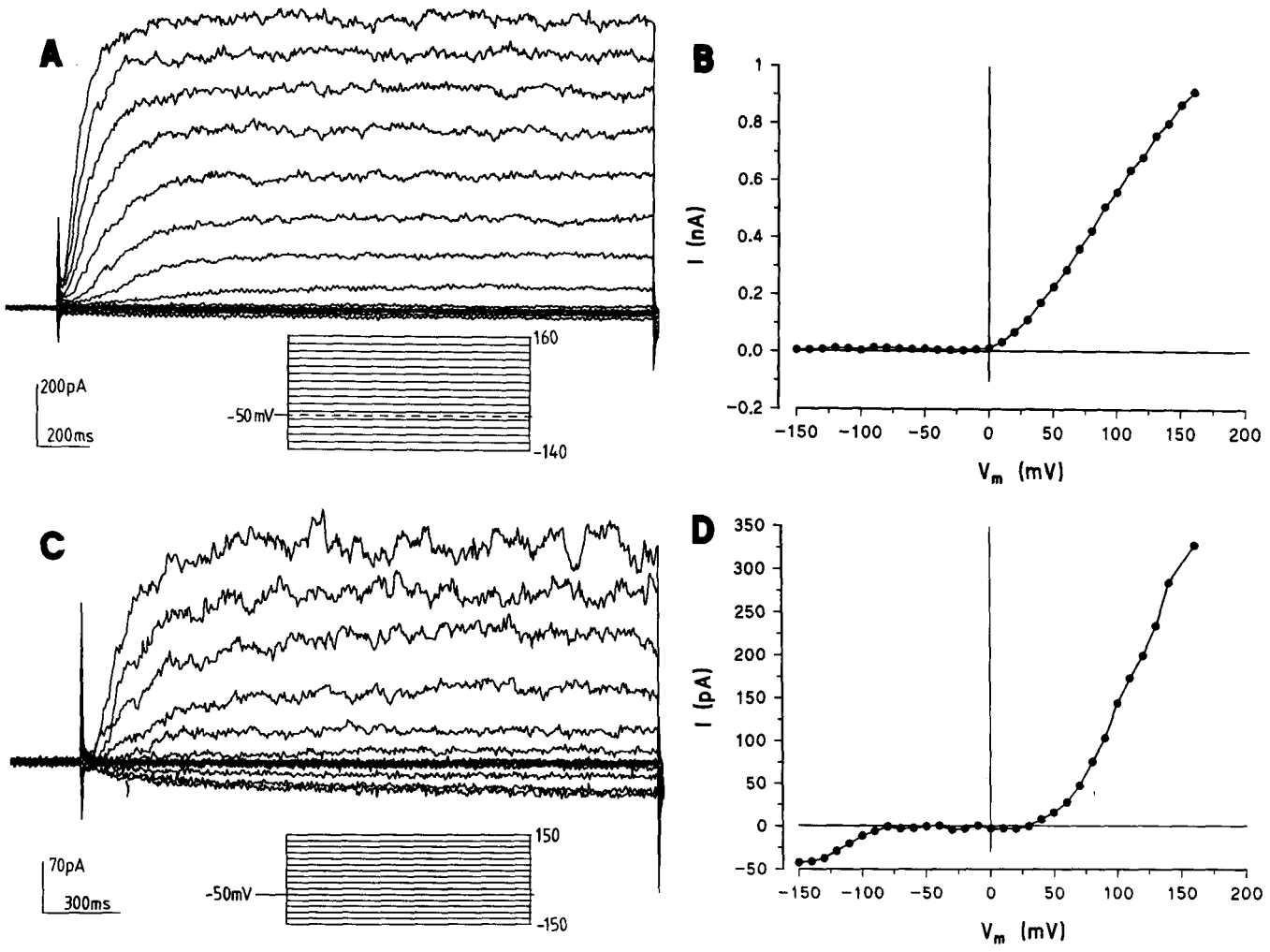

Figure 1. Currents measured in the whole-cell configuration of the patch-clamp technique across the plasma membrane of two types of tobacco protoplasts (A, B and C, D). The bath solution was ECS (i.e. 11 mM K^+) in both cases and the pipette solution was ICS (i.e. 106 mM K^+) in both cases. V_H was −50 mV. A, Plasma membrane current responses upon voltage pulses of 2.3 s ranging from −150 to +160 mV (10-mV steps were made; however, for reasons of clarity, current responses are shown for 20-mV steps) applied to the pipette interior, according to the voltage protocol shown in the inset. The displayed currents are corrected for the leak conductance. B, *I-V* relationship of the currents displayed in A. C, Plasma membrane current responses (corrected for the leak conductance) upon the same voltage protocol as in A. D, *I-V* relationship of the currents displayed in C.

maintained. From the *I-V* relationship (Fig. 1, B and D), it is clear that a depolarizing voltage step evokes a large increase in membrane conductance. A hyperpolarizing voltage step of similar magnitude did not induce this membrane conductance increase (Fig. 1, B and D) (except for large hyperpolarizing steps in cells showing inward current as well) (Fig. 1D). The activation of the outward current followed a sigmoidal time course (Fig. 1, A and C) and the current reached a steady-state level with time. The depolarization-induced outward current did not show time-dependent inactivation (for depolarizing voltage steps lasting up to 20 s), and the current magnitude was independent of the preceding E_H (data not shown).

Application of a negative potential after current-activating depolarizations of increasing duration showed rapidly decaying inward currents of increasing amplitude (Fig. 2). This response is due to deactivation of the depolarization-activated ion channels at hyperpolarized potentials. The increase of the inward current at hyperpolarized potentials with increasing depolarization duration illustrates the time-dependent increase in plasma membrane conductance upon depolarization.

Determination of the major ion passing through the ion channels underlying this depolarization-induced membrane conductance increase was performed by measuring the shift in E_{rev} upon changing the extracellular ionic conditions. E_{rev} was measured in tail-current experiments. In these experiments, activating depolarizing voltage steps to +100 mV were followed by steps to different less positive potentials (Fig. 3A, inset). Stepping back to less positive potentials gives rise

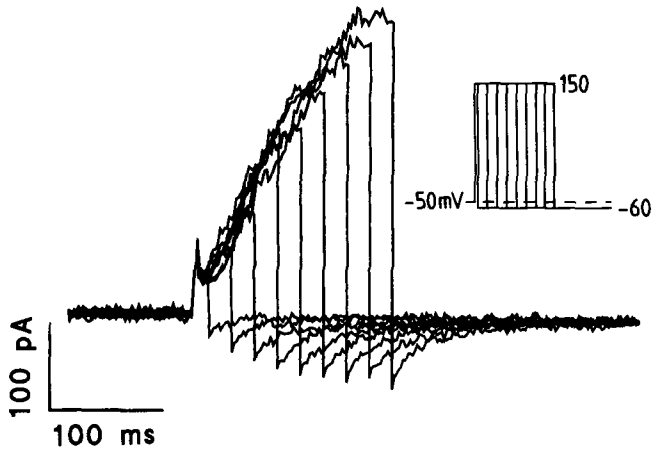

Figure 2. Depolarization-activated currents in the whole-cell configuration. Bath solution is ECS (11 mM K^+) and pipette solution is ICS (106 mM K^+). V_H = −50 mV. Voltage pulses of increasing duration to +150 mV were applied to the pipette interior. At the end of each pulse, the voltage was set to −60 mV. The inset shows a schematic representation of the applied voltage protocol. Upon stepping back from +150 to −60 mV, the current reverses (inward directed current), which is subsequently followed by a decrease of current amplitude in time due to deactivation. The instantaneous (tail) current increases with increasing duration of the depolarizing voltage step. The displayed currents are corrected for the leak conductance.

to deactivation of the conductance that can be seen in the appearance of tail-currents (Fig. 3A). The E_{rev} of the conductance is equal to the "back-step" potential where the polarity of the tail-currents reverses (i.e. where the instantaneous current after stepping to E_{rev} is 0). The E_{rev} can be easily obtained from the instantaneous *I-V* relationship after stepping back to less positive potential values (Fig. 3B). The mean E_{rev} of the outward currents for cells bathed in ECS and ICS in the pipette was −29.3 ± 9.6 mV (n = 15). In this measurement condition, E_{Cl} is about −44 mV and E_K is about −59.0 mV. To determine the main ion passing the conductance, the shift in E_{rev} was measured upon increasing both the extracellular K^+ and Cl^- bath concentration (K-ECS). The calculated shift in E_{Cl} is about −26 mV, and that for E_K is about +44 mV. Experimentally, the increase in both K^+ and Cl^- concentration caused a mean shift in E_{rev} of +48.7 ± 11.0 mV (n = 5). From the fact that the shift of E_{rev} is close to the shift of E_K, it can be concluded that K^+ is passing freely through the depolarization-activated conductance. However, the fact that E_{rev} is more positive than E_K indicates that some other ion with a more positive equilibrium potential must contribute. Tail-current experiments with an additional 5 mM NaCl in the intracellular solution and ECS with different extracellular Na^+ concentrations (0, 33.3, and 50 mM) showed that the E_{rev} of the outward current was only slightly sensitive to changes in E_{Na}. Assuming low permeability for chloride, the permeability ratio for K^+ and Na^+ (P_{K+}/P_{Na+}) was calculated by the Goldmann equation (see e.g. Schroeder, 1988). The mean P_{K+}/P_{Na+} was 11.6 ± 2.9 (n = 3).

From the steady-state *I-V* relationship (Fig. 1, B and D) of the outward current, the conductance (G_K) of the membrane at a given E_m can be determined from the relationship $G_K = I/(V_m - E_{rev})$. The G_K of the outward current saturated at potentials more positive than +100 mV for cells bathed in ECS. The mean $G_{K,max}$ of the outward current was 2.3 ± 1.4 nS (n = 16) for cells bathed in ECS. From the normalized conductance ($G_K/G_{K,max}$) as a function of V_m, a mean $V_{0.25}$ (potential at which $G_K/G_{K,max}$ = 0.25) of 31.6 ± 10.1 mV (n = 12) was found. Increase of the extracellular K^+ concentration did not alter $G_{K,max}$ of the outward current (in K-ECS, $G_{K,max}$ = 2.4 ± 1.1 nS [n = 5]). However, $V_{0.25}$ increased with about 30 mV when ECS was replaced by K-ECS (in K-ECS, $V_{0.25}$ = 59.1 ± 20.8 mV, n = 5).

Slow voltage ramps (from −180 mV to +180 and back to −180 mV in 5 s) in which the maximal conductance reached was equal to values reached during voltage-step protocols, were used to test the effect of different K^+ channel blockers on the amplitude of the outward current. Extracellular appli-

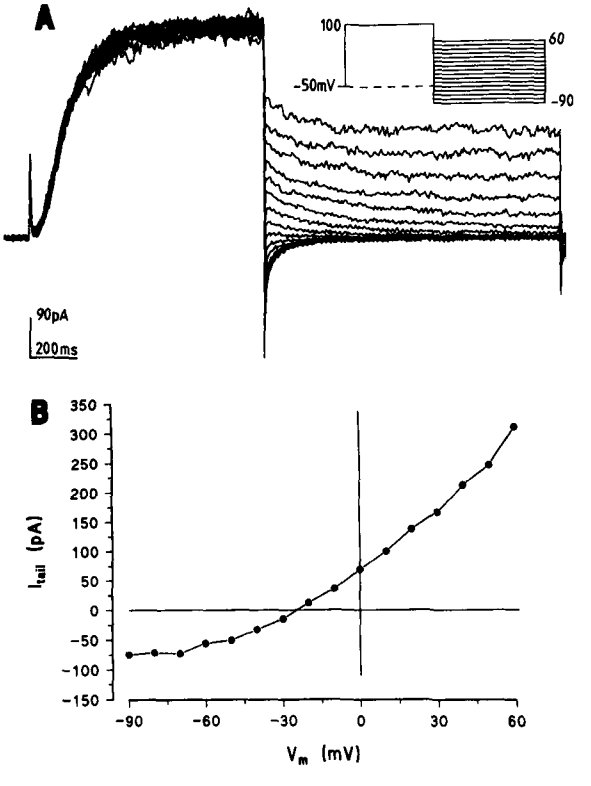

Figure 3. Measurement of E_{rev} of the outward current with ECS (11 mM K^+) in the bath and ICS (106 mM K^+) in the pipette. A, Current responses upon application of the voltage protocol shown in the inset in the whole-cell configuration. After application of activating depolarizing pulses to +100 mV to the pipette interior, repolarizing pulses ranging from +60 to −90 mV were applied. The current responses on the repolarizing test pulse show a reversal of the instantaneous "tail" current at an applied voltage of about −30 mV. Furthermore, voltage- and time-dependent deactivation of the conductance can be seen in the current responses upon the repolarizing test pulses. Displayed currents are corrected for the leak conductance. B, *I-V* relationship of the instantaneous tail current upon repolarizing voltage pulses as displayed in A.

cation of the K^+ channel-inhibiting agents TEA, quinidine, and 4-AP reduced the magnitude of the outward current. The mean percent reduction at V_m = +160 mV was: 18 ± 7 (n = 3) for 19 mM TEA; 32 ± 10 (n = 4) for 0.17 mM quinidine; and 32 ± 5 (n = 3) for 5 mM 4-AP. These measurements support the conclusion that the outward current is carried mainly by K^+ ions.

Identification of Inward Current

In about 30% of the cells, step-wise hyperpolarizations from a holding potential of −50 mV evoked time- and voltage-dependent membrane conductance changes resulting in inward currents (Fig. 1, C and D). Because the inward current was only present in a small part of all the cells tested, it was not possible to analyze this current to the same extent as the outward current. In addition, the inward current amplitude in many cases ran down with time. The data presented, therefore, are from whole-cell measurements obtained within 5 min after achieving the whole-cell configuration. The I-V relationship shows that the inward current can be detected for V_m values more negative than about −90 mV (Fig. 1D). In contrast to the outward current (Fig. 1, A and C) the activation of the inward current did not show a clear sigmoidal time course, but a more exponential time course (Fig. 1C). This suggests different voltage dependent kinetics of the ion channels conducting the outward and inward current. Like the outward current the inward current reached a steady-state level with time and did not show inactivation (Fig. 1C).

E_{rev} of the inward current was determined in a similar way as for the outward current (see above). Activating hyperpolarizing voltage pulses were followed by different steps to less negative potentials (Fig. 4A, inset). The deactivation of the conductance could be seen in the "tail-currents" in these experiments (Fig. 4A). For protoplasts bathed in ECS, the E_{rev} determined from the tail-currents was −53 ± 3 mV (n = 3) (see Fig. 4B). Increasing the extracellular K^+ concentration resulted in an E_{rev} of −22 ± 10 mV (n = 3). This shift in E_{rev} in combination with the fact that E_{rev} is close to E_K for both ECS and K-ECS indicate that K^+ is the major ion carrying the inward current.

As for the outward current, the G_K of the membrane at different V_m was determined for the inward rectifying conductance from the steady-state I-V relationship. Saturation of the inward conductance occurred at potentials more negative than −150 mV for protoplasts bathed in ECS. The mean $G_{K,max}$ was 0.60 ± 0.26 nS (n = 7) in ECS. From the normalized conductance ($G_K/G_{K,max}$) as a function of V_m (Fig. 5), a mean $V_{0.25}$ of −95.5 ± 17.7 mV (n = 7) was found for cells bathed in ECS. Increase of the K^+ concentration in the bath did not much change the mean $G_{K,max}$ ($G_{K,max}$= 0.67 ± 0.37 nS [n = 3]). The mean $V_{0.25}$ was not significantly different after changing the bath solution from ECS to K-ECS ($V_{0.25}$ = −94.0 ± 35.1 mV, n = 3).

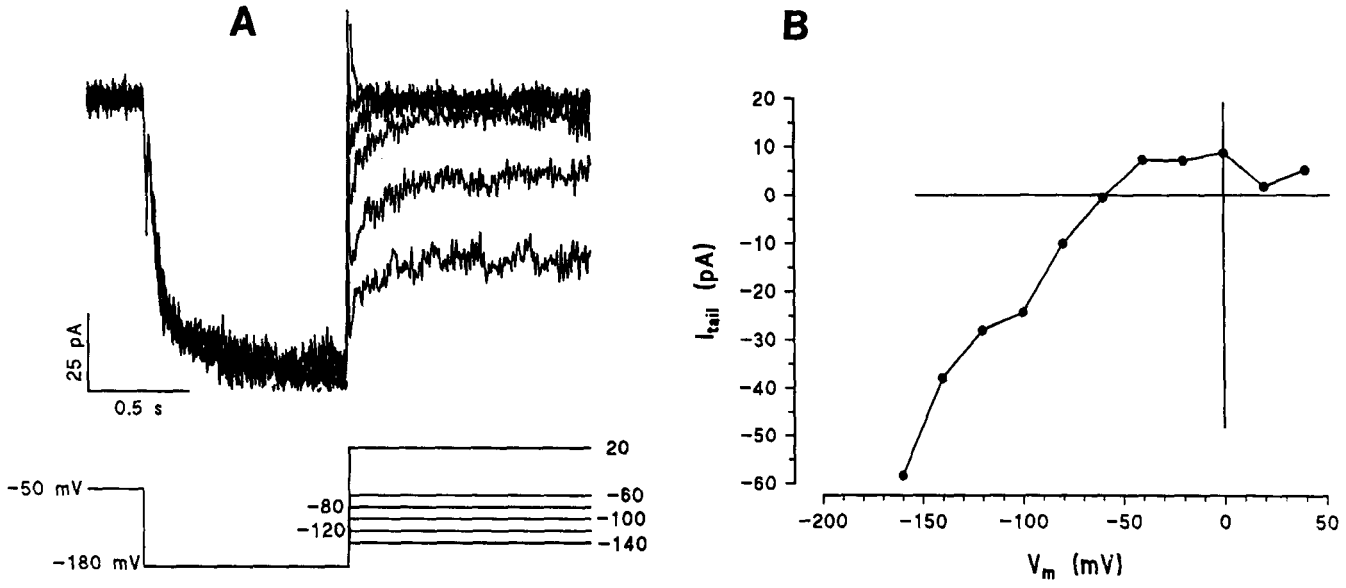

Figure 4. Measurement of E_{rev} of the inward current with ECS (11 mM K^+) in the bath and ICS (106 mM K^+) in the pipette. A, Current responses upon application of the voltage protocol shown in the lower part in the whole-cell configuration. After application of activating hyperpolarizing pulses to −180 mV to the pipette interior, repolarizing pulses ranging from −160 to +40 mV (with a 20-mV interval) were applied. Because the current traces for repolarizing pulses more positive than −60 mV show overlap, not all traces for these potential values are shown for reasons of clarity. The current responses on the repolarizing test pulse show a reversal of the instantaneous tail current at an applied voltage of about −60 mV. Furthermore, voltage- and time-dependent deactivation of the conductance can be seen in the current responses upon the repolarizing test pulses. Displayed currents are corrected for the leak conductance. B, I-V relationship of the instantaneous tail current upon repolarizing voltage pulses as displayed in A.

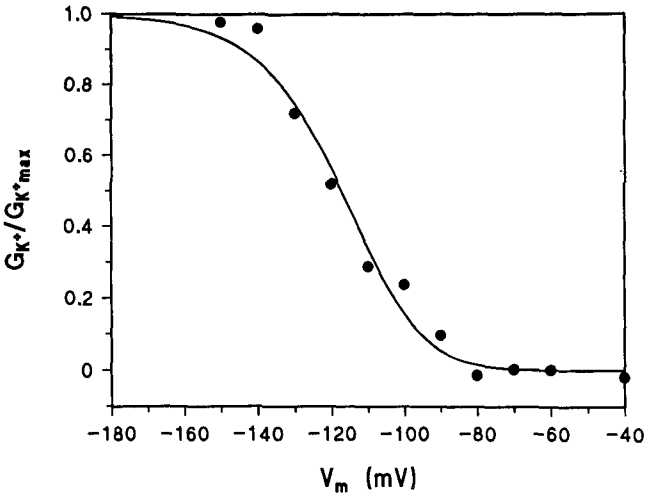

Figure 5. Steady-state activation (●) of the inward current (ECS [11 mM K^+] in bath, ICS [106 mM K^+] in pipette). The activation degree ($G_K/G_{K,max}$) was calculated from the *I-V* relationship with respect to the determined E_{rev} (-55 mV). $G_{K,max}$ for this protoplast was 0.64 nS.

DISCUSSION

We found two different ion conductances in the tobacco protoplasts: an outward rectifying conductance and an inward rectifying conductance. The outward rectifying conductance was activated upon plasma membrane depolarization (more positive than about 0 mV for cells bathed in ECS), whereas the inward rectifying conductance was activated upon plasma membrane hyperpolarization (more negative than about −90 mV for cells bathed in ECS). Similar looking hyperpolarization- and depolarization-activated K^+ currents have been shown to be present in other plants (Iijima and Hagiwara, 1987; Schroeder et al., 1987; Bush et al., 1988; Moran et al., 1988; Schroeder, 1988; Ketchum et al., 1989; Colombo and Cerana, 1991; Fairley et al., 1991; Fairley-Grenot and Assmann, 1992; Kouri and Goldsmith, 1992). Some of these conductances have been shown to be under control of the cytoplasmic and external calcium concentration, pH, or G-proteins (Schroeder and Hagiwara, 1989; Ketchum et al., 1991; Blatt, 1992; Fairley-Grenot and Assmann, 1992). Except for the ion channels detected in guard cells and pulvinar cells, which play an important role in cell shrinking and swelling (e.g. Schroeder et al., 1987; Moran et al., 1988; Blatt, 1991; Schroeder and Fang, 1991), no clear function in plant cells has been assigned to the ion channels described.

All the tobacco protoplasts used in our experiments showed the presence of the outward rectifying conductance, whereas an inward directed current was present in only 30% of the protoplasts as well. This suggests that the population of protoplasts from suspension-cultured tobacco cells in the stationary phase is a heterogenous population that consists of at least two different responding cell types. This heterogeneity may be due to asynchronicity in the cell cycles of different cells in the culture. However, damaging of inward rectifying channels during protoplast preparation cannot be ruled out. Similar heterogeneity in the presence of different ion channels in different cells has been reported for corn suspension-cultured cells (Ketchum et al., 1989; Fairley et al., 1991) and cultured cells from *Arabidopsis thaliana* (Colombo and Cerana, 1991).

K^+ ions contribute largely to the outward-directed, depolarization-activated current in tobacco protoplasts described in the present study. Evidence for K^+ selectivity comes from (a) the extracellular K^+-sensitive shifts of the measured E_{rev}, which are in agreement with the shifts in E_K. The P_K/P_{Na} value of about 12 is close to the value reported for the outward rectifying K^+ current in *Vicia faba* guard cells (Schroeder et al., 1987; Schroeder, 1988). (b) Potassium channel inhibitors decrease the magnitude of the outward rectifying current. Both TEA and quinidine, which reduce the outward rectifying K^+ current tobacco protoplasts, have been reported to block depolarization-activated K^+ currents in different plant cells (e.g. Blatt, 1988; Ketchum et al., 1989; Fairley et al., 1991; White and Tester, 1992). However, although at lower concentrations, 4-AP and TEA failed to block depolarization-activated (quinidine blockable) K^+ channels from rye roots (White and Tester, 1992), in contrast to the effect in tobacco protoplasts.

Because E_{rev} of the outward current is more positive than E_K, some other ion with a more positive equilibrium potential must contribute to the conductance as well. This ion could be Mg^{2+} and/or Ca^{2+}, as was found for the outward K^+ conductance in guard cells (Schroeder and Hagiwara, 1989).

The activation potential of the current ($V_{0.25}$) shifts in agreement with changes in E_K. This results in currents of smaller magnitude for more positive E_K at equal V_m. The maximal conductance is not affected by changes in E_K. Similar E_K dependence of outward rectifying K^+ currents has been reported for intact guard cells (Blatt, 1988), guard cell protoplasts (Schroeder, 1988), corn root (Ketchum et al., 1989), and corn shoot (Fairley et al., 1991) suspension cells. The outward K^+ current shows a sigmoidal activation time course, which is relatively fast compared with the activation of the outward K^+ currents reported for suspension-cultured cells from other species (Colombo and Cerana, 1991; Fairley et al., 1991) or wheat root cells (Schachtman et al., 1991). However, the time course of activation is of the order of magnitude of the outward K^+ current measured in guard cells of different species (Schroeder et al., 1987; Fairley-Grenot and Assmann, 1992). The activation potential of the outward current, as represented by the value of $V_{0.25}$, is somewhat more positive than was found for the outward K^+ current in *V. faba* guard cell protoplasts (Schroeder, 1989), but is about the same as was found in *Zea mays* guard cell protoplasts and root suspension cells (Ketchum and Poole, 1991; Fairley-Grenot and Assmann, 1992). However, it must be noted that the differences in reported activation potentials may be due to differences in free (local) concentrations of cytosolic calcium or other ions in the different experiments (Ketchum and Poole, 1991).

The outward rectifying K^+ conductance seems to be a common conductance in plant cells. It has been reported to exist in many different species, different tissues, and undifferentiated cells (Iijima and Hagiwara, 1987; Moran et al., 1988; Schroeder, 1988, 1989; Fairley et al., 1991; Fairley-Grenot and Assmann, 1992). This, however, does not rule

out a role for this conductance in specific cell functions or transduction of hormone signals. The outward rectifying K^+ conductance in guard cells acts as a K^+ release channel during stomatal closure (Schroeder et al., 1987; Blatt, 1991). The extreme depolarized E_ms (>+100 mV) needed to fully activate the outward rectifying K^+ conductance presumably indicates that activation of this conductance is regulated by a cytoplasmic component besides voltage control.

The plasma membrane hyperpolarization-activated inward current was present only in about 30% of the protoplasts. In addition, the current magnitude, in many cases, decreased in time after formation of the whole-cell configuration. This run down of the current indicates that cytoplasmic factors are important for the maintenance of this current. It has been reported that inward K^+ currents in guard cell protoplasts are under calcium and G-protein control (Schroeder and Hagiwara, 1989; Fairley-Grenot and Assmann, 1991). Therefore, the inward current could not be studied in the same detail as was the outward current. The K^+ selectivity of the inward current was shown by the shift in E_{rev}, as measured in tail-current experiments, in agreement with the shift in E_K. In addition, the mean value of E_{rev} itself is close to the calculated value of E_K. The inward rectifying K^+ current activated at strongly hyperpolarized plasma E_ms with an exponential time course. Time course of activation is relatively slow and is of about the same order of magnitude as the inward rectifying K^+ conductance in other cells, as is the activation potential (Schroeder et al., 1987; Colombo and Cerana, 1991; Fairley-Grenot and Assmann, 1991, 1992). Neither the activation potential ($V_{0.25}$) nor the $G_{K,max}$ were sensitive to changes in E_K. A similar independence, for extracellular K^+ concentrations between 3 and 100 mM, of the E_K was found for the inward rectifying K^+ conductance from guard cells (Schroeder et al., 1987; Schroeder, 1988; Blatt, 1992). However, at lower extracellular K^+ concentrations (1–11 mM), K^+-dependent shifts in activation potential of the inward rectifying K^+ conductance have been reported (Schroeder and Fang, 1991). In this case, inward rectifying K^+ channels may provide a K^+-sensing voltage-dependent K^+ uptake mechanism (Schroeder and Fang, 1991). In guard cells, the inward rectifying K^+ conductance plays an important role in the regulation of stomatal function (Schroeder et al., 1987; Blatt, 1991; Schachtman et al., 1991; Schroeder and Fang, 1991; Blatt, 1992). The inward K^+ current may be important to ensure effective H^+ transport by the electrogenic H^+-ATPases by preventing the occurrence of extreme membrane hyperpolarization (which will inhibit H^+ efflux).

Because the outward rectifying conductance can be present in the absence of the inward rectifying conductance, it appears that the channels underlying the inward current are different from the channels conducting the outward current. In addition, the activation kinetics of both conductances, the $G_{K,max}$, and E_{rev} of the inward and outward rectifying conductance are different.

The data presented show that voltage- and time-dependent potassium conductances are present in the plasma membrane of tobacco protoplasts from cell suspension cultures. Because application of molecular biological techniques to tobacco cells is relatively easy and much biochemical and molecular biological knowledge is already available for these cells, a combination of a biochemical, molecular biological, and electrophysiological approach in this system may provide detailed information on the role and regulation of ion channels in plant cells.

ACKNOWLEDGMENTS

The authors are grateful to Drs. L.G. Van der Molen and M. Wang for support and for critical reading of the manuscript.

Received July 28, 1992; accepted September 8, 1992.

LITERATURE CITED

An G (1985) High efficiency transformation of cultured tobacco cells. Plant Physiol **79:** 568–570

Blatt MR (1988) Potassium-dependent bipolar gating of potassium channels in guard cells. J Membr Biol **102:** 235–246

Blatt MR (1991) Ion channel gating in plants: physiological implications and integration for stomatal function. J Membr Biol **124:** 95–112

Blatt MR (1992) K^+channels of stomatal guard cells. Characteristics of the inward rectifier and its control by pH. J Gen Physiol **99:** 615–644

Briskin DP, Leonard RT (1979) Ion transport in isolated protoplasts from tobacco suspension cells. III. Membrane potential. Plant Physiol **64:** 959–962

Bush DS, Hedrich R, Schroeder JI, Jones RL (1988) Channel-mediated K^+flux in barley aleurone protoplasts. Planta **176:** 368–377

Colombo R, Cerana R (1991) Inward rectifying K^+ channels in the plasma membrane of *Arabidopsis thaliana*. Plant Physiol **97:** 1130–1135

Dangle JL, Hauffe KD, Lipphardt S, Hahlbrock K, Scheel D (1987) Parsley protoplasts retain differential responsiveness to u.v. light and fungal elicitor. EMBO J **6:** 2551–2556

Fairley K, Laver D, Walker NA (1991) Whole-cell and single-channel currents across the plasmalemma of corn shoot suspension cells. J Membr Biol **121:** 11–22

Fairley-Grenot K, Assmann SM (1991) Evidence for G-protein regulation of inward K^+ channel current in guard cells of fava bean. Plant Cell **1:** 1037–1044

Fairley-Grenot KA, Assmann SM (1992) Whole-cell K^+ current across the plasma membrane of guard cells from a grass: *Zea mays*. Planta **186:** 282–293

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 Eur. J. Physiol. **391:** 85–100

Iijima T, Hagiwara S (1987) Voltage-dependent K channels in protoplasts of trap-lobe cells of *Dionaea muscipula*. J Membr Biol **100:** 73–81

Ketchum KA, Poole RJ (1991) Cytosolic calcium regulates a potassium current in corn (*Zea mays*) protoplasts. J Membr Biol **119:** 277–288

Ketchum KA, Shrier A, Poole RJ (1989) Characterization of potassium-dependent currents in protoplasts of corn suspension cells. Plant Physiol **89:** 1184–1192

Kourie J, Goldsmith MHM (1992) K^+ channels are responsible for an inwardly rectifying current in the plasma membrane of mesophyll protoplasts of *Avena sativa*. Plant Physiol **98:** 1087–1097

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant **18:** 100–127

Marten I, Lohse G, Hedrich R (1991) Plant growth hormones control voltage-dependent activity of anion channels in plasma membrane of guard cells. Nature (Lond) **353:** 758–762

Moran N, Ehrenstein G, Iwasa K, Mischke C, Bare C, Satter RL (1988) Potassium channels in motor cells of *Samanea saman*: a patch-clamp study. Plant Physiol **88:** 643–648

Schachtman DP, Tyerman SD, Terry BR (1991) The K^+/Na^+ selec-

tivity of a cation channel in the plasma membrane of root cells does not differ in salt-tolerant and salt-sensitive wheat species. Plant Physiol **97:** 598–605

Schroeder JI (1988) K^+ transport properties of K^+ channels in plasma membrane of *Vicia faba* guard cells. J Gen Physiol **92:** 667–683

Schroeder JI (1989) Quantitative analysis of outward rectifying K^+ channel currents in guard cell protoplasts from *Vicia faba*. J Membr Biol **107:** 229–235

Schroeder JI, Fang HH (1991) Inward-rectifying K^+ channels in guard cells provide a mechanism for low-affinity K^+ uptake. Proc Natl Acad Sci (USA) **88:** 11583–11587

Schroeder JI, Hagiwara S (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. Nature (Lond) **338:** 427–430

Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K^+ channels in guard-cell protoplasts. Proc Natl Acad Sci USA **84:** 4108–4112

Van der Zaal EJ, Mennes AM, Libbenga, KR (1987) Auxin-induced rapid changes in translatable mRNAs in tobacco cell suspensions. Planta **172:** 514–519

Van Duijn B, Ypey DL, Van der Molen LG, Libbenga KR (1992) Whole-cell patch-clamp experiments on tobacco protoplasts. *In* CM Karssen, LC van Loon, D Vreugdenhil, eds, Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 668–674

White PJ, Tester MA (1992) Potassium channels from the plasma membrane of rye roots characterized following incorporation into planar bilayers. Planta **186:** 188–202