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Whole-Cell and Single-Channel Currents across the Plasmalemma of Corn Shoot Suspension Cells

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Summary. Whole-cell sealed-on pipettes have been used to measure electrical properties of the plasmalemma surrounding protoplasts isolated from Black Mexican sweet corn shoot cells from suspension culture. In these protoplasts the membrane resting potential (V_m) was found to be -59 ± 23 mV ($n = 23$) in 1 mM K_o^+. The mean V_m became more negative as $[K^+]_o$ decreased, but was more positive than the K^+ equilibrium potential. There was no evidence of electrogenic pump activity. We describe four features of the current-voltage characteristic of the plasmalemma of these protoplasts which show voltage-gated channel activity. Depolarization of the whole-cell membrane from the resting potential activates time- and voltage-dependent outward current through K^+-selective channels. A local minimum in the outward current-voltage curve near $V_m = 150$ mV suggests that these currents are mediated by two populations of K^+-selective channels. The absence of this minimum in the presence of verapamil suggests that the activation of one channel population depends on the influx of Ca^{2+} into the cytoplasm. We identify unitary currents from two K^+-selective channel populations (40 and 125 pS) which open when the membrane is depolarized; it is possible that these mediate the outward whole-cell current. Hyperpolarization of the membrane from the resting potential produces time- and voltage-dependent inward whole-cell current. Current activation is fast and follows an exponential time course. The current saturates and in some cases decreases at membrane potentials more negative than -175 mV. This current is conducted by poorly selective K^+ channels, where $P_{Cl}/P_K = 0.43 \pm 0.15$. We describe a low conductance (20 pS) channel population of unknown selectivity which opens when the membrane is hyperpolarized. It is possible that these channels mediate inward whole-cell current. When the membrane is hyperpolarized to potentials more negative than -250 mV large, irregular inward current is activated. A third type of inward whole-cell current is briefly described. This activates slowly and with a U-shaped current-voltage curve over the range of membrane potentials $-90 < V_m < 0$ mV.

Key Words channel · protoplast · K^+ current · patch clamp · corn · maize

Introduction

It has been recognized since the '70s that electrical difficulties often exist when using inserted microelectrodes to measure potential differences across the membranes bounding small cells, especially those without cell walls and without turgor. These difficulties include electrical leakage at the point of insertion and salt leakage from the inserted electrode. They have been overcome in a limited number of cases (*see* Blatt, 1987; Parsons & Sanders, 1989).

The development of the patch-clamp method (Hamill et al., 1981) provided a new way of measuring the electrical properties of the plasmalemma of small wall-less cells such as plant protoplasts. This method depends on the formation of a high resistance seal between a glass micropipette and the cell membrane. When seals of gigaohm resistance are obtained leak currents are small and current through individual ion-conducting channels (patch mode) or through populations of ion channels in the cell membrane (whole-cell mode) can be measured. The formation of seals of such high resistance is far from easy with plant plasmalemma, but it has been achieved by a number of workers (*see* review by Tester, 1990).

In patch mode there can be no salt leakage from the pipette tip as it is sealed by the membrane. In whole-cell mode leakage becomes an advantage: the pipette solution perfuses the cell cytoplasm and hence the internal face of the plasmalemma. Whole-cell recording therefore allows both sides of the plasmalemma to be perfused with experimentally defined ionic solutions. Equilibrium potentials for ions on either side of the membrane can then be calculated on the basis of known rather than estimated concentrations. This facilitates easier identification of the ions conducting the current. It has the disad-

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vantage that the normal constitution of the cytoplasm is disturbed and replaced with what is, at best, a sketchy imitation. The method of slow whole-cell clamping addresses this problem, permeabilizing the patch of membrane under the pipette tip instead of breaking through it (Lindau & Fernandez, 1986). This allows only partial control of cytoplasmic contents, however.

This work began with the intention to identify features of the whole-cell current-voltage curve of a higher plant cell and to identify, as far as possible, the single channels responsible. As the majority of plant patch-clamp work to date focuses on currents in stomatal guard cells it was decided to instead measure current across the plasmalemma of cells from ground tissue. Suspension cells isolated from corn mesocotyl were chosen for this purpose. The whole-cell recording approach has been independently adopted by Ketchum, Shrier and Poole (1989) who used suspension cultures of corn root cells. They have been able to identify inward and outward time-dependent currents in protoplasts isolated from those cells.

Materials and Methods

Corn Cell Cultures and Protoplast Isolation

Black Mexican sweet (BMS) corn cell suspension cultures were obtained from the C.S.I.R.O. Division of Plant Industry, Canberra. These were originally derived from the mesocotyl of BMS corn seedlings according to the method of Chourey and Zurawaski (1981). Cell cultures were maintained as described in Fairley and Walker (1989).

Protoplasts were isolated from suspension cell cultures using a procedure adapted from Somers et al. (1987). Cells from cultures aged 3–6 days were washed in a solution (PWS) containing 80 mM $CaCl_2$, 0.5% MES [2(N-morpholino)-ethanesulfonic acid] and 0.125 mM dithiothreitol at pH 5.1 with osmolality adjusted to ca. 480 mOsm/kg (D-mannitol). They were then placed in PWS containing 0.5% protease-free bovine serum albumin, 1% hemicellulase, 1% pectinase and 3% cellulase. All chemicals and enzymes were obtained from Sigma Chemical (St. Louis, MO) except for the cellulase (Onozuka R-10) which was obtained from Yakult Honsha (Japan). Digestion of the cell wall was allowed to proceed for 4 hr at 24–26°C on a rotary shaker at 80 rpm. Protoplasts thus liberated were washed three times in PWS and then filtered through a 125 μm screen into a bath containing a chosen bathing solution.

Current Recording

Protoplasts were allowed to settle in the bath which was kept at ground potential by an immersed Ag/AgCl electrode connected to earth via the headstage of a patch-clamp amplifier (List EPC-7). Protoplasts were approached with pipettes which were made from either soda glass (Clay Adams, Parsippany, NJ) or borosilicate glass (Modulohm I/S, Denmark) using a two-stage vertical electrode puller (Kopf Model 720). Hard glass pipettes were fire-polished prior to use. The electrode offset was compensated when each pipette was immersed in the bathing solution.

The resistance across the tip of each patch pipette was calculated by measuring the current which flowed when a 1-mV square wave was applied through the patch-clamp amplifier and was typically within the range 1–8 MΩ after being filled with one of the solutions listed below. Seal formation was monitored by observing the increase in this resistance as a pipette was brought in contact with a protoplast surface and suction applied. Protoplasts were approached with pipettes between 10 min and 5 hr after isolation. All experiments were carried out at 22 ± 2°C.

When cell-attached patches were obtained the membrane potential (V_m) was held at the protoplast resting potential (V_r) by imposing zero command voltage (V_c) ($V_m = -V_c + V_r$). The membrane was then depolarized and hyperpolarized in step-like jumps to potentials in the range $V_m = \pm 250$ mV.

When whole-cell seals were obtained V_r was measured immediately and the holding potential set to this value. In whole-cell mode $V_m = V_c$. Measurements of membrane current were made during and after perfusion of the cytoplasm by the pipette-filling solution. V_r was checked at later stages in order to see whether or not it had drifted from its original value due to this perfusion.

In both single-channel and whole-cell recording the transmembrane current was displayed on a cathode ray oscilloscope (CRO) and simultaneously digitized and recorded (along with the pipette potential, V_c) on video tape using a digital P.C.M. unit (Sony 501-ES) and a video recorder (Sony SL-HF150). Positive current was defined as the movement of positive charge out of the protoplast. A computer (IBM-AT) was interfaced to the patch-clamp amplifier through a multipurpose I/O device (DT-2801-A) and provided one way of setting V_c via the program COMVOLT. The computer and I/O device were later used to redigitize the current and voltage time courses using the program ADCIN (Dr. J. Pumplin, Michigan State University). These were then redisplayed on the CRO using the program SCOPE (Dr. J. Pumplin). The recording and playback system was calibrated by recording a square waveform with 5 V amplitude at the start or end of each experiment. Hard copies of current data were obtained using the program AMPLOT (Dr. R. Reid, Adelaide University).

Solutions

Three sets of pipette/bathing solutions were used (concentrations in mM): (1) CYTOPIP: 9.6 KCl, 45.2 K_2SO_4, 0.2 $CaCl_2$, 1 EGTA, 5 HEPES, pH = 7.5 (free $Ca^{2+} = 1.04 \times 10^{-5}$ mM; pCa = 8); 1BATH: 1 KCl, 2 $CaCl_2$, 5 HEPES, pH = 7.0. (2) PUMPPIP: as for CYTOPIP plus 3 ATP and 3 $MgSO_4$; PUMPBATH: 0.1 KCl, 2 $CaCl_2$, 5 HEPES, pH = 7.5. (3) 75PIP: 75 KCl, 2 $CaCl_2$, 10 MES, pH = 5.1 (pCa = 3); 75BATH: 75 KCl, 2 $CaCl_2$, 10 MES, pH = 5.1.

The osmolality of all solutions was measured with a vapor pressure osmometer and adjusted to 480–500 mOsm/kg with D-mannitol. The pH of all solutions was adjusted with KOH except for 1BATH and PUMPBATH where NaOH was used as $[K^+]$ was low. Set 1 was intended to emulate in vivo cytoplasmic and extracellular ionic conditions as reported in the literature (*see* Hajibagheri et al., 1988; Okihara & Kiyosawa, 1988; Bush, Biswas & Jones, 1989; Harvey, 1989). By providing ATP and lowering the external K^+ concentration it was intended in Set 2 to

Table. Effect of external K^+ concentration, $[K^+]_o$, on protoplast resting potential (V_r)

$[K^+]_o$ (mM)	Initial V_r (mean ± SD) (mV)	n	E_{Cl}* (mV)	E_K* (mV)
0.1	−98 ± 24	8	23	−166
1.0	−59 ± 23	23	17	−109
75	−36 ± 19	10	0	0

* Before perfusion of the cytoplasm by the pipette solution. Calculated from published values for cytoplasmic $[K^+]$: 75 mM (Hajibagheri et al., 1988) and $[Cl^-]$: 10 mM (Okihara & Kiyosawa, 1988).

provide conditions under which ATPases might be active, as in a charophyte-like 'pump' state (Beilby, 1984). In Set 3 it was intended to increase and therefore highlight any inward potassium or chloride currents.

Results

Membrane-Pipette Seals

Lasting whole-cell seals (seal resistance > 100 mΩ) were obtained in an average of one in four attempts. However, lasting cell-attached patches or excised patches (seal resistance > 1 GΩ) were obtained infrequently. We were unable to improve the frequency of gigaohm seal formation by using inhibitors of cell wall regeneration (Fairley & Walker, 1989).

Whole-Cell Potential

The effect of varying the external K^+ concentration, $[K^+]_o$, on the protoplast resting potential, V_r, measured immediately after seal formation is shown in the Table. At lower $[K^+]_o$, V_r was more negative. It was never more negative than E_K or more positive than E_{Cl}.

Whole-Cell Currents

Membrane Depolarization Activates Outward Currents

Depolarization of the protoplast membrane from a holding potential set at V_r activated time- and voltage-dependent outward current. The depolarizing potential at which these currents were of a discernable magnitude (the 'activation potential') was ca. −40 mV when E_K = −116 mV and E_{Cl} = +17 mV (n = 11), ca. 0 mV when E_K = E_{Cl} = 0 mV (n = 4) and ca. −90 mV when E_K = −174 mV and E_{Cl} = +23 mV (n = 6).

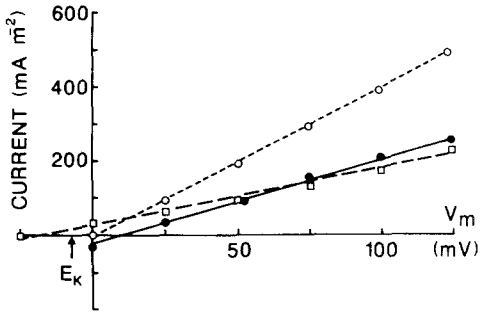

Fig. 1. Instantaneous whole-cell current as a function of membrane potential (V_m) from a holding potential of −80 mV (open squares) and from a holding potential of +125 mV (open circles). The difference between these sets of values gives the current-voltage relationship of channels open at V_m = 125 mV (filled circles). The arrow labeled 'E_K' shows the electrochemical reversal potential for K^+ (75BATH/CYTOPIP)

The current-voltage relationship for the channels which open in response to membrane depolarization was determined from two measurements of instantaneous current. The instantaneous current at any given potential is the sum of current leaking through the membrane-pipette seal and current through channels open at the moment before the voltage is changed to the given value. The instantaneous current-voltage relationship was measured (*i*) from a holding potential of −80 mV, where very little current passes through these channels, and (*ii*) from a holding potential of +125 mV, where the magnitude of outward current is large. Instantaneous current was measured 10 msec after the voltage change when it could be clearly resolved from the associated capacitance spike. By subtracting (*i*) from (*ii*) the seal leak component of the instantaneous current was removed, assuming that the seal integrity remained constant over the (4 min) time course of the experiment. The reversal potential obtained in this way was 6 ± 3 mV when E_K = −7 mV and E_{Cl} = −52 mV (75BATH/CYTOPIP; Fig. 1). The reversal potential for the outward current was also determined from the direction of tail current when repolarizing the membrane from V_m = +100 mV to a range of membrane potentials. In this way it was found that the reversal potential was 0 ± 3 mV when 75BATH/CYTOPIP were used.

The time-activated current reached a steady magnitude with time in most cases (Fig. 2). The magnitude of the steady-state current increased with increasing depolarization except for a reproducible decrease at or near V_m = +160 mV (Fig. 3). This decrease was associated with the time-activated current as there was no corresponding 'notch' in the

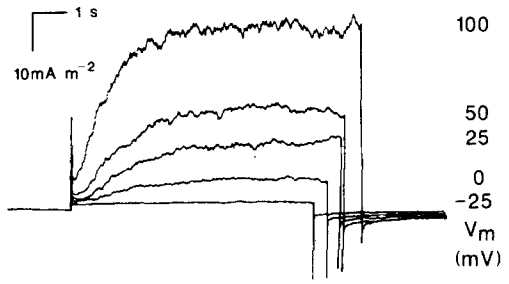

Fig. 2. Typical whole-cell current time courses after depolarization of a protoplast membrane from a holding potential of −90 mV to a series of membrane potentials (V_m). Results are from one whole-cell seal on a protoplast bathed in 1BATH and using CYTOPIP

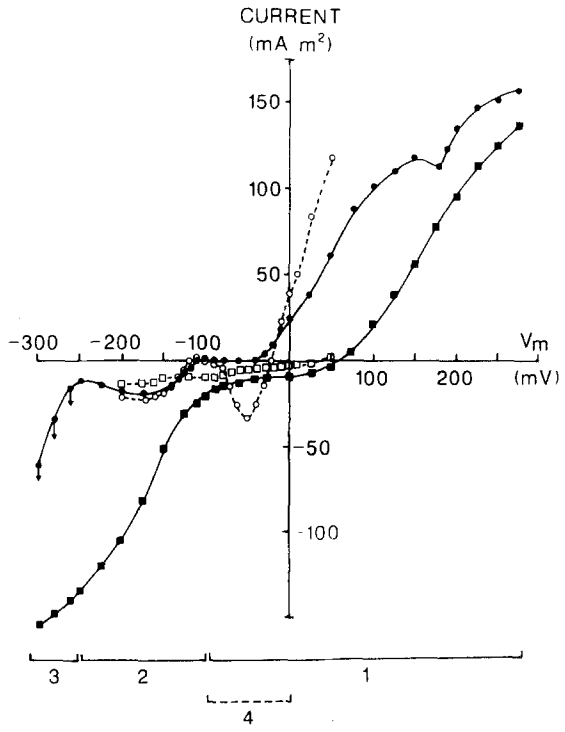

Fig. 3. Instantaneous and time-activated steady-state whole-cell current as a function of membrane potential (V_m). Instantaneous current was measured 10 msec after the change in membrane potential. It was then subtracted from the steady-state whole-cell current to give the time-activated value at a given membrane potential. Results shown are from whole-cell seals on two protoplasts: (*i*) from a holding potential of −60 mV in 1BATH/CYTOPIP: instantaneous (×½, filled squares) and time-activated (filled circles); (*ii*) from a holding potential of −90 mV in PUMPBATH/PUMPPIP: instantaneous (×½, open squares) and time-dependent (open circles). *1–4* correspond to different types of whole-cell currents described in the text. The arrows on the points at extreme negative membrane potentials are used to indicate the way in which inward current continued to increase with time at these potentials

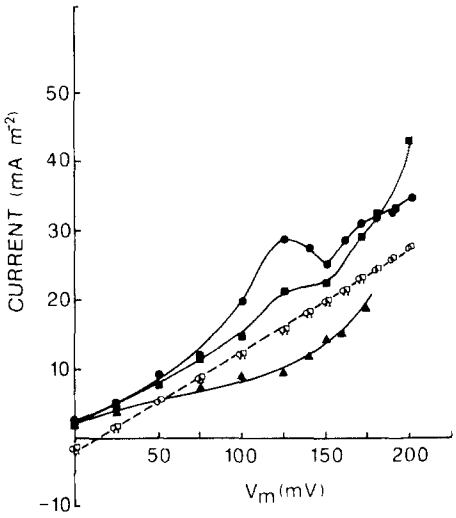

Fig. 4. Effect of verapamil (10 μM) on time-independent and time-activated outward whole-cell current. Results are for a protoplast in 1BATH/CYTOPIP after depolarization from a holding potential of −100 mV. Current was measured before verapamil (circles), 4 min after the perfusion of the bathing medium with verapamil (squares) and 12 min after verapamil addition (triangles). Open symbols (dotted line) refer to time-independent current (×$\frac{1}{10}$); filled symbols refer to time-activated current, obtained by subtracting the time-independent current from the total steady-state current at a given membrane potential

instantaneous current-voltage curve. The notch was present in all cases where the membrane potential could be taken to such extreme positive potentials without causing protoplast lysis. Reducing the internal calcium concentration from $p\text{Ca} = 3$ to $p\text{Ca} = 8$ shifted the membrane potential at which the notch occurred from 130 ± 10 mV to 160 ± 10 mV. The addition to the bath of verapamil (10 μM), an organic blocker of L-type Ca^{2+} channels in the plasmalemma of animal cells (Fox, Nowycky & Tsien, 1987) reduced the amplitude of whole-cell currents and eliminated the notch (Fig. 4). Both of these effects were reversible.

A reduction in outward current amplitude in the presence of the inorganic Ca^{2+} channel blocker, Cd^{2+}, has also been observed: addition of 400 μM Cd^{2+} to the bath reduces the current by approximately 55% (measured at $V_m < 125$ mV). The putative K^+ channel blocker, TEA (20 mM), was also found to block outward current.

Activation of the outward current followed a sigmoidal time course in low $[K^+]_o$ and low $[Cl^-]_o$ (1BATH/CYTOPIP and PUMPBATH/PUMPPIP; Fig. 2). Variations in the initial 'lag' phase of the time course were estimated by measuring the time taken for the current to reach half its steady-state magnitude. This 'activation half-time' decreased as the depolarized potential became more positive (up

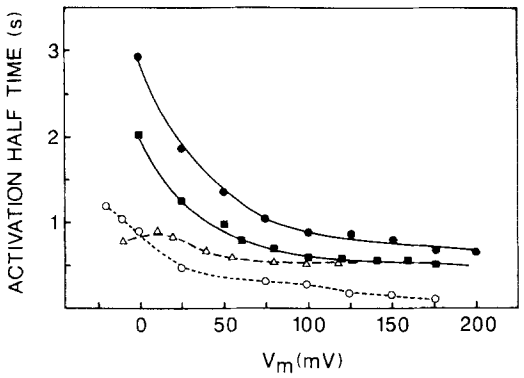

Fig. 5. Activation half-time as a function of membrane potential (V_m). Activation half-time was measured as the point in time at which the time-activated current had reached half of its steady-state value. Mean half-time values (n = 2) are for protoplasts in 1BATH depolarized from a holding potential of −50 mV (filled circles) and −90 mV (filled squares); (open circles) values are from another protoplast patched immediately after (and under identical conditions to) one of those used for (filled squares), and also from a holding potential of −90 mV; (triangles) half-time are for currents after depolarization from a holding potential of −50 mV in a protoplast bathed in 75BATH. CYTOPIP was used in all cases

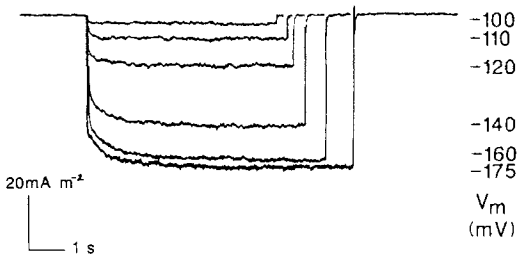

Fig. 6. Typical whole-cell current time courses after hyperpolarization from a holding potential of −90 mV to a series of membrane potentials (V_m). Results are from a whole-cell patch using 1BATH/CYTOPIP

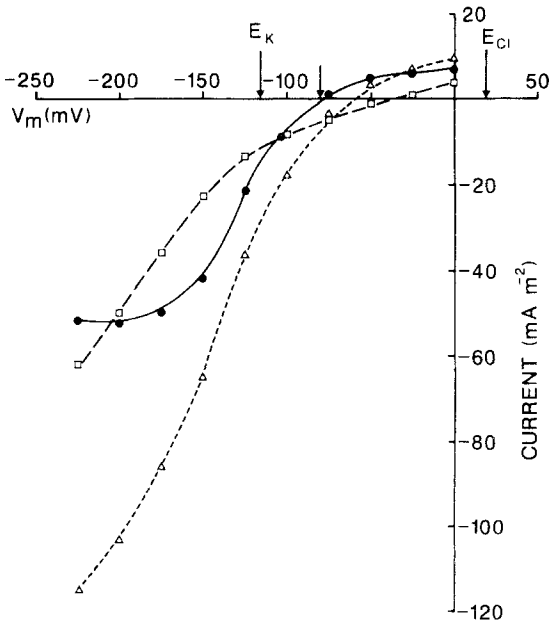

Fig. 7. Time-independent whole-cell current as a function of membrane potential (V_m) from a holding potential of −80 mV (open squares) and from a holding potential of −200 mV (open triangles). The difference between these sets of values gives the current-voltage relationship of channels open at V_m = −200 mV (filled circles). The unlabeled arrow signifies the reversal potential for the current flowing through these channels. The arrows labeled 'E_K' and 'E_{Cl}' refer to the electrochemical reversal potentials for K^+ and Cl^- (1BATH/CYTOPIP)

to V_m = 125 mV) or as the holding potential became more negative (Fig. 5, filled symbols). In some protoplasts the activation half-time was greatly reduced (Fig. 5, open circles). In high $[K^+]_o$ and high $[Cl^-]_o$ (75BATH/CYTOPIP) the time course of the outward current show a much shorter lag phase and the activation half-time was reduced by a factor of 4 at V_m = 0 mV and less so at more positive V_m (Fig. 5, triangles). There was no obvious difference in activation half-time on either side of the membrane potential at which the notch occurred.

Membrane Hyperpolarization Activates Inward Currents

Hyperpolarization of the protoplast membrane activated an inward current with a fast exponential time course in approximately 60% of protoplasts (Figs. 3(2) and 6). Activation of this current was much faster than activation of the outward current described above: activation half-time decreased from 120 to 60 msec over the range of V_m from −120 to −170 mV. Inactivation of the current was also fast; inactivation half-times were around 40 msec (Fig. 6). The magnitude of the current decreased at V_m more negative than −175 mV (Fig. 3(2)). The hyperpolarizing potential beyond which this current occurred (the 'activation potential') was −110 mV when E_K = −116 mV and E_{Cl} = +17 mV (n = 4) and −130 mV when E_K = −174 mV and E_{Cl} = +23 mV (n = 4). These results are recorded for protoplasts where the sampling voltages allowed resolution of the activation potential to within 10 mV.

The current-voltage relationship for the channels which open when the membrane is hyperpolarized was obtained by subtracting the instantaneous current-voltage curve obtained from a holding potential of −80 mV from that obtained from a holding potential of −200 mV (Fig. 7). Where E_K = −116 mV and E_{Cl} = 17 mV (CYTOPIP/1BATH) this gave

values for the reversal potential of -75 ± 8 mV and -80 ± 2 mV in two different experiments.

External TEA (20 mM) did not change the position of this reversal potential even though TEA blocked the fast inward current with a K_M of ca. 10 mM. Approximately 75% of the current was restored after removal of the TEA.

Currents after Extreme Membrane Hyperpolarization

Hyperpolarization of the corn plasmalemma to potentials more negative than -250 mV caused some protoplasts to lyse. Others showed reproducible inward current (Fig. 3(*3*)) with time courses displaying at least two of three distinct phases: (*i*) an early phase of variable length (3–20 sec) occupied by the fast-activating inward current described above, and (*ii*) a second phase during which the magnitude of the total current increased slowly with time (up to 60 sec). This was often followed by (*iii*), a sudden and rapid increase in the current. In some protoplasts this rapid current occurred immediately after the extreme hyperpolarization. This did not result in degradation of the membrane, however, as the current-voltage features of 'normal' protoplasts could be reproduced after such behavior.

Slow-Activating Inward Current

In one protoplast inward current was repeatedly observed at $-90 < V_m < 0$ mV (Figs. 3(*4*) and 8). In this protoplast the outward and fast inward currents described above were also observed. This anomaly could not be reproduced despite several attempts to do so. The amplitude of the current showed a distinctive voltage dependence, with maximum current flowing at $V_m = -50$ mV (Fig. 3(*4*)). Current activation was slow: the activation half-time was 4 to 5 sec at $V_m = -40$ to -60 mV (Fig. 8). There was a sudden decrease in activation time when V_m was more negative than this, however.

Large tail currents were observed after repolarization of the membrane potential (Fig. 8). The magnitude of the tail current greatly increased with increasing depolarization of the membrane over the range -80 to -10 mV. The tail current decreased after depolarization to potentials more positive than this. Decay of the current was slow (Fig. 8): inactivation half-times were around 1.5 sec after repolarization from potentials between -30 and -70 mV.

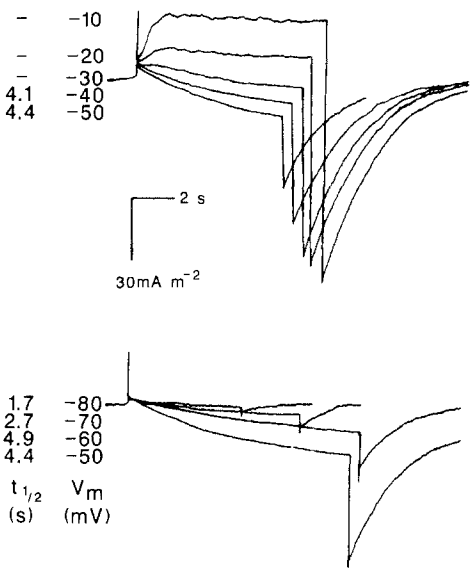

Fig. 8. Whole-cell current time courses after depolarization from a holding potential of -90 mV to a series of membrane potentials (V_m). Results are from a whole-cell seal on a protoplast using PUMPBATH/PUMPPIP. Activation half-time ($t_{1/2}$) is listed where only the slow inward current dominates the total whole-cell current

Single-Channel Activity

The most commonly seen single-channel activity in cell-attached patches was that of a population of low conductance channels (40 pS) with a reversal potential at -4 ± 5 mV (75PIP in pipette; Fig. 9*A*). They were rarely seen to open at negative membrane potentials. Repeated depolarization of the membrane resulted in repeated activation of these channels. Figure 10 shows the result of the addition of repeated activation after depolarization from $V_m = -70$ to $V_m = +130$ or $+180$ mV. The summed activation has a sigmoidal time course with a half-activation time of 250 msec and shows signs of slowly deactivating with time.

A second channel type has been observed, also mainly at positive membrane potentials. These channels have a conductance of 125 pS and exhibit a conductance substrate of ca. 100 pS. They have a reversal potential ca. 0 mV (conditions as for 40-pS channel; Fig. 9*B*) and a mean open time of 6 msec; this is voltage independent over the range $V_m = 70$–150 mV.

The activity of a third, low conductance channel type has been observed when V_m is more negative than -150 mV (Fig. 9*C*). This range did not encompass a reversal potential. The single-channel conductance is 20 pS when 75PIP is used. These chan-

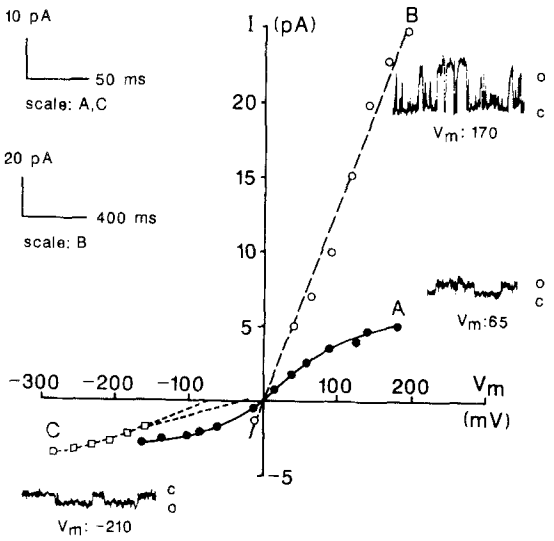

Fig. 9. Single-channel current (I) for three different channel types, *A, B, C,* as a function of membrane potential (V_m) in one cell-attached patch when using 75PIP. The current amplitude was measured from digitized current records displayed on a cathode ray oscilloscope. The inserts show samples of current recorded through each type of channel. All currents shown were filtered at ca. 350 Hz.

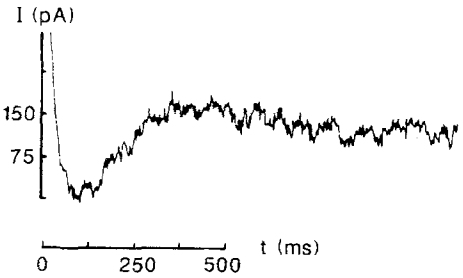

Fig. 10. Summation of 29 records showing the activation of current (I) with time (t) through 40-pS channels in a cell-attached patch after repeated depolarization from a membrane potential of −70 mV to a potential of either +130 or +180 mV in PUMPBATH/PUMPPIP

nels were sometimes seen to open for tens of milliseconds.

The data shown in Fig. 9 is from a patch where the activity of all three channel types was seen.

Discussion

Whole-Cell Potential

The dependence of the resting potential, V_r, on external $[K^+]_o$ (the Table) suggests that it is partly determined by the distribution of K^+ across the membrane and therefore that some K^+-conducting channels are open in the resting state. V_r has a similar K_o^+ dependence in corn root protoplasts (Ketchum et al., 1989), in *Nitella* protoplasts (Abe & Takeda, 1986) and in *Vicia* guard cells (Blatt, 1988).

The difference between V_r and V_K may be due to the contribution of other electrogenic transport systems (e.g., for Cl^-) and/or to leakage through the membrane-pipette seal.

The fact that V_r is less negative than E_K even in the presence of ATP and Mg^{2+} suggests that electrogenic pumps presumed to be present in the membrane are not actively extruding H^+ from the cytoplasm. Low negative resting potentials have been reported from patch-clamp measurements on protoplasts of other cell types, for example barley cells (−45 mM in 10 mM K_o^+, Bush et al., 1988) and corn root cells (−64 ± 23 mV in 10 mM K_o; Ketchum et al., 1989). Resting potentials measured in this way tend to be less negative than resting potentials recorded in intact plant cells in similar $[K^+]_o$ using intracellular electrodes: for example, −112 ± 17 mV in 1 mM K_o^+ in *Lemna gibba* (Abe, Takeda & Senda, 1980); −121 ± 4 mV in 0.6 mM K_o^+ in soybean suspension cells (Parsons & Sanders, 1989); and −130 ± 3 mV in 0.1 M K_o^+ in *Vicia* guard cells (Blatt, 1988). It is possible that this is because the functioning of the pumps is impaired by the enzyme treatment used to isolated protoplasts from intact cells. This possibility has previously been suggested by Schroeder (1988) who found that outward currents activated by blue light and fusicoccin are smaller in *Vicia* protoplasts than currents reported under the same conditions in intact *Vicia* cells (Blatt & Clint, 1989). Blue light and fusicoccin reputedly stimulate the activity of H^+-ATPases in the plant plasmalemma (Marré, 1985; Shimazaki, Iino & Zeiger, 1986).

Outward Current

Ion Selectivity

Four independent results indicate that the channels which mediate the outward whole-cell current are K^+ selective:

i) The reversal potential for current through the open channel (6 ± 4 mV, Fig. 1) is close to V_K (−7 mV) and well removed from V_{Cl} (−52 mV).

ii) The reversal potential for tail currents (0 ± 3 mV) is also close to V_K (−7 mV).

iii) The activation potential of the current changes in the same way that V_K changes in different

sets of solutions. Similarly, in corn root cells the activation potential shifts from −60 to −30 mV on changing from 1 to 10 mM external K^+ (Ketchum et al., 1989). The magnitude of the whole-cell current at a given membrane potential depends on the unitary channel conductance, the probability of channel opening at that potential and on the driving force on the ion ($V_m - V_K$). Thus, a shift in activation potential results from a shift in reversal potential provided that the channel gating is not affected by the change in K^+ concentration.

iv) This current has a smaller magnitude in the presence of TEA. Stoeckel and Takeda (1989*b*) found that 25 mM TEA reversibly blocks whole-cell K^+-dependent currents in *Mimosa pudica* and 10 mM TEA almost completely blocks outward currents in corn root cells (Ketchum et al., 1989). These authors also concluded that the outward current in corn root cells is carried by K^+. The TEA result presented here is not conclusive on its own, however, as TEACl was used. This would give the appearance of channel block by shifting the reversal potential to more positive potentials if it is an anion-selective channel.

Current Magnitude and Activation

The magnitude of outward whole-cell current measured at various values of V_m (Fig. 3(*1*)) is comparable with the magnitudes of outward current reported in *Hydrodictyon* (Findlay & Coleman, 1983) and *Chara* (Beilby, 1986). For example, depolarization of the plasmalemma of *Hydrodictyon* by 160 mV in 1 mM K_o^+ gives rise to an outward current of ca. 100 mA m^{-2} (Findlay & Coleman, 1983). This is interesting given the major differences between cells of these species and corn cells. These differences include larger cell size (about 3000 times larger), an aquatic growth environment and a relatively small proportion of cell cytoplasm (less than 10%, *cf.* approximately 30% in corn cells).

The current magnitude also resembles that of outward current in depolarized protoplasts from corn root suspension cells (Ketchum et al., 1989). It is smaller than outward current in *Vicia* guard cells: for example, ca. 280 mA m^{-2} after a 140-mV depolarization in 1 mM K_o^+ (Blatt, 1988), where large ion fluxes are important for the regulation of cell volume and hence stomatal aperture (*see also* Schauf & Wilson, 1987*a*; Schroeder, Raschke & Neher, 1987; Hosoi, Iino & Shimazaki, 1988; Schroeder, 1988, 1989). Time- and voltage-dependent outward current is also activated by depolarization of the plasmalemma surrounding protoplasts from *Dionaea* trap lobe cells (Iijima & Hagiwara, 1987), *Mimosa* pulvinar cells (Stoeckel & Takeda, 1989*b*), barley aleurone cells (Bush et al., 1988) and *Asclepias* suspension cells (Schauf & Wilson, 1987*b*).

The sigmoidal time course of the outward current (Fig. 2) suggests that the K^+ channels conducting the current may be modeled as having more than one closed state (C) in series with the open state(s) (O), where transitions between those states are governed by the rate constants k_1 and k_2:

$$C_2 \overset{k_2}{\rightleftharpoons} C_1 \overset{k_1}{\rightleftharpoons} O$$

(*see* Colquhoun & Hawkes, 1981). The voltage dependence of the activation half-time (Fig. 5) suggests that k_1 and/or k_2 are greater at more positive V_m and also depend on the holding potential. Outward current with similar activation kinetics has been observed in corn root protoplasts (Ketchum et al., 1989: 2.5–1.0 sec over the range V_m = 0 to −80 mV) and in protoplasts isolated from *Samanea* (Moran, Ehrenstein & Iwasa, 1984). Outward current activates with a faster sigmoidal time course in *Hydrodictyon* (Findlay & Coleman, 1983), in *Vicia* guard cells and protoplasts (Blatt, 1987; Schroeder et al., 1987) and in *Dionaea* protoplasts (Iijima & Hagiwara, 1987).

One or Two K^+ Channel Populations?

The notch in the outward current-voltage curve around V_m = 160 mV is a feature of particular interest. A similar current-voltage notch has been reported in the current-voltage behavior of the plasmalemma of some animal cells (Hille, 1984). It has been found in snail pacemaker neurones, for example, that the notch occurs because the total outward current consists of two major components: current through Ca^{2+}-dependent K^+-selective channels and current through Ca^+-independent K^+-selective channels (Heyer & Lux, 1976). When Ca^{2+} influx is stopped the outward current is reduced in magnitude and the notch disappears as current is only conducted by Ca^{2+}-independent (outwardly rectifying) channels.

The addition of the Ca^{2+} channel blocker, verapamil, to the bath in the present work has the same two effects: the magnitude of the outward current is reduced and the notch disappears (Fig. 4). This suggests that depolarization of the corn plasmalemma activates outward current through two populations of K^+-selective channels where the operation of one of those populations is stopped in the presence of verapamil at the external face of the membrane. We suggest that verapamil has its effect

by blocking the movement of Ca^{2+} into the cytoplasm through Ca^{2+} channels hence stopping the activity of Ca^{2+}-activated K^+ channels. Ca^{2+}-independent channels then conduct that proportion of the outward current which remains after verapamil treatment. Previously outward current across the depolarized plasmalemma of small plant cells has been considered to result from only one population of K^+-selective channels.

The partial block of the outward current by Cd^{2+} reported here (at $V_m \leq 125$ mV) further suggests that Ca^{2+} is required for activation of some of the outward current. Outward K^+ currents in corn root protoplasts are also reduced in the presence of verapamil (Ketchum & Poole, 1990). The shift in the notch to less positive membrane potentials with increasing internal Ca^{2+} is another indicator of the Ca^{2+} dependence of the outward current described here. Ketchum and Poole (1990) report a similar shift in the 'voltage activation range' of the outward current in corn root protoplasts to less positive membrane potentials as the internal Ca^{2+} concentration is increased.

There is increasing evidence that Ca^{2+} is involved in the activation of outward currents in depolarized plant cells. Outward currents through Ca^{2+}-dependent K^+-selective channels have recently been reported in the plasmalemma of *Eremosphaera viridis* (Thaler et al., 1987; Förster et al., 1989), and cation channels in the plasmalemma of endosperm cells have a much higher probability of opening as pCa is increased from 3 to 8 (Stoeckel & Takeda, 1989*b*).

The only report of a higher plant plasmalemma being clamped to high positive membrane potentials ($V_m > 120$ mV) is in *Dionaea* protoplasts, where the plasmalemma was clamped to 160 mV (Iijima & Hagiwara, 1987). These currents were recorded in the absence of internal Ca^{2+} and no notch was seen. According to our findings, a notch, if present, would occur at even more positive membrane potentials under these conditions. A region of negative slope conductance has been reported in the current-voltage characteristic of *Vicia* guard cells near $V_m = 100$ mV (Blatt, 1988, 1990). Blatt (1988) shows that the outward current magnitude does rise at more positive membrane potentials in these cells and that the resulting notch is removed, and the current magnitude reduced, after metabolic block by cyanide. It is possible that this block has its effect by reducing the cytoplasmic Ca^{2+} concentration. However, Hosoi et al. (1988) found that the internal Ca^{2+} concentration did not affect outward currents in *Vicia* guard cell protoplasts.

The absence of a noticeable change in the activating current time course as the depolarized potential becomes more positive than the notch potential suggests that if two K^+ channel populations are activating at potentials less positive than the notch potential, they are doing so with very similar activation kinetics. Intuitively this would seem improbable, although Yellen (1987) comments that many biological membranes contain a variety of K^+ channels, some of which have similar voltage dependencies and kinetics. In contrast, Blatt (1988) does see a change in the time course of outward current activation at 'post-notch' potentials: activation is slower, especially in low K_o^+ (0.1 mM). Clearly the notch is a feature worthy of further investigation.

Two Types of K^+ Channels in Cell-Attached Patches

Two types of single-channel activity (40 and 125 pS conductance) were activated by depolarization of the membrane and rarely by hyperpolarization of the membrane (Fig. 9). It is possible that these conduct the outward whole-cell current.

The reversal potential for the 40 pS channels shown in Fig. 9*A* indicates that these are K^+ selective, where $P_{Cl}/P_K = 0.10 \pm 0.05$ if the cytoplasmic K^+ concentration, $[K^+]_c$ is around 75 mM. This is within the reported range of values: 60–90 mM for $[K^+]_c$ in maize cells (Hajibagheri et al., 1988). Potassium-selective channels of a similar amplitude have been identified in the plasmalemma surrounding protoplasts from barley (35 pS, $K^+_{pip} = 100$ mM; Bush et al., 1988), and *Vicia* (40 pS, $K^+_{pip} = 100$ mM; Schauf & Wilson, 1987*a*). Lower conductance K^+-selective channels have also been identified in *Vicia* (20 pS, 100 mM symmetric K^+; Schroeder, Hedrich & Fernandez (1984)); the activity of this channel population is Ca^{2+} dependent (Schroeder & Hagiwara, 1989). Calcium-activated cation-selective channels with 40 pS conductance have recently been identified in endosperm cells (Stoeckel & Takeda, 1989*a*).

The reversal potential for the 125 pS channels shown in Fig. 9*B* indicates that these are also K^+-selective channels. Channels of this magnitude resemble large-conductance K^+-selective channels (maxi-K channels) in the plasmalemma of animal cells. The contribution of maxi-K channels in the animal plasmalemma to outward currents is well documented (Rudy, 1988). Animal maxi-K channels are activated by Ca^{2+} at the cytoplasmic face (Blatz & Magleby, 1984). The notch in the outward current-voltage curve of the snail neurone described above (Heyer & Lux, 1976) is because of the inhibition of Ca^{2+}-dependent maxi-K channels.

In order to show that these single-channel popu-

lations mediate the outward current it is necessary to show that their combined activation and inactivation kinetics resemble the kinetics of the whole-cell current. At present the only available data of this kind are for the 40 pS channels. A group of these channels activates with a sigmoidal time course (Fig. 10). The activation half-time of the summed single-channel activation records is faster than that of whole-cell currents. This suggests that either these channels are not solely responsible for whole-cell outward current (as in a two-channel population system) or that perfusing the cytoplasm has altered their kinetics.

The activation half-time derived by summation of summed single-channel currents (summed activation) closely resembles that for summed K^+-selective channels in the plasmalemma of *Vicia* cells (Schroeder et al., 1987). As mentioned above, whole-cell currents activate more quickly in *Vicia* than in corn. The summed activation rate is slower than the summed activation for low conductance cation-selective channels in endosperm protoplasts (Stoeckel & Takeda, 1989*a*) where the activation half-time was ca. 500 msec. It will be interesting to see how different types of plant cells use different combinations of K^+ channels with similar kinetics and conductances to mediate outward whole-cell current.

Fast-Activating Inward Current

The faster activation of the inward whole-cell current observed on hyperpolarization of the protoplast membrane (Figs. 3(*2*) and 6) indicates that this current is conducted through different channels from those responsible for the whole-cell current described above. The observation of inward current in only 60% of protoplasts suggests that such current activation depends on something other than just hyperpolarization of the membrane. Inward current with a similar time- and voltage-dependence has been observed in *Vicia* guard cell protoplasts (Schroeder et al., 1987) and in barley aleurone protoplasts (Bush et al., 1988).

The tendency for the current to saturate and in some cases decrease when V_m is more negative than -175 mV is a feature which has not previously been reported in a higher plant cell. It may be due to blocking by external Ca^{2+} at extreme negative potentials. A similar feature has been described in *Chara* (Beilby, 1985).

The activation potential for fast inward current varies with, but does not equal, V_K. This may suggest that the current is mediated by K^+ ions, which would be consistent with the observation of similar inward current in corn root protoplasts (Ketchum et al., 1989), even in the absence of external chloride. However, the results presented here show that in corn shoot protoplasts the inward current can be conducted by both K^+ and Cl^- as the experimentally derived instantaneous reversal potential (ca. -78 mV; Fig. 7) lies between V_K (-116 mV) and V_{Cl} (-17 mV). The selectivity ratio calculated from these values using the generalized null potential equation (Barry & Gage, 1984) is $P_{Cl}/P_K = 0.43 \pm 0.15$. The observation that TEA can block the current and not affect the reversal potential suggests that these ions cross via one channel population. Alternatively, the selectivity ratio indicates the relative contributions of anion- and cation-selective channel populations to the whole-cell inward current. Both anion and cation currents are activated by hyperpolarization of the plasmalemma surrounding *Vicia* guard cell protoplasts, for example (Schroeder & Hagiwara, 1990).

It is possible that the fast inward current is mediated by the 20 pS channel population as these channels were only activated at negative membrane potentials. As they were never seen to open at positive membrane potentials the current reversal potential can only be estimated from an extrapolation of the data (Fig. 9*C*). Extrapolation gives a reversal potential between -60 mV in which case the channel would be Cl^- selective and 0 mV in which case the channel would be K^+ selective. A value within this range would suggest permeability to both K^+ and Cl^-. It is noted that reversal potential values obtained by extrapolation are not conclusive.

Currents After Extreme Hyperpolarization

Whole-cell currents at extreme membrane potentials have not yet been characterized in higher plant cells. The variable and sometimes suddenly increasing current seen in this work suggests that many channels may be activated by extreme hyperpolarization. This area is worthy of further investigation. Currents at extreme negative membrane potentials have been well characterized in charophyte cells. In *Chara* and *Nitella* cells, large, sudden inward currents flow across the plasmalemma at sufficiently hyperpolarizing potentials. This 'punchthrough' phenomenon was originally attributed to the current-voltage characteristic of a double-fixed charge system in the membrane. It has since been shown to involve Cl^- channels (Coster, 1965; Ohkawa & Kishimoto, 1977; Tyerman, Findlay & Paterson, 1986). Chloride channels in the plasmalemma of *Chara* have been identified by Coleman (1986).

Slow-Activating Inward Current

Inward whole-cell currents which activate slowly (Fig. 8) and with such a distinct, U-shaped voltage dependence (Fig. 3(*4*)) have not previously been reported in small plant cells. It is not yet clear what ions are responsible. The fact that the channels responsible for this current pass inward current simultaneously with the outward K^+-dependent current (Fig. 8) rules out the possibility that they are K^+-selective channels. The possibility that these are Ca^{2+}- or Ca^{2+}-dependent anion currents has been considered.

The shape of the steady-state current-voltage curve (Fig. 3(*4*)) resembles that of current-voltage curves for some types of calcium channels opening in the plasmalemma of animal cells (Carbone & Lux, 1976; Hille, 1984). The large, slowly inactivating tail current (Fig. 8) also resembles (in shape) current during the closure of Ca^{2+} channels in animal cells (Eckert & Ewald, 1983). Both activation and inactivation occur more slowly in the plant membrane, however. The presence of L-type Ca^{2+} channels in this membrane is already implied by the effect of verapamil on the outward current here and in corn root cells, where nifedipine also blocks the outward current (Ketchum & Poole, 1990). Nifedipine is another organic L-type Ca^{2+} channel blocker (Fox et al., 1987). Further, a protein which binds verapamil has recently been partially purified from maize coleoptiles (Harvey, 1989). Verapamil and some related organic compounds have been shown to block $^{45}Ca^{2+}$ uptake into carrot protoplasts, apparently by blocking calcium channels (Graziana et al., 1988). It is possible that this whole-cell inward current is mediated by calcium channels in the corn plasmalemma. A similar feature has been reported in perfused charophyte cells (Lunevskii, Zherelova & Berestovskii, 1983; Shiina & Tazawa, 1987). These currents were Mg^{2+} and ATP-dependent and allowed Ca^{2+} into the cytoplasm. They were inhibited by nifedipine. Mg^{2+} and ATP were present when the currents reported here were seen.

Keller, Hedrich and Raschke (1989) have recently reported a whole-cell current in *Vicia* protoplasts which occurs over a similar range of membrane potentials with a current maximum at $V_m = -40$ mV. They do not show the time course for this current, however, which they attribute to anion-selective channels. Anion-dependent whole-cell current in *Vicia* in this voltage range is further described by Schroeder and Hagiwara (1990): current is Ca^{2+} dependent and activates more quickly than the current described here.

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