J. Membrane Biol. 142, 381–393 (1994)

The Journal of
Membrane
Biology

Ion Channels in the Plasma Membrane of Protoplasts from the Halophytic Angiosperm *Zostera muelleri*

A. Garrill, S.D. Tyerman, G.P. Findlay
School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, South Australia 5001

Received: 23 May 1994/Revised: 19 August 1994

Abstract. Patch clamp studies show that there may be as many as seven different channel types in the plasma membrane of protoplasts derived from young leaves of the halophytic angiosperm *Zostera muelleri.* In whole-cell preparations, both outward and inward rectifying currents that activate in a time- and voltage-dependent manner are observed as the membrane is either depolarized or hyperpolarized. Current voltage plots of the tail currents indicate that both currents are carried by K^+. The channels responsible for the outward currents have a unit conductance of approximately 70 pS and are five times more permeable to K^+ than to Na^+. In outside-out patches we have identified a stretch-activated channel with a conductance of 100 pS and a channel that inwardly rectifies with a conductance of 6 pS. The reversal potentials of these channels indicate a significant permeability to K^+. In addition, the plasma membrane contains a much larger K^+ channel with a conductance of 300 pS. Single channel recordings also indicate the existence of two Cl^- channels, with conductances of 20 and 80 pS with distinct substates. The membrane potential difference of perfused protoplasts showed rapid action potentials of up to 50 mV from the resting level. The frequency of these action potentials increased as the external osmolarity was decreased. The action potentials disappeared with the addition of Gd^{3+}, an effect that is reversible upon washout.

Key words: Halophyte — Seagrass — Patch clamp — Ion channel — Turgor regulation — Action potential

Introduction

There are a growing number of studies of ion channels in the plasma membranes of plant cells (*reviewed by* Hedrich & Schroeder, 1989; Tester, 1990; Tyerman, 1992), all of which have been done on protoplasts derived from the leaves and roots of glycophytic plants with little attention being paid to halophytic species. The plasma membrane of halophyte cells could contain ion channels quite different to those of glycophytes. Large fluxes occur during turgor regulation and in general the cells accumulate, in addition to K^+, Na^+ and Cl^- in the vacuole as osmotica.

The seagrasses are a group of halophytic angiosperms with specific physiological systems enabling them to survive in marine environments, particularly estuaries (Tyerman, 1989). In estuarine environments the principal problem facing the plants is not lack of water but high salt concentrations and, in addition, large changes in salinity and therefore external osmolarity (π_o).

Seagrass leaf cells have been shown to regulate turgor and also to exchange K^+, Na^+ and Cl^- with the surrounding seawater medium (Tyerman, 1989). In *Zostera* and *Posidonia* an osmotic gradient occurs along the strap-like leaves that are enclosed within the leaf-sheath. The leaf cells at the base of the sheath are exposed to only 30–50% seawater and accumulate K^+, Na^+ and Cl^- as the cells expand. As the cells grow, they move into regions of increasing osmotic pressure but nonetheless maintain turgor pressure (Tyerman et al., 1984; Tyerman, 1989). We have used the patch clamp technique to study ion channels operating as an ensemble in the whole plasma membrane or singly in isolated membrane patches in these cells, and the results are described in this paper.

Materials and Methods

Zostera muelleri was collected about 1 km upstream from the mouth of the Onkaparinga river at Port Noarlunga, South Australia. Plants were

Correspondence to: A. Garrill

then kept for no more than two weeks with their roots anchored in an aerated aquarium tank filled with water collected from the river.

For protoplast isolation, we used a modification of the methods of Arai et al. (1991). One-centimeter lengths of tissue from about 1 cm below the sheath of the innermost leaves in the base of the plant were used. About 0.01 g of such tissue was then finely chopped in a small volume of solution A (mM: 50 sodium acetate, 10 ascorbic acid, 1 $CaCl_2$, 700 sorbitol, pH 5.5) containing 0.8% cellulase (Onozuka RS; Yakult Honsha, Tokyo) and 0.08% pectolyase (Sigma Chemical). The sections were transferred to a 50 ml Ehrlenmeyer flask to which was added 10 ml of the above mixture. After incubation for 2 hr and 15 min in a 30°C shaking water bath in the dark, the digest was filtered through fine muslin and centrifuged at 300 × *g* for 5 min. The pellet was resuspended in 5 ml of 50 mM sodium acetate, 10 mM ascorbic acid, 1 mM $CaCl_2 \cdot 2H_2O$, 700 mM sucrose, pH 5.5. On top of this was layered 2 ml of 50 mM sodium acetate, 1 mM $CaCl_2 \cdot 2H_2O$, 140 mM sorbitol, 560 mM sucrose, pH 5.5 followed by 1 ml of solution A. All solutions were chilled and filtered (0.2 μm Millipore) before use. After centrifugation for 5 min at 200 × *g*, protoplasts were collected at the interface between the uppermost layers. The protoplasts were then washed by mixing with 5 ml solution A and centrifuged for 5 min at 60 × *g*. The final pellet was resuspended in 0.5 ml solution A.

Protoplasts used for patch clamping were derived from epidermal tissue. These could be readily distinguished from mesophyll tissue-derived protoplasts in that they were much smaller and contained greater numbers of chloroplasts (Kuo & McComb, 1989). These protoplasts stuck readily to the glass bottom of the experimental chamber, usually a good indicator that patch pipettes will form high resistance seals with the membrane.

Patch Clamping

Patch pipettes were constructed from borosilicate glass blanks (Clark Electromedical, Reading, UK) using a double-pull method, coated with Sylgard (Dow Corning) and fire-polished to a bubble number of 5 (measured with a 10 ml syringe (Corey & Stevens, 1983)) for whole cell and 4–4.5 for isolated patch studies immediately before use.

Experiments were carried out using the whole-cell and outside-out patch recording modes of the patch clamp technique (Hamill et al., 1981). Whole-cell preparations were obtained by forming a gigaseal (resistance >5 GΩ) in the cell-attached mode and then applying a short burst of extra suction to rupture the membrane. A substantial increase in capacitance indicated the attainment of the whole-cell mode. In the experiments described we have used the standard convention that outward current is positive. For calculating the current density (mA m^{-2}) for whole-cell preparations, we have assumed that the protoplasts are spherical. For outside-out patches, the actual current flow through the patch is given.

The voltage across the patch was controlled and current measured using either a List EPC7 or a Dagan 3900A patch clamp amplifier. Cell-attached, whole-cell and outside-out recording modes of the patch clamp technique were used (Hamill et al., 1981). Series resistance was compensated to about 50% for both amplifiers and capacitance compensation was used with the List but not with the Dagan where capacitance transients were only subtracted if data were required for kinetic analysis. Current and voltage data were initiated and saved directly to computer using 12-bit D/A or A/D converters. For some experiments, a STROBES acquisition unit was used (Strobes Engineering, New Zealand) with pulse commands generated by an analog pulse generator. Single channel data were recorded using a Sony PCM 701 pulse code modulator and stored on VHS video tape. Prior to analysis, data were digitized (at about 5× the filter frequency) using Adcin (Dr. J. Pumplin, Department of Physics, Michigan State University). Single channel data were analyzed using IPROC (F. Sachs, J. Neil, State University of New York at Buffalo) or TRAMP (Tyerman et al., 1992). Whole-cell data were collected and analyzed using the pCLAMP suite of programs. Whole-cell current-voltage curves were fitted with third-order polynomials.

A fast voltage ramping protocol was used to obtain the current-voltage curve for single channels in detached patches (Tyerman & Findlay, 1989). The membrane potential difference (PD) was held at a value at which the channel opened and then repeatedly scanned to a positive PD (if the patch was held at a negative PD) or a negative PD (if the patch was held at a positive PD) with 25 msec linear ramps. Subtraction of a scan in which the channel was closed from a neighboring scan (temporally) in which the channel was open gave the single channel current-voltage curve. Single channel current-voltage curves were fitted with first-order regressions.

Junction potentials were calculated and corrected for, using the program JPCalc (P.H. Barry, University of New South Wales, Sydney, Australia). All experiments were carried out at 22–25°C.

Solutions

Ionic compositions of solutions used are given in the appropriate figure legends. Using sorbitol, we osmotically balanced solutions to 700 mOsM for bath and 720 mOsM for pipette solutions, respectively. All solutions were filtered with a 0.22 μm filter immediately prior to use. The program Buffa (Dr. R.G. Ryall, Flinders Medical Centre, South Australia) was used to calculate free calcium concentrations.

Results

Time-dependent Outward Current

Whole cells displayed outward currents which activated in a time- and voltage-dependent manner (Fig. 1*a*). The outward current was present in 34% of whole cells studied (total $n = 74$). The currents began to appear as the membrane potential was pulsed positive of the reversal potential for K^+. Activation of these currents, in comparison with time-dependent outward currents in other plant species (for examples, *see* Discussion), was rapid.

The whole-cell outward currents in response to positive going steps in PD from a holding PD of 0 mV could be best fitted with an equation consisting of a constant leak and the sum of two exponentials. With 100 mM KCl in the external solution and 90 mM K glutamate and 10 mM KCl in the pipette solution, the fast time constants (mean ± SD) (n = number of cells) were 40 ± 21 msec ($n = 5$) at +40 mV, 90 ± 62 msec ($n = 6$) at +60 mV and 277 ± 128 msec ($n = 6$) at +80 mV constituting 30, 34 and 51% of the time-dependent current, respectively. Slow time constants were 1,049 ± 478 msec ($n = 5$) at +40 mV, 1,177 ± 299 msec ($n = 6$) at +60 mV and 4,578 ± 3,610 msec ($n = 6$) at +80 mV.

We obtained evidence for the specificity of these channels for K^+ using deactivating or "tail" currents.

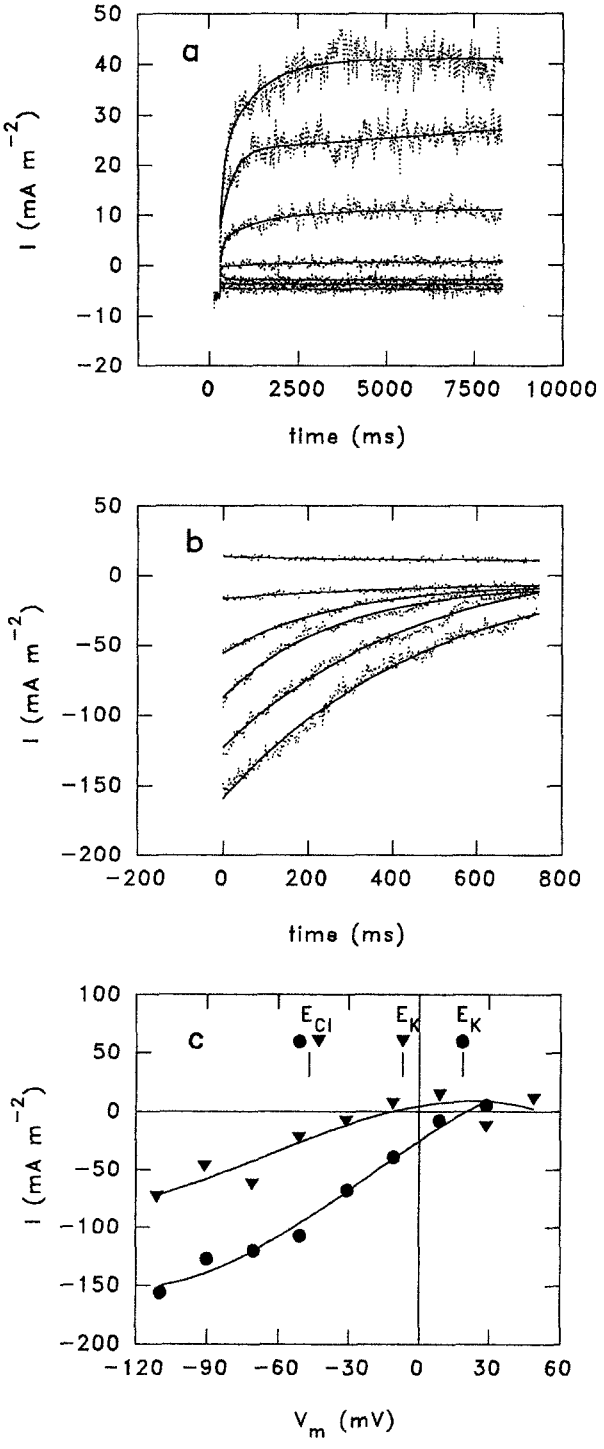

Fig. 1. Activation of the time-dependent outward current. (*a*) Current flowing across the plasma membrane of a protoplast during seven voltage clamp steps in V_m from a holding PD of −71 mV, to −53, −33, −13, +5, +25, +45 and +65 mV, respectively. Data were fitted to the sum of a constant leak and two exponential components. (*b*) The outward current deactivates as the membrane potential is pulsed from a PD at which the current is on (+47 mV) to various PD's negative to this value (+7, −13, −33, −53, −73, −93 mV) to give tail currents. Data were fitted with the sum of a constant leak and a single exponential component. (*c*) Current-voltage curve for the tail currents from two experiments using different pipette solutions. The reversal potentials close to E_K indicate that the current is carried by K^+ ions. The pipette solution contained (in mM): (*a, b, c* (▼)) K glutamate 90, KCl 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2ATP 2, EGTA 10, KOH 36, HEPES 10, sorbitol 481, pH 7.2, (*c*) (●): K glutamate 10, NaCl 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2ATP 2, EGTA 10, KOH 35, HEPES 10, sorbitol 641, pH 7.2; in the bath: (*a, b, c* (●, ▼)) KCl 100, $CaCl_2$ 10, KOH 2.5, MES 5, sorbitol 465, pH 6.

For these experiments, the voltage was held slightly negative of the PD at which the outward current activates. It was then taken to a more positive value that activated the currents. Once in the steady-state, i.e. after about 5 sec, the PD was then taken to a less positive value to deactivate the current. The deactivating currents were observed over a range of PD's and are shown in Fig. 1*b*. The data were fitted to a single exponential decay. In most experiments, some currents could be better fitted with a double exponential decay although there is no clear trend with the voltages, the bath or pipette solutions at which this discrepancy arises. We could discern no voltage dependence of the time constants (*data not shown*). A current-voltage plot of these currents revealed a reversal PD that was close to E_K (Fig. 1*c*).

The channels responsible for these currents were

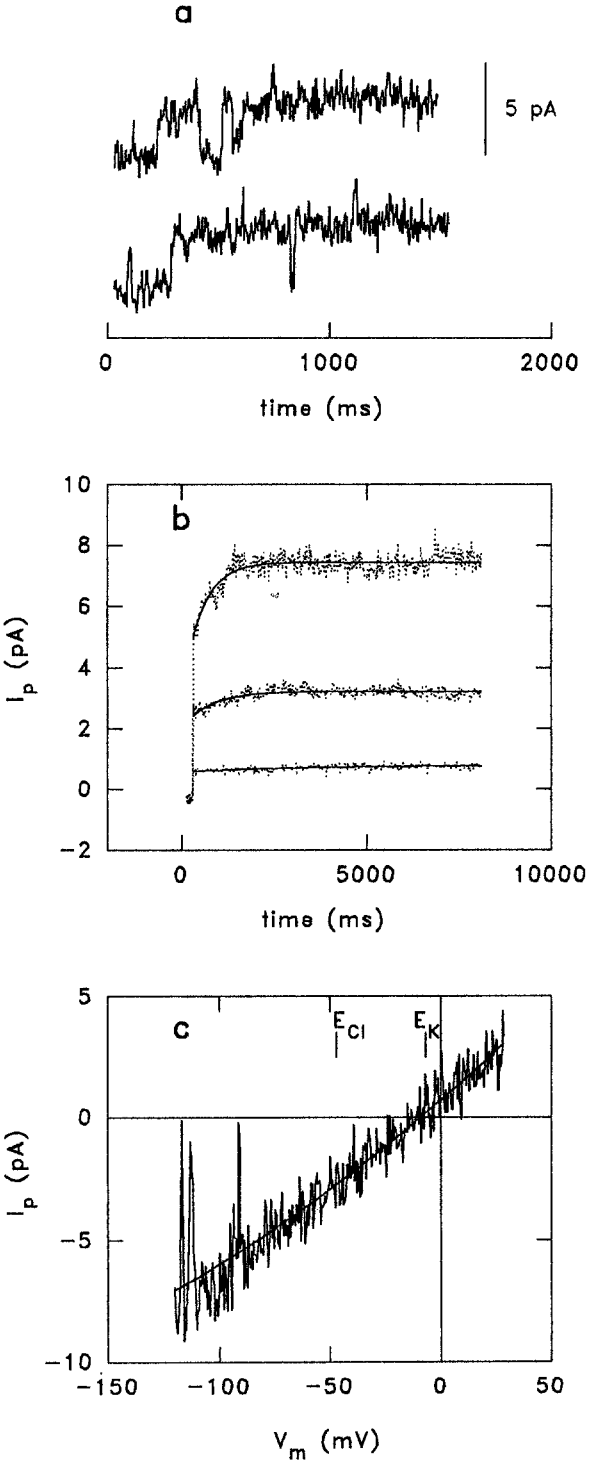

Fig. 2. K^+ outward rectifier in an outside-out patch. (*a*) Single channel records of two consecutive pulses that were used to reconstruct the whole-cell current at +49. For clarity, the zeros of the traces were displaced. (*b*) Reconstruction of the time-dependent outward current. Fifteen consecutive pulses of PD to +49 mV and 30 consecutive pulses to +29 and +9 mV, all from a holding PD of −11 mV were averaged. For comparison, *see* Fig. 1. Data were fitted to the sum of a constant leak and two exponential components. (*c*) Current-voltage curve for a single channel from data shown in *b*, obtained by rapidly scanning the PD from +29 to −119 mV. The channel had a conductance of 74 pS. The electrochemical equilibrium potentials for K^+ and Cl^- are shown by the lines. The pipette solution contained (in mM): K glutamate 90, KCl 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2ATP 2, EGTA 10, KOH 36, HEPES 10, sorbitol 481, pH 7.2; in the bath: KCl 100, $CaCl_2$ 10, KOH 2.5, MES 5, sorbitol 465, pH 6.

identified by reconstructing equivalent whole-cell currents by averaging at least 15 currents from outside-out patches in response to voltage pulses (Fig. 2*a, b*). Fitting of the currents to a constant leak conductance and sum of two exponentials gave values of 80 ± 95 msec ($n = 3$) and 933 ± 744 msec at 49 mV ($n = 3$) for the fast and slow time constants. The kinetics are therefore comparable to those obtained with whole cells although it should be noted that the fast time component only constituted 8% of the time-dependent current in the outside-out patches. Current-voltage curves obtained from fast voltage ramping protocols on these single channels in detached

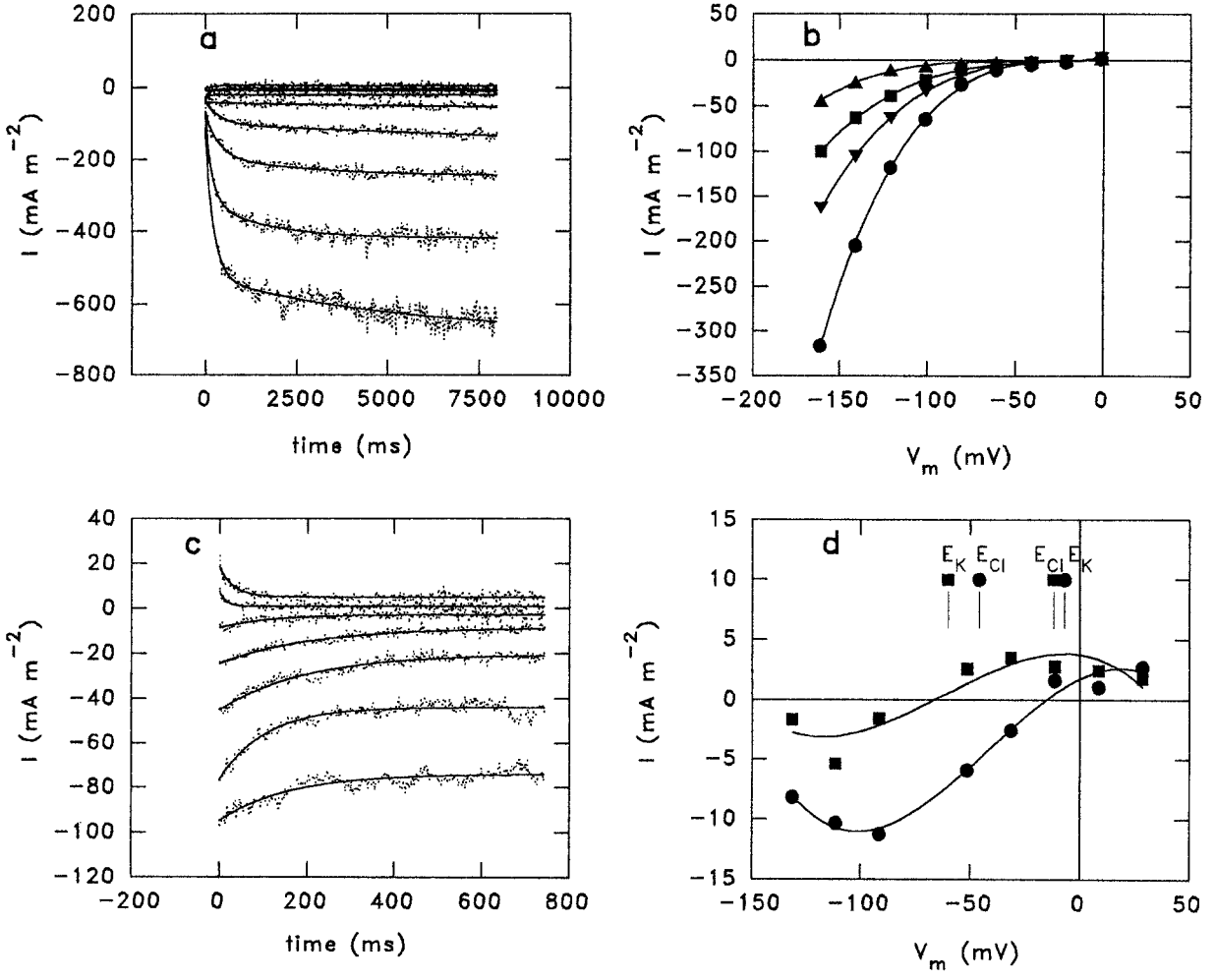

Fig. 3. Activation of the time-dependent inward current. (*a*) Current flowing across the plasma membrane of a protoplast during voltage clamp steps in V_m from a holding PD of +20 mV, to −21, −41, −61, −81, −101, −121, −141 and −161 mV, respectively. Data were fitted with the sum of a constant leak and single exponential component (*b*). Effect of changing the bath KCl concentration on the inward current. KCl concentrations were (in mM): (●) 100, (▼) 30, (■) 10, (▲) 1. (*c*) If the current is activated (at −153 mV) and then the voltage is stepped to less negative potentials (−113, −93, −73, −53, −33, +27, +47 mV) tail currents are observed. (*d*) Current-voltage curve of the deactivating current with two different bath solutions. KCl concentrations were (in mM): (●) 100, (■) 10. The reversal potential follows E_K indicating that the currents are carried by K^+. Pipette (*a–d*) and bath (*a, c*) solutions were as for Fig. 2. Bath solutions (*b, d*) were as for Fig. 2 except that the KCl concentrations were as detailed above. In addition, the sorbitol and KOH concentrations were adjusted to make the solution 700 mOsM and pH 6.

patches indicate that the channels are 70 pS K^+ channels (Fig. 2*c*).

The permeabilities of the outward rectifier to Na^+ were calculated in two outside-out patches from the change in reversal potential with changing bath solutions (Eq. 10–16 from Hille, 1984). Shifts of −39 and −41 mV changing from a bath solution containing 100 mM KCl to one containing 100 mM NaCl give a K^+/Na^+ permeability ratio of 1.0/0.2.

Time-dependent Inward Currents

The application of negative-going voltage-clamped steps in PD to the plasmalemma in whole cells caused an inward current flow which activated in a time-dependent manner (Fig. 3*a*). This current was present in 39% of the whole-cell preparations ($n = 74$). An increase in inward current with increasing external K^+ is shown in the current-voltage curves for the steady-state current (Fig. 3*b*).

It was not possible to ascertain the reversal PD of the inward currents using a current-voltage curve constructed from the steady-state currents because inward rectifying K^+ channels normally deactivate at PD's negative of E_K. The ions responsible for these whole-cell currents were therefore characterized using the deactivating "tail" currents (Fig. 3*c*). These currents, like those of the outward tail currents, showed kinetic variability and could be fitted by single or double exponential decays, with no discernible relationship between the type of fit and voltage/solutions used. In a bath solution

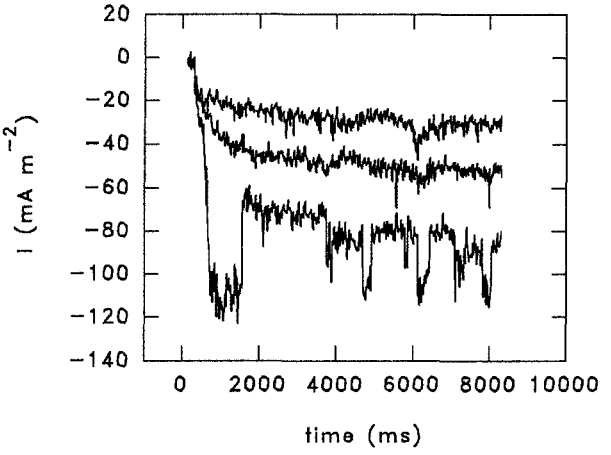

Fig. 4. Activation of the inward "spiky" current. Current flowing across the plasma membrane of a protoplast during three voltage clamp steps in V_m from a holding PD of −11, to −121, −151, and −171 mV, respectively. In this particular instance, the "spiky" current was present with the time-dependent inward current. Pipette and bath solutions were as for Fig. 2.

containing 100 KCl, the reversal potential of these "tail" currents fell between E_K and E_{Cl} ($n = 4$). As the external KCl was decreased (making E_K more, and E_{Cl} less, negative) the reversal potential was seen to follow E_K and not E_{Cl} (Fig. 3*d*); the current was thus carried by an influx of K^+. The reversal potential of the decaying inward current in 100 mM KCl solution also falls between E_K and E_{Cl} in protoplasts isolated from wheat root cortical cells (Findlay et al., 1994). This might be accounted for, once the PD has been stepped to a less negative value after the current has been activated, by a fast activating Cl^- current (Skerrett & Tyerman, 1994).

The whole-cell inward currents, like the outward currents, could be best fitted as the sum of a constant leak and two exponential components. With 100 mM KCl in the external and 90 mM K glutamate and 10 mM KCl in the pipette solution, the fast time constants (mean ± SD) (n = number of cells) were 217 ± 67 msec ($n = 5$) at −160 mV, 451 ± 332 msec ($n = 5$) at −140 mV constituting 40 and 35% of the total time-dependent current, respectively. Slow time constants of 2,098 ± 1,013 msec ($n = 5$) at −160 mV and 13,548 ± 20,629 msec ($n = 5$) at −140 mV were obtained. All of these cells were held at a PD of 0 mV before the voltage steps were applied.

Spiky Inward Current

A spiky inward current was evident in some whole-cell preparations (Fig. 4). This current appeared as large, apparent "single channel" openings, usually when the membrane PD was −140 mV but occasionally at less negative values, and was present in 38% of the whole-cell preparations ($n = 74$). The spiky current appeared to be present whether or not the time-dependent inward current was present (*cf.* Findlay et al., 1994).

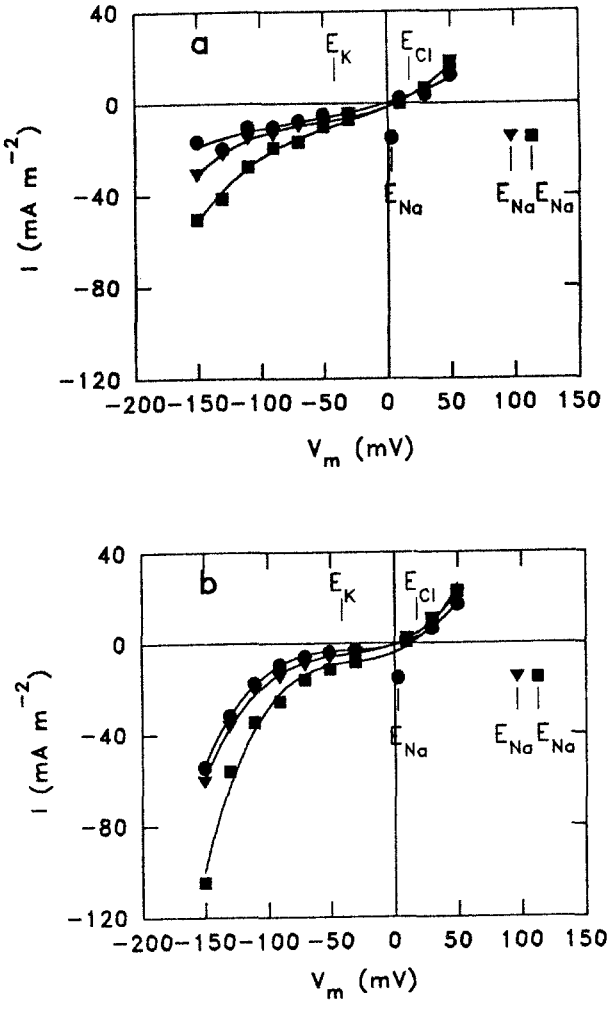

Fig. 5. Inward current in the presence of Na^+. (*a*) Initial and (*b*) steady-state current-voltage curves for the inward current in a whole-cell preparation. The membrane PD was held at −12 mV. There was an increase in both the initial and steady-state current in response to increasing external Na^+: (●) 2 mM Na^+, (▼) 93 mM Na^+, (■) 181 mM Na^+. The reversal potentials for K^+, Cl^- and Na^+ are shown by the lines. The solution in the pipette contained (in mM): NaCl 10, KCl 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2 ATP 2, EGTA 10, KOH 37.2, HEPES 10, sorbitol 632, pH 7.2; in the bath; (●) KCl 10, $CaSO_4$ 0.1, MES 5, sorbitol 671, NaOH 2.4, pH 6; (▼) KCl 10, Na_2SO_4 45, $CaSO_4$ 0.1, MES 5, sorbitol 536, NaOH 2.9, pH 6; (■) KCl 10, Na_2SO_4 90, $CaSO_4$ 0.1, MES 5, sorbitol 401, NaOH 1, pH 6.

Effect of Na^+ on Whole-Cell Inward Currents

Time-dependent inward currents were still seen in whole-cell preparations when K^+ in the bathing medium was replaced with Na^+. With an increase in external Na^+, there was an increase in both the initial (Fig. 5*a*) and steady-state (Fig. 5*b*) inward current. The reversal

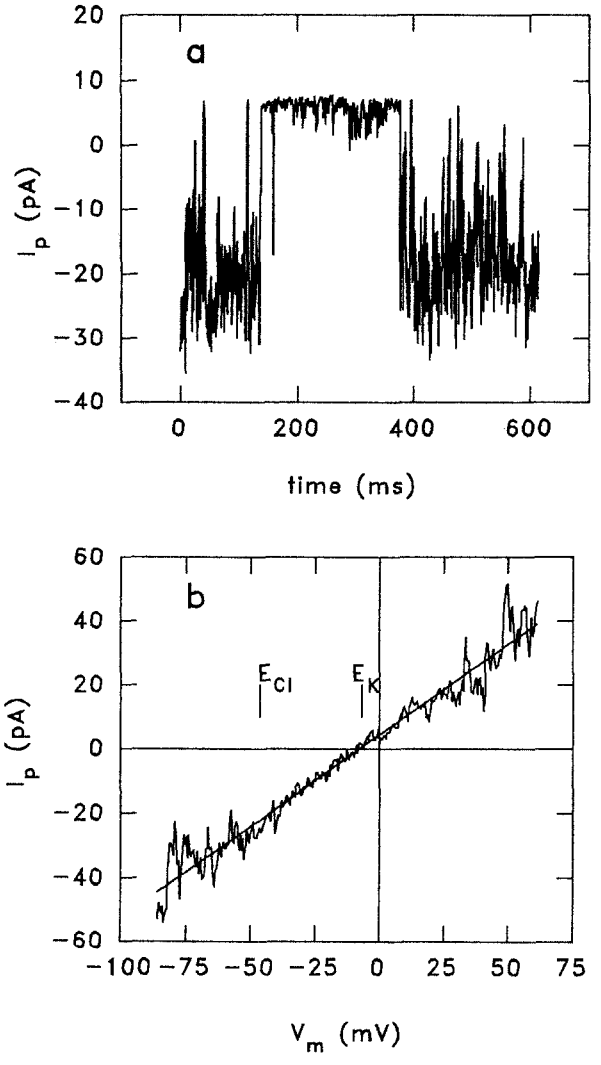

Fig. 6. Large conductance K^+ channel. (*a*) Inward current through a channel in an outside-out patch held at a PD of −84 mV. The patch also contains smaller unidentified channels. (*b*) Current-voltage curve for the type of channel shown in *a*, obtained by rapidly scanning the PD from −87 to +61 mV. The channel had a conductance of 300 pS. The electrochemical equilibrium potentials for K^+ and Cl^- are shown by the lines. The pipette and bath solutions are as for Fig. 2.

potential of these currents was close to E_{Cl}, positive of E_K and showed little change as E_{Na} was taken more positive with increasing external Na^+.

Large Conductance K^+ Channel

In detached patches we frequently observed the type of channel activity shown in Fig. 6*a*. This type of conductance may be explained by one large noisy channel displaying several levels of conductance or a number of channels that display rapid opening and closing. Despite the noisy nature, we were unable to identify any distinct substates when the data were analyzed using the TRAMP program (*cf.* large conductance Cl^- channel described below). The maximum conductance level observed was around 300 pS. Current-voltage plots from a fast voltage ramping protocol indicated that these channels are permeable to K^+ (Fig. 6*b*). The large conductance of this channel coupled with the membrane PD at which it is activated suggests that it may be responsible for the large "single channel" openings seen in whole-cell recordings.

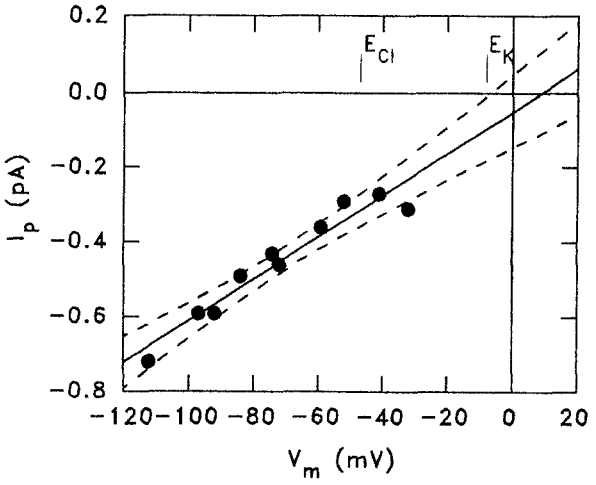

Fig. 7. Small conductance K^+ channel. (*a*) Current-voltage curve of the combined mean channel currents obtained from single channel data from three outside-out patches. The channel had a conductance of 6 pS. The data were fit with a first-order regression and are shown with 95% confidence limits. The electrochemical equilibrium potentials for K^+ and Cl^- are shown by the lines.

Small Conductance Channel

In several outside-out patches we have observed a small conductance channel of approximately 6 pS. Similar to the stretch-activated channel described above, the reversal PD is positive of E_K indicating that the channel is permeable to K^+ but may pass other cations (Fig. 7).

Stretch-activated Channel

In cell attached, inside-out and outside-out patches, we frequently observed a stretch-activated channel (Fig. 8*a*). We identified the channel as a 100 pS channel from the current-voltage plot (Fig. 8*b*). The current reverses positive of E_K which suggests a significant permeability to K^+ although the channel may be permeable to other ions such as Ca^{2+} for which the reversal PD in the experiment shown is positive of E_K. The mean current used to construct current-voltage curves was obtained using distinct single channel openings as opposed to the multiple openings evident in parts of Fig. 8*b*. Although the channels were often present without apparent perturbation of the

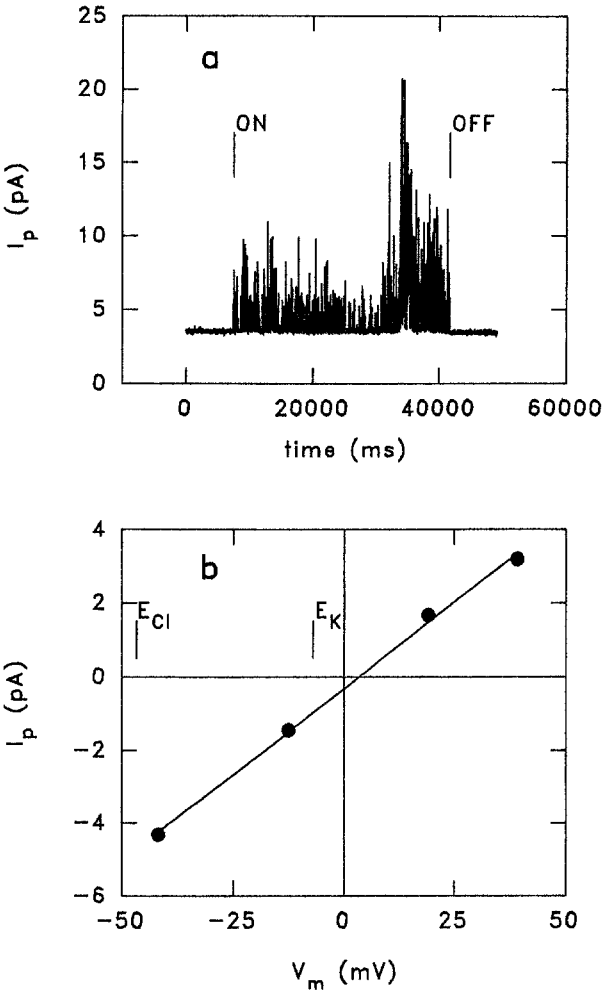

Fig. 8. Stretch-activated 100 pS K^+ channel. (*a*) Stretch activation of channels in an outside-out patch with approximately 8 KPa suction via the patch pipette. The patch was held at a PD of +58 mV. The lines represent suction on and suction off. (*b*) Current-voltage curve of the mean channel current obtained from the single channel data. The channel has a conductance of 100 pS. The electrochemical equilibrium potentials for K^+ and Cl^- are shown by the lines. Pipette and bath solutions are as for Fig. 2.

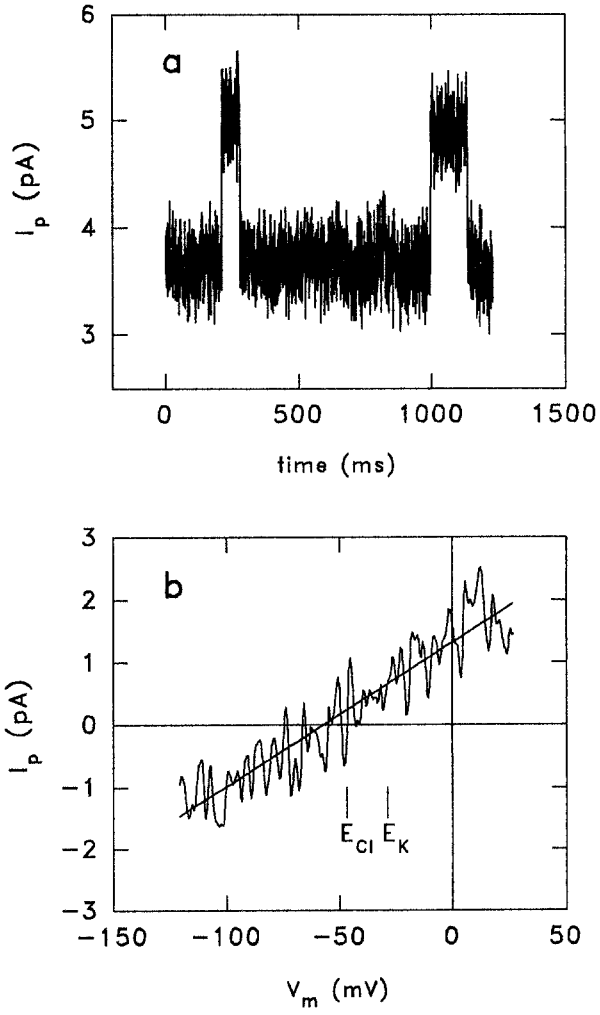

Fig. 9. Small conductance Cl^- channel. (*a*) Outward current (Cl^- influx) through the channel in an outside-out patch held at a PD of +27 mV. (*b*) Current-voltage curve for the type of channel shown in *a* obtained by rapidly scanning the PD from +27 to −123 mV. The conductance of the channel was 20 pS. The electrochemical equilibrium potentials for K^+ and Cl^- are shown by the lines. For the experiments shown, the pipette solution contained (in mM) NaCl 10, K glutamate 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2ATP 2, EGTA 10, KOH 35, HEPES 10 sorbitol 641, pH 7.2; the bath solution KCl 10, NaCl 90, $CaCl_2$ 10, KOH 2.5, MES 5, sorbitol 465, pH 6.

membrane patch, they were more evident if tension was applied to the isolated patch via suction through the patch pipette.

Chloride Channels

In outside-out patches we observed two different types of chloride channels; a small conductance channel of 20 pS (Fig. 9*a, b*) and a larger channel which appears to show distinct substates and long open times of up to 25 sec (Fig. 10*a, b*). To analyze the substates, we used the TRAMP software program (Tyerman, Findlay & Terry, 1992). With TRAMP, a plot is made of the rate of change of membrane current (*dI/dt*) at every point in a record against the amplitude of the current at that point. The plot is a phase diagram that indicates the current levels to which a channel opens and the directions and magnitude of transitions from other levels. By superimposing a limit on *dI/dt* (shown by the horizontal lines in Fig. 10*c*), it is possible to window points which represent steady amplitude levels, the contours within the window indicating the number of points at a particular level. The phase diagram in Fig. 10*c* shows four states, closed, 50, 75 and 100% with the majority of transitions occurring between the closed and 100% state and the 100 and 50/75% states. Very few transitions occur between the 50/75% states and the closed state.

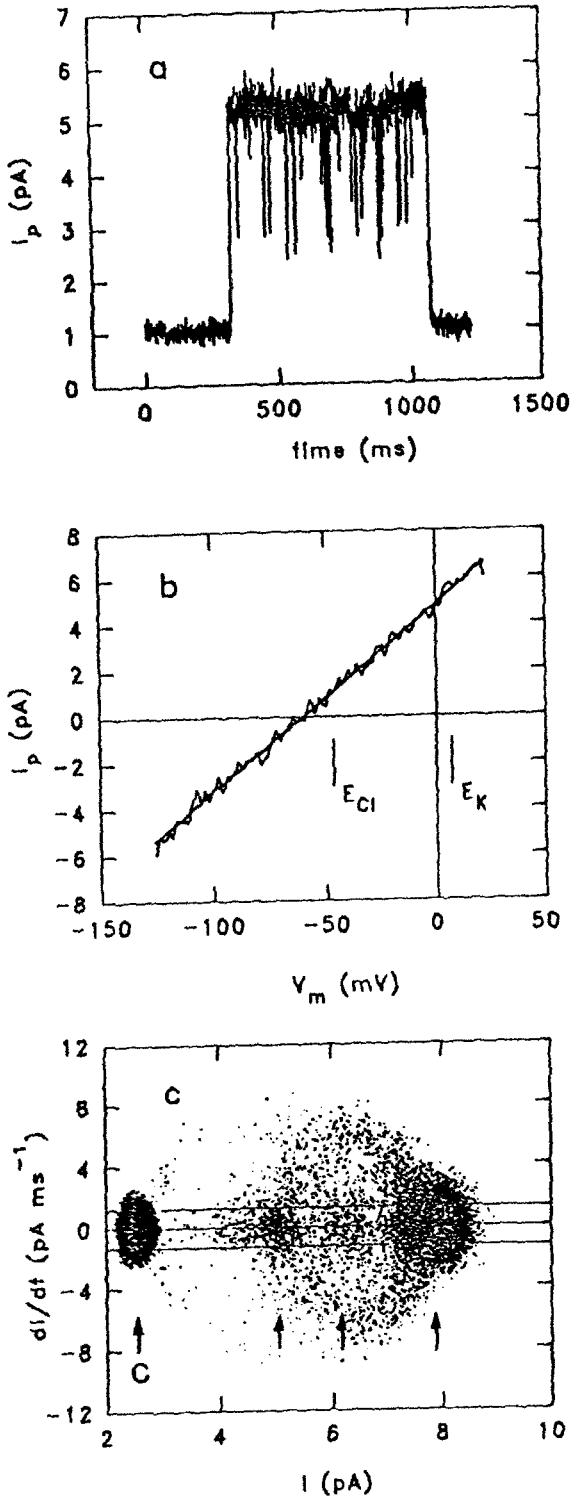

Fig. 10. Large conductance Cl^- channel. (*a*) Outward current (Cl^- *influx*) through the channel in an outside-out patch held at a PD of +52 mV. The channel showed distinct substates and open times of up to 25 sec. (*b*) Current-voltage curve for the type of channel shown in *a*, obtained by rapidly scanning the PD from +22 to −127 mV. The conductance of the main open state was 80 pS. The electrochemical equilibrium potentials for K^+ and Cl^- are shown by the lines. (*c*) Rate amplitude plot for the large conductance Cl^- channel in an outside-out patch held at +72 mV with arrows indicating the closed state (labeled *C*) and approximate 50, 75 and 100% conductance levels. The thresholds in *dI/dt* used to detect transitions are shown by the upper and lower horizontal lines. For the experiments shown, the pipette solution contained (in mM) NaCl 10, K glutamate 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2ATP 2, EGTA 10, KOH 35, HEPES 10, sorbitol 641, pH 7.2; the bath solution KCl 100, $CaCl_2$ 10, KOH 2.5, MES 5, sorbitol 465, pH 6.

Action Potentials

In the whole-cell mode of recording it was possible, if the cells were current clamped to zero current, to observe action potentials ($n = 8$ cells) (Fig. 11). The magnitude of the action potentials varied from cell to cell ranging from 50 mV (Fig. 11*a*) to approximately 2 mV (Fig. 11*c*). The action potentials usually occurred in cells which were hyperpolarized with respect to the equilibrium potentials for the dominant cation (K^+) and anion (Cl^-) present although we also observed them over a range of membrane potentials and occasionally in the

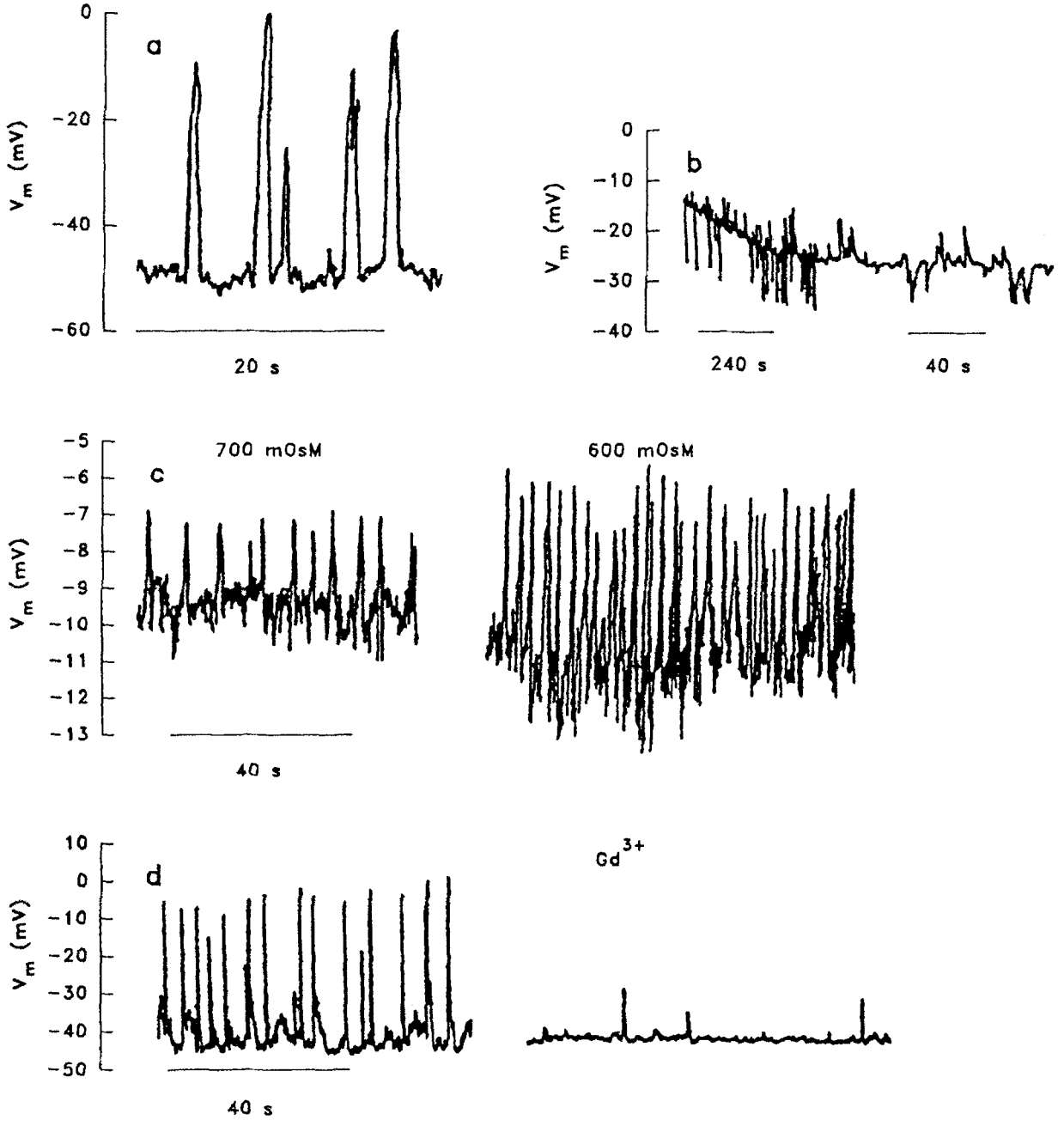

Fig. 11. Action potentials from a whole cell held in the current clamp configuration. (*a*) The action potentials varied in their magnitude but could reach 50 mV positive of the resting potential and usually occurred over a time period of 1–2 sec. (*b*) In this cell in which the membrane is hyperpolarizing, action potentials are seen in both directions. (*c*) The frequency of the action potentials increased as the osmolarity of the bathing solution was decreased from 700 to 600 mOsM. (*d*) Addition of 100 μM gadolinium reduced the frequency of the action potentials. For the experiments shown, the pipette solution contained (in mM) NaCl 10, KCl 10, $CaCl_2$ 2.3, $MgCl_2$ 2, K_2ATP 2, EGTA 10, KOH 35, HEPES 10, sorbitol 641, pH 7.2; the bath solution KCl 100, $CaCl_2$ 10, KOH 2.5, MES 5, sorbitol 465, pH 6 (700 mOsM solution); KCl 100, $CaCl_2$ 10, KOH 2.5, MES 5, sorbitol 365, pH 6 (600 mOsM solution).

opposite direction (Fig. 11*b*). In the example shown in Fig. 11*b* the cell is hyperpolarizing, the peak of the action potentials shifts with the baseline potential possibly due to the effect of other conductances in the membrane that change as the membrane hyperpolarizes. The duration of the action potentials was about 1 sec and therefore in the same range as action potentials of other higher plant species (Pickard, 1973). Since the action potentials could be observed in opposite directions at the same PD there may be two different types.

To determine the possible function of these action potentials, we looked at the effect of decreasing π_o,

thereby assessing any role in turgor regulation. The frequency and magnitude of the action potentials increased as π_o was reduced from 700 to 600 mOsM ($n = 4$ cells) (Fig. 11*c*). A subsequent increase in π_o resulted in a return to the original frequency and magnitude (*data not shown*).

Given that Ca^{2+}-activated channels are involved in the action potentials of *Chara,* we investigated the effect of the Ca^{2+} and stretch-activated channel blocker Gd^{3+}. Addition of Gd^{3+} at a concentration of 100 μM greatly reduced both the magnitude and frequency of the action potentials (Fig. 11*d*). Upon washout of the Gd^{3+}, the action potentials return ($n = 3$ cells).

Discussion

We have described the first patch clamp studies on the plasma membrane of a halophytic angiosperm. In whole-cell studies we show time-dependent outward and inward rectifying currents as well as a spiky inward current. Single channel studies on isolated patches indicate several K^+ channels with a range of conductances from 6 to 300 pS and two Cl^- channels with conductances of 20 and 80 pS. From these data, it appears that a greater variety of channels are present in this halophyte in comparison to glycophytic species we have studied such as wheat (Schachtman, Tyerman & Terry, 1991; Findlay et al., 1994; Skerrett & Tyerman, 1994).

There have been demonstrations of an outward current which activates in response to depolarizing voltage pulses in a time- and voltage-dependent manner in whole-cell preparations of almost all plant species patched to date. However, detailed analysis indicates that there may be species-specific differences in the kinetics of these conductances (Fairley-Grenot & Assman, 1993). In *Z. muelleri,* the outward current could be best fitted with a sum of a constant leak and two exponential components. This is similar to the outward current in *Vicia faba* guard cells (Fairley-Grenot & Assman, 1993) and suggests the existence of two closed states. In other species, the time-dependent outward current activates sigmoidally according to the Hodgkin and Huxley model (Hodgkin & Huxley, 1952) (e.g., in intact cells of *Hydrodictyon africanum* (Findlay & Coleman, 1983) and in protoplasts isolated from *Zea mays* suspension cells (Ketchum, Schrier & Poole, 1989; Fairley, Laver & Walker, 1991), *Zea mays* guard cells (Fairley-Grenot & Assman, 1993), tobacco cell suspension (Van Duijn, 1993) and wheat root cortex (Schachtman et al., 1991)), suggesting that in these species the channels may have more than two closed states. The deactivation of the outward current of *Zostera* may be fitted with either one or two exponential decays, indicating a channel with two open states or two populations of channels with one open state. In other plant species the deactivation of the outward current can usually be fitted with a single exponential decay (Fairley-Grenot & Assmann, 1993).

The selectivity of the outward rectifier channels correlates with those obtained for the K^+ outward rectifier in protoplasts derived from *Amaranthus* cotyledons (which is six times more permeable to K^+ than to Na^+ (Terry, Tyerman & Findlay, 1991)) but differs markedly to those obtained from wheat root cortical cells (20 times more permeable to K^+ than to Na^+ (Schachtman et al., 1991)). It appears, therefore, that the differences in the K^+/Na^+ permeabilities of the outward rectifier occur between those in leaf and root cells, with no difference in those from the leaves of glycophyte and halophyte plants. It seems likely that the K^+ outward rectifier is used to clamp the PD at negative values since cytoplasmic K^+ is likely to be of the order of 100 mM or more, while in seawater K^+ is approximately 10 mM. Under conditions where the electrogenic pump is inhibited (possibly when ATP is limited), the K^+ outward rectifier would ensure a negative PD is maintained such that some transport of nutrients will occur at the expense of previously stored K^+ (Skulachev, 1978).

Inward currents have been less frequently reported in plant cell protoplasts (guard cells of *V. faba* (Schroeder, Raschke & Neher, 1987); barley aleurone cells (Bush et al., 1988); corn root suspension cells (Ketchum et al., 1989); motor cells of *Samanea saman* (Moran & Satter, 1989); *Avena sativa* mesophyll (Kourie & Goldsmith, 1992); wheat root cortical cells (Findlay et al., 1994)). Like the outward current, the kinetics of activation of the inward current can be best fitted with two exponentials and an instantaneous leak. The data are consistent with two closed states of a channel or two populations of channel each with a single closed state. Similar results have been obtained with wheat root cortex (Findlay et al., 1994) and *Avena sativa* mesophyll (Kourie & Goldsmith, 1992) protoplasts. The deactivation of the inward rectifier could be fitted with either single or double exponential decays suggestive of two open states or two populations of channel with a single open state. Similar channels may exist in *Avena* in which deactivating currents could be best fitted by two exponential components (Kourie & Goldsmith, 1992). In contrast, the inward rectifier of wheat root cortical cells (Findlay et al., 1994) and guard cells of *Vicia* and *Zea* (Fairley-Grenot & Assman, 1993) show simpler kinetics, fitting to a single exponential which suggests a single open state. It is likely that the time-dependent inward current functions in K^+ uptake.

The presence of a time-dependent current when external K^+ was replaced by Na^+ and the increase in current with increasing external $[Na^+]$ suggests that the inward rectifying K^+ channels may also be permeable to Na^+. Given the low Na^+ permeability of *Arabidopsis* inward rectifying K^+ channels (Schachtman et al., 1992), it is possible that an additional population of Na^+ permeable

channels exists which displays different kinetics as evidenced by the fast activating currents in Fig. 5*a*. However, the data do not exclude the possibility that the inward currents are carried by Cl^- permeable channels stimulated by Na^+.

It could be argued that the 300 pS K^+ channel may be an artifact caused by the electrical breakdown of the membrane patch. Recently, Sachs and Qin (1993) reported what appears to be gated, ion-specific channel activity if a patch pipette is pushed against Sylgard. However, in the present system we see transitions of the channel from its closed to open and open to closed states, respectively, and like the spiky conductance of wheat root cortical cells (Findlay et al., 1994) the conductance is very different in magnitude and form from that described by Sachs and Qin (1993). In addition, large conductance channels have been observed in other plant cells including a 200 pS (in 100 mM KCl) Ca^{2+} channel in charophyte plasmalemma (for references, *see* Tester, 1990). In terms of conductance and the voltages at which the channel opens, there are similarities in the channels of wheat and seagrass. Interestingly, both species show the spiky inward currents at hyperpolarized PD's in whole-cell preparations. This channel could also function in the uptake of K^+ from the external medium.

Stretch-activated channels have been described in several types of plant cell (for review, *see* Garrill, Findlay & Tyerman, 1994). In studies on *V. faba* guard cell protoplasts (Cosgrove & Hedrich, 1992), three types of channel were observed, a cation channel, an anion channel and a highly selective calcium channel, all thought to function in osmoregulation. Similarly, the stretch-activated K^+ channel in seagrass could function in very rapid responses to changes in π_o, releasing K^+ as a sort of "pressure outlet" mechanism as π_o decreases. Also of interest is the possibility of the channel being permeable to other ions, suggested by the reversal PD falling positive of E_K. While we have yet to investigate their permeability to other ions, stretch-activated channels show little discrimination between cations in plant cells in general (*see* Garrill et al., 1994). The channels may therefore be a pathway for Na^+ release. In addition, the permeability of such channels to Ca^{2+} may indicate a role in the induction of signal transduction pathways necessary for responses to changing environmental parameters such as π_o. The possibility of this channel functioning in turgor regulation suggests it may play some role in the triggering of the action potentials described below.

The larger conductance Cl^- channel shows distinct substates. This seems to be a common feature of anion channels in plant plasma membranes (Coleman, 1986; Schauf & Wilson, 1987; Keller, Hedrich & Raschke, 1989; Terry et al., 1991; Tyerman, 1992). It is interesting to note the similarity in the pattern of substates for the *Zostera* large conductance Cl^- channel and that for the K^+ channel described in the tonoplast of *Chara corallina* (Tyerman, Terry & Findlay, 1992). Both channels show distinct closed, open and mid-states with the vast majority of transitions occurring between the open and mid-states. In both cases only rarely does the channel close from or open to the mid-state. It is possible that either or both of the Cl^- channels described in the present study function in turgor regulation. As described above, the marine alga *Acetabularia mediterranea* shows a correlation between turgor regulation, Cl^- efflux and action potentials (Wendler, Zimmerman & Bentrup, 1983).

Z. muelleri lives in estuarine habitats and therefore has to respond to both high levels of and appreciable changes in the levels of salinity and thus π_o. While no direct studies have been made on *Z. muelleri,* experiments with the other seagrasses *Zostera capricorna* and *Posidonia australis* (Tyerman, 1989) indicate that these species maintain a fairly constant level of turgor (P) over a range of salinities. We observed action potentials in the membrane potential of protoplasts of *Z. muelleri,* the frequency of which increases in response to a decrease in π_o. These action potentials may be involved in the changes of π_i required to maintain P at a constant value. The role of action potentials in turgor regulation is evident from work on marine species (Mummert & Gradmann, 1976; Wendler et al., 1983). In the latter study, the frequency of spontaneous action potentials increased with rising cell turgor. In addition, studies on euryhaline species suggest that increases in cytoplasmic Ca^{2+} trigger a sequence of events which gives rise to turgor regulation via the activity of K^+ and Cl^- channels in the plasmalemma and tonoplast (*reviewed by* Tazawa, Shimmen & Mimura, 1987). In contrast, however, the changes in intracellular osmolarity (π_i) necessary for turgor regulation in seagrass are brought about largely by plasmalemma fluxes of Na^+ and Cl^-, with the concentration of K^+ remaining relatively constant (Tyerman, 1989). It should be remembered, however, that the data in the present study are from protoplasts which are at zero turgor. There is clearly a need to see whether action potentials occur in intact cells although these may be difficult to measure because of the relatively small size of the epidermal cells.

We would like to thank K. Morris and D. McKenzie for technical assistance and the Australian Research Council for financial support.

References

Arai, M., Pak, J.Y., Nomura, K., Nitta, T. 1991. Seawater-resistant, non-spherical protoplasts from seagrass leaves. *Physiol. Plant* **83**:551–559

Bush, D.S., Hedrich, R., Schroeder, J.I., Jones, R.L. 1988. Channel-mediated K^+ flux in barley aleurone protoplasts. *Planta* **176**:368–377

Coleman, H.A. 1986. Chloride currents in *Chara*—A patch-clamp study. *J. Membrane Biol.* **93**:55–61

Corey, D.P., Stevens, C.F. 1983. Science and technology of patch-

recording electrodes. *In:* Single-channel recording. B. Sakmann and E. Neher, editors. pp. 53–68. Plenum, New York and London

Cosgrove, D.J., Hedrich, R. 1991. Stretch-activated chloride, potassium, and calcium channels coexisting in plasma membranes of guard cells of *Vicia faba* L. *Planta* **186:**143–153

Fairley-Grenot, K.A., Assman, S.M. 1993. Comparison of K^+-channel activation and deactivation in guard cells from a dicotyledon (*Vicia faba* L.) and a graminaceous monocotyledon (*Zea mays*). *Planta* **189:**410–419

Fairley, K.A., Laver, D.R., Walker, N.A. 1989. Single-channel and whole-cell currents across the corn plasmalemma. *J. Membrane Biol.* **108:**153–164

Findlay, G.P., Coleman, H.A. 1983. Potassium channels in the membrane of *Hydrodictyon africanum*. *J. Membrane Biol.* **75:**241–251

Findlay, G.P., Tyerman, S.D., Garrill, A., Skerrett, M. 1994. Pump and K^+ inward rectifiers in the plasmalemma of wheat root protoplasts. *J. Membrane Biol.* **139:**105–118

Garrill, A., Findlay, G.P., Tyerman, S.D. 1994. Mechanosensitive ion channels. *In:* Membranes, Specialised Functions in Plants. M. Smallwood, P. Knox and D. Bowles, editors. JAI, Greenwich (*in press*)

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hedrich, R., Schroeder, J.I. 1988. The physiology of ion channels and electrogenic pumps in higher plants. *Annu. Rev. Plant Physiol.* **40:**539–569

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer, Sunderland, MA

Hodgkin, A.L., Huxley, A.F. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117:**500–544

Keller, B.U., Hedrich, R., Raschke, K. 1989. Voltage-dependent anion channels in the plasma membrane of guard cells. *Nature* **341:**450–453

Ketchum, K.A., Shrier, A., Poole, R.J. 1989. Characterisation of potassium dependent currents in protoplasts of corn suspension cells. *Plant Physiol.* **89:**1184–1192

Kourie, J., Goldsmith, M.H.M. 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

Kuo, J., McComb, A.J. 1989. Seagrass taxonomy, structure and development. *In:* Biology of Seagrasses. A.W.D. Larkum, A.J. McComb and S.A. Shepherd, editors. pp. 6–56. Elsevier Science Publishers, Amsterdam

Moran, N., Satter, R.L. 1989. K^+ channels in the plasmalemma of motor cells of *Samanea saman*. *In:* Plant Membrane Transport: The Current Position. J. Dainty, M.I. De Michelis, E. Marre and F. Rasi Caldogno, editors. pp. 529–530. Elsevier Science Publishers, Amsterdam

Mummert, H., Gradmann, D. 1976. Voltage dependent potassium fluxes and the significance of action potentials in *Acetabularia*. *Biochim. Biophys. Acta* **443:**443–450

Pickard, B.G. 1973. Action potentials in higher plants. *Bot. Rev.* **39:**172–201

Sachs, F., Qin, F. 1993. Gated ion-selective channels observed with patch pipettes—novel properties of a gigaseal. *Biophys. J.* **65:**1101–1107

Schauf, C.L., Wilson, K.J. 1987. Properties of single K^+ and Cl^- channels in *Asclepsias tuberosa* protoplasts. *Plant Physiol.* **85:**413–418

Schachtman, D.P., Schroeder, J.I., Lucas, W.J., Anderson, J.A., Gaber, R.F. 1992. Expression of an inward-rectifying potassium channel by the *Arabidopsis KAT1* cDNA. *Science* **258:**1654–1658

Schachtman, D.P., Tyerman, S.D., Terry, B.R. 1991. The K^+/Na^+ selectivity 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, J.L., Raschke, K., Neher, E. 1987. Voltage dependence of K^+ channels in guard cell protoplasts. *Proc. Natl. Acad. Sci. USA* **84:**4108–4112

Skerrett, M., Tyerman, S.D. 1994. A channel that allows inwardly directed fluxes of anions in protoplasts derived from wheat roots. *Planta* **192:**295–305

Skulachev, V.P. 1978. Membrane-linked energy buffering as the biological function of Na^+/K^+ gradient. *FEBS Lett.* **87:**171–179

Tazawa, M., Shimmen, T., Mimura, T. 1987. Membrane control in the Characeae. *Annu. Rev. Plant Physiol.* **38:**95–117

Terry, B.R., Tyerman, S.D., Findlay, G.P. 1991. Ion channels in the plasma membrane of Amaranthus protoplasts: One cation channel and one anion channel dominate the conductance. *J. Membrane Biol.* **121:**223–236

Tester, M. 1990. Plant ion channels: whole-cell and single-channel studies. *New Phytologist* **114:**305–340

Tyerman, S.D. 1989. Solute and water relations of seagrasses. *In:* Biology of Seagrasses. A.W.D. Larkum, A.J. McComb and S.A. Shepherd, editors. pp. 729–759. Elsevier Science Publishers, Amsterdam

Tyerman, S.D. 1992. Anion channels in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43:**351–373

Tyerman, S.D., Findlay, G.P. 1989. Current-voltage curves of single Cl^- channels which coexist with two types of K^+ channel in the tonoplast of *Chara corallina*. *J. Exp. Bot.* **40:**105–117

Tyerman, S.D., Hatcher, A.I., West, R.J., Larkum, A.W.D. 1984. *Posidonia australis* growing in altered salinities: leaf growth, regulation of turgor and the development of osmotic gradients. *Aust. J. Plant Physiol.* **11:**35–47

Tyerman, S.D., Terry, B.R., Findlay, G.P. 1992. Multiple conductances in the large K^+ channel from *Chara corallina* shown by a transient analysis method. *Biophys. J.* **61:**736–749

Van Duijn, B. 1993. Hodgkin-Huxley analysis of whole-cell outward rectifying K^+ currents in protoplasts from tobacco cell suspension cultures. *J. Membrane Biol.* **132:**77–85

Wendler, S., Zimmermann, U., Bentrup, F.-P. 1983. Relationship between cell turgor pressure, electrical membrane potential and chloride efflux in *Acetabularia mediterranea*. *J. Membrane Biol.* **72:**75–84