J. Membrane Biol. 121, 223–236 (1991)
0022263191000698

The Journal of
Membrane Biology

Ion Channels in the Plasma Membrane of *Amaranthus* Protoplasts: One Cation and One Anion Channel Dominate the Conductance

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Summary. This report details preliminary findings for ion channels in the plasma membrane of protoplasts derived from the cotyledons of *Amaranthus* seedlings. The conductance properties of the membrane can be described almost entirely by the behavior of two types of ion channel observed as single channels in attached and detached patches. The first is a cation-selective outward rectifier, and the second a multistate anion-selective channel which, under physiological conditions, acts as an inward rectifier.

The cation channel has unit conductance of approx. 30 pS (symmetrical 100 K^+) and relative permeability sequence $K^+ > Na^+ \gg Cl^-$ (1 : 0.16 : 0.03); whole-cell currents activate in a time-dependent manner, and both activation and deactivation kinetics are voltage dependent. The anion channel opens for hyperpolarized membrane potentials, has a full-level conductance of approx. 200 pS and multiple subconductance states. The number of subconductances does not appear to be fixed. When activated the channel is open for long periods, though shuts if the membrane potential (V_m) is depolarized; at millimolar levels of $[Ca^{2+}]_{cyt}$ this voltage dependency disappears. Inward current attributable to the anion channel is not observed in whole-cell recordings when MgATP (2 mM) is present in the intracellular solution. By contrast the channel is active in most detached patches, whether MgATP is present or not on the cytoplasmic face of the membrane. The anion channel has a significant permeability to cations, the sequence being $NO_3^- > Cl^- > K^+ >$ Aspartate (2.04 : 1 : 0.18 to 0.09 : 0.04). The relative permeability for K^+ decreased at progressively lower conductance states. In the absence of permeant anions this channel could be mistaken for a cation inward rectifier. The anion and cation channels could serve to clamp V_m at a preferred value in the face of events which would otherwise perturb V_m.

Key Words plant ion channels · patch clamp · plasma membrane · anion channel · cation channel · multistate channel

Introduction

Protoplasts prepared from the cotyledons of *Amaranthus tricolor* seedlings are known to respond to exogenously applied cytokinins and a calcium influx is an early part of that response (Elliott & Yao, 1989). Conveniently, certain cells of *Amaranthus* cotyledons signal their reaction to cytokinins with the production of betacyanins, while others undergo considerable cell expansion (Elliott, 1983); cells can be selected which are in the process of cytokinin-induced change. Thus the *Amaranthus* system offers opportunities to investigate effects that this family of growth regulators have at the cellular level, including their effects on the plasma membrane ion channels. It is for this reason that we now add *Amaranthus* protoplasts to the list of types of plant cell whose plasma membrane ion channels have been studied by means of the patch-clamp method. This paper describes the behavior and characteristics of the channels in the plasma membrane as we find them in freshly prepared protoplasts, prior to any treatment with exogenous cytokinins.

The plasma membrane of most plant cells continues to be an awkward experimental subject for patch-clamp studies, the problem being the need to remove the cell wall, cleanly, before the membrane can be patched; as a result there are relatively few descriptions of plasma membrane channels. However, the channels that have been described can be divided between three classes. The first class of ion channels seems to be ubiquitous in plant plasma membranes; these are cation-selective channels which act as efficient outward rectifiers, allowing current to flow only when the membrane potential (V_m) becomes more positive than the equilibrium potential of the predominant monovalent cation present (K^+ in normal conditions). These channels dominate the membrane conductance at depolarized values of V_m. The outward rectifiers are characterized by high cation-to-anion permeability ratios, activation and deactivation kinetics which are voltage sensitive, and unit conductance values of between 20 to 40 pS (in symmetrical 100 mM K^+): *see* review by Tester (1990). The uniformity of the outward rectifiers found in plants is in sharp contrast to the

variety found in their animal cell counterparts, the delayed rectifiers (e.g., *see* Hille, 1984; Rudy, 1988; Brown, 1990).

Inward rectifying cation channels have also been reported for some patch-clamped plasma membranes (Schroeder, Raschke & Neher, 1987; Bush et al., 1988; Ketchum, Shrier & Poole, 1989; Moran & Satter, 1989), but they are far from being a consistent feature of the plasma membrane conductance, either because they are absent from most plant cells, or are inactive in most patch-clamp preparations. However, for guard cells, Schroeder et al. (1987) demonstrated voltage- and time-dependent inward current in whole-cell preparations, together with evidence that this current was carried by a set of channels distinct from the outward rectifiers (Schroeder, 1988). Single-channel evidence for inward rectifiers, such as it is, suggests that their unit conductances (in symmetrical K^+ solutions, at least) are similar to those for outward rectifiers (Schroeder et al., 1987; Bush et al., 1988; Moran & Satter, 1989). The principal differences between inward and outward rectifiers, as far as are known, are their reversed voltage gating and the sensitivity of inward rectifiers (in *Vicia* guard cells) to the level of free calcium on the cytoplasmic side; 1.5×10^{-6} M Ca^{2+}, or greater, will shift their activation potential to very negative values, but is reported to have no appreciable effects on the outward rectifiers (Schroeder & Hagiwara, 1989).

Anion-selective channels form the third class, although to date only Coleman (1986), Schauf and Wilson (1987) and Keller, Hedrich and Raschke (1989) have presented single-channel data for plasma membrane anion channels. A fourth paper, by Schroeder and Hagiwara (1989), indicates the presence of a large, but short-lived anion conductance in some whole-cell preparations. However, anion channels have been well documented in the plasma membranes of some of the giant algae by means of classical whole-cell electrophysiology and flux studies (e.g., Coster, 1965, 1969; Findlay & Coleman, 1983; Lunevsky et al., 1983; Kataev, Zherelova & Berestovsky, 1984; Coleman & Findlay, 1985; Tyerman, Findlay & Paterson, 1986*a*,*b*; Shiina & Tazawa, 1987; Zherelova, 1989). From these studies it could be expected that plasma membrane anion channels should activate upon hyperpolarization of V_m in a voltage- and time-dependent way (Coleman & Findlay, 1985; Tyerman et al., 1986*a*) and also be activated by elevated levels of Ca^{2+} in the cytoplasm (Kataev et al., 1984; Shiina & Tazawa, 1987; Zherelova, 1989). From the information available on animal anion channels (*see* Fox, 1987), a high unit conductance (compared to the cation channels) and the presence of well-defined substates might also be predicted for plant anion channels. With the exception of the currents reported by Keller et al. (1989), plant anion channels do indeed open only upon hyperpolarization of V_m (Coleman, 1986; Schauf & Wilson, 1987; Schroeder & Hagiwara, 1989), and are apparently activated by elevated levels of cytoplasmic Ca^{2+} (Keller et al., 1989; Schroeder & Hagiwara, 1989), but it is also clear that if these papers are reporting on the same type of channel (and two groups, Schroeder and Hagiwara (1989) and Keller et al., (1989) have described anion currents for guard cell protoplasts from *Vicia faba*), then the channel has presented a very different aspect of its character to each team of investigators: a major discrepancy exists for the values reported for unit conductances of the channels, which vary from 7 to 44 pS for *Chara australis* (Coleman, 1986), to 100 pS in *Asclepias tuberosa* (Schauf & Wilson, 1987) and 39 pS for *Vicia* (Keller et al., 1989). All single-channel recordings have shown multiple subconductance levels for these anion channels, but the values of the principal states appear to be widely different.

In this paper we describe our findings for *Amaranthus* protoplasts; first an outward rectifier-type cation channel, and second a multistate anion channel. Both channels are voltage gated under physiological conditions. The activity of the anion channel appears to be modulated by the presence of intracellular ATP and may be affected by changing levels of free cytoplasmic calcium. A brief account of some of these results has already appeared elsewhere (Boult et al., 1989).

Materials and Methods

Data for this paper are from attached patches, excised inside-out and outside-out patches (Hamill et al., 1981) of plasma membrane from freshly prepared protoplasts of *Amaranthus tricolor*. All data presented here come from recordings made at 22 to 25°C.

Preparation of Protoplasts

Our standard protoplast preparation is as follows: Approximately 0.5 g of tissue is harvested from seedlings of *A. tricolor* (Yates; var. Flying Colors) which have been grown for 3.5 days at 27°C without light. This material consists of a mixture of hypocotyls and cotyledons. The tissue is chopped finely in a small volume of solution A (500 mM sorbitol, 5 mM MES,[1] 1 mM $CaCl_2$, 0.5% wt/vol PVP,[2] 0.5% BSA,[3] pH 5.5) then exposed to partial vacuum to remove air from spaces between the cells. Suspended in 10 ml of solution A containing 0.8% cellulase (Onozuka RS; Yakult Honsha, Tokyo) and 0.08% pectolyase (Sigma Chemical), pH 5.5,

[1] MES: (2-[N-morpholino]ethane-sulfonic acid).

[2] PVP: (polyvinylpyrrolidone, average mol wt 360,000).

[3] BSA: (bovine serum albumin, fraction V).

the tissue is agitated at 30°C in the dark for 2 hr, followed by a further 1 hr after the addition of another 10 ml of solution A containing 0.8% cellulase only, at pH 5.8. The digest is filtered through fine muslin, centrifuged at 60 × *g* for 5 min and the pellet resuspended in 5 ml of ice-cold 500 mM sucrose, 5 mM MES, 1 mM $CaCl_2$, pH 6.0. On top of this is layered 2 ml of 400 mM sucrose, 100 mM sorbitol, 5 mM MES, 1 mM $CaCl_2$, pH 6.0, followed by a 1-ml layer of 500 mM sorbitol, 5 mM MES, 1 mM $CaCl_2$, pH 6.0. After 5 min centrifugation at 200 × *g* clean protoplasts are collected from the interface between the top two layers. For a last wash the protoplasts are mixed with 5 ml of the top gradient solution, spun at 60 × *g* for 5 min, and resuspended in 2 ml of the top gradient solution. Throughout the purification procedure the protoplasts are kept at 4°C; this period of low temperature treatment seems to delay synthesis of new wall material even when the cells are returned to 22 to 25°C. The protoplasts remain patchable for over 12 hr.

Patch-Clamp Procedure

Protoplasts are transferred to a flow chamber of less than 0.3 ml volume for patching; solutions can be exchanged rapidly without undue disturbance to whole-cell or cell-attached preparations. The chamber has a thin glass base to which the clean protoplasts adhere firmly. Only cells with streaming cytoplasm are selected for patching.

Patch pipettes are pulled from borosilicate glass blanks (Clark Electromedical, Reading, UK), coated with Sylgard® (Dow-Corning) and fire-polished immediately before use. Pipettes for detached patches are usually polished to between 20 and 25 MΩ (measured in 100 mM KCl); pipettes with lower resistance are used for whole-cell recordings, polished to about 7 to 12 MΩ. Gentle and prolonged suction is required to form giga-seals on the plasma membrane of these protoplasts. Solution in the flow chamber is earthed via an agar salt-bridge connected to a silver/silver chloride bath electrode. Patch and bath electrodes are filled with the same, or similar solutions to minimize offset potentials; unbalanced tip potentials, if present, are corrected for as described in Tyerman and Findlay (1989). The potential difference across a patch is expressed in terms of conventional membrane potential (V_m); the resting membrane potential (E_m), pipette potential (V_p) and tip potential (E_1, pipette with respect to bath) are included (where applicable) in calculations of V_m.

The amplifier used is a List EPC-7 (List Electronic, Darmstadt, FRG); signals from the unfiltered output are fed through a modified pulse code modulator (PCM 701ES; Sony) and stored on VHS video tape. The amplifier is connected via a 12-bit A/D converter to a control computer which can be used to monitor the pipette resistance, initiate linear (triangular) ramps of the pipette potential from any chosen holding potential, generate sequences of voltage command pulses from a given holding potential, of from 2 to 12 sec duration, and record the current and voltage data resulting from the procedures described, at sampling rates of between 10 to 2 kHz.

Solutions

All solutions are osmotically balanced with sorbitol, to 700 mOsM for most and 720 mOsM for intracellular solution. Details of particular solutions are listed in the appropriate figure legends and are labeled *Ext.* or *Cyt.* to indicate which face of the membrane is exposed to them in each situation. All solutions are filtered (0.22 μm Millipore) before use. The standard intracellular solution contains in (mM): 100 KCl, 2 $MgCl_2$, 2 EGTA,[4] 2 Na_2ATP + 2 $MgCl_2$, 490 sorbitol, 10 HEPES,[5] and 13 KOH, pH 7.2.

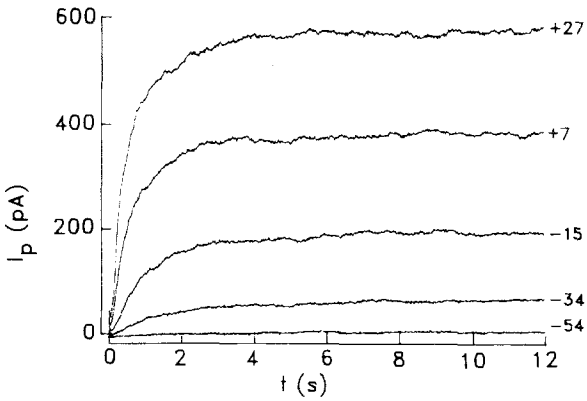

Fig. 1. Time-dependent outward currents from a whole-cell preparation. The cell membrane potential was pulsed sequentially from a common holding potential of −80 mV to progressively more positive potentials (millivolt levels shown adjacent to curves). Cyt: standard intracellular solution. Ext. (in mM): 3 KOH, 1 $CaCl_2$, 2 $MgCl_2$, and 10 MES, pH 5.7

Results

The sign of pipette currents (I_p) and pipette command potentials (V_p) have been reversed where necessary so that the usual convention applies to all measurements regardless of the patch configuration used; potentials are from the point of view of the cytoplasmic side of the membrane, and outward current is taken as positive charge moving out of the cytoplasm.

Cation Channel

Whole Cell

The characteristic feature of the whole-cell preparations is that depolarizing pulses of the clamp voltage elicit outward currents which increase to maximum values within 3 to 6 sec. A typical series of such pulses is shown in Fig. 1. A small instantaneous current at the start of each trace is assumed to be a nonspecific component of the membrane conductance and will be referred to hereafter as the "leak component," although it must be understood that

[4] EGTA: (ethyleneglycol-bis-(2-aminoethylether); N,N,N′,N′-tetraacetic acid).

[5] HEPES: (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid).

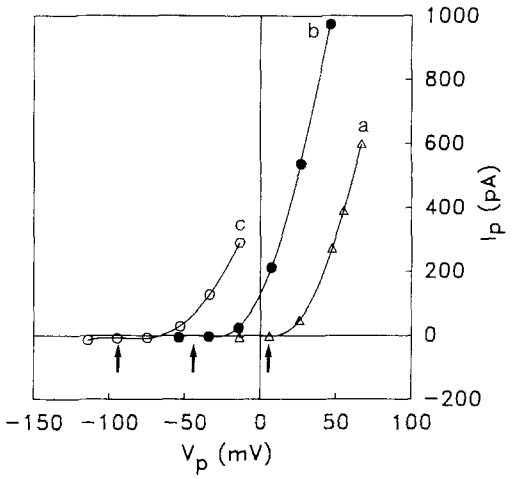

Fig. 2. Current/voltage (I/V) curves for the steady-state current (at 12 sec) obtained for a whole cell in different external concentrations of K^+. The arrows indicate the reversal potentials for the time-dependent component and were obtained from intersects with the I/V curves for the steady-state inward current (*not shown*). Cyt.: Standard intracellular solution. Ext. in (mM): (*a*, 100 K-aspartate, 10 KCl, 45 $CaCl_2$, and 5 Tris-MES, pH 6.5; (*b*, 10 KCl, 45 $CaCl_2$, and 5 Tris-MES, pH 6.5; and (*c*, 1 KCl. 49 $CaCl_2$, and 5 Tris-MES, pH 6.5. The Nernst potentials for K^+ and reversal potentials for the time-dependent outward current were respectively (*a*, −3, +6 mV (▲); (*b*, −61, −45 mV (●); (*c*, −119, −94 mV (○). $CaCl_2$ was used to maintain $[Cl^-]_{ext}$ at 100 mM for each solution. Whole-cell currents did not appear to be affected by the high concentration of extracellular Ca^{2+}

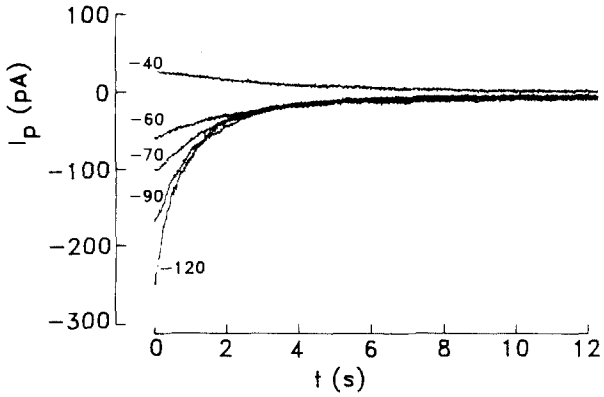

Fig. 3. Inward current as a function of time after the whole-cell membrane was pulsed from a holding potential of +10 mV to progressively more negative potentials (millivolt levels shown adjacent to curves). Cyt.: standard intracellular. Ext. (in mM): 3 KOH, 7 K-aspartate, 1 $CaCl_2$, 2 $MgCl_2$, and 10 MES, pH 5.7

this current has not yet been fully characterized. That the current elicited by these depolarizing pulses is carried by cations, in this case K^+, is demonstrated by the current-voltage (I/V) curves for a single cell bathed successively in three different concentrations of potassium ions, shown in Fig. 2. The curves in Fig. 2 describe total current for the whole cell, and therefore include the leak component. The arrows indicate the reversal potentials for the time-dependent component after the leak component has been subtracted. These potentials were determined from the points at which instantaneous I/V curves derived from sets of negative-going pulses (as in Fig. 3) intersect the steady-state I/V curves; in this way the leak component, common to each pair of curves, is subtracted to reveal the null potential (V_r) for the steady-state time-dependent component alone. The I/V curves and the reversal potentials are those expected for a potassium-selective conductance.

Average values (±SEM) of whole membrane parameters from 22 measurements are as follows: membrane capacitance, 8.24 ± 0.26 mF · m^{-2}; leak conductance, 0.006 S · m^{-2} (from voltage ramps); voltage- and time-dependent cation conductance, 50 mV positive of reversal potential, 0.67 ± 0.06 S · m^{-2}; maximum voltage- and time-dependent cation conductance (approx. 100 mV positive of reversal potential), 2.06 ± 0.21 S · m^{-2}.

When the outward rectifiers are open, at some depolarized V_m, and the clamp potential is pulsed to a more negative value, the channels deactivate and the current can be seen to decrease with time (Fig. 3). As the pulse potential is shifted further negative of the reversal potential (about −45 mV in Fig. 3), so the time constants for deactivation decrease. Similarly, activation of the outward current by depolarizing pulses is voltage dependent. The general trends of activation and deactivation time constants plotted against ($V_m - V_r$) are constant from cell to cell, and treatment to treatment, but actual values of time constant for a particular voltage pulse can show a wide range (±50% or more), even for the same cell at different stages of an experiment (Fig. 4).

Once the outward rectifiers have shut, it is unusual to see inward current of any kind (other than that carried by the leak) in whole-cell preparations (Figs. 2 and 3). It could be argued that the potentials used may not be extreme enough to activate such inward rectifying channels as might be present, although negative pulse sets are generally extended 70 to 100 mV beyond the reversal potential of the outward rectifiers. Under certain specific conditions both inward and outward currents have been seen (*see* Figs. 12–14) in the same preparation, at different voltages. Under these various conditions the inward current is usually activated at 30 to 40 mV negative of the Nernst potential for K^+ (E_K), although where identified it has always been carried via the anion channels and does not represent the activation of inward rectifying cation channels.

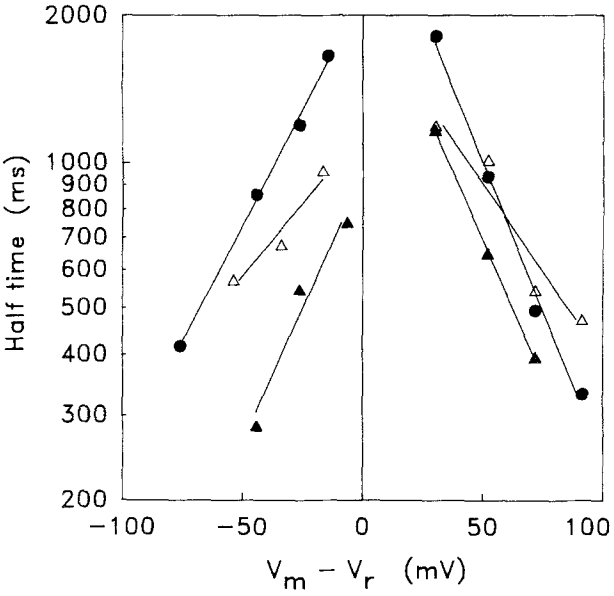

Fig. 4. Half-times for activation and deactivation of the K^+-outward current as a function of the holding potential minus the reversal potential ($V_m - V_r$). Data for three separate pulse sequences on one whole-cell preparation are shown to illustrate the variability. Each sequence of pulses was performed in the same ionic conditions, but at different times during the course of the experiment. Solutions as in Fig. 3. Times after forming whole-cell configuration: −5 min (▲); 30 min (●); 60 min (△)

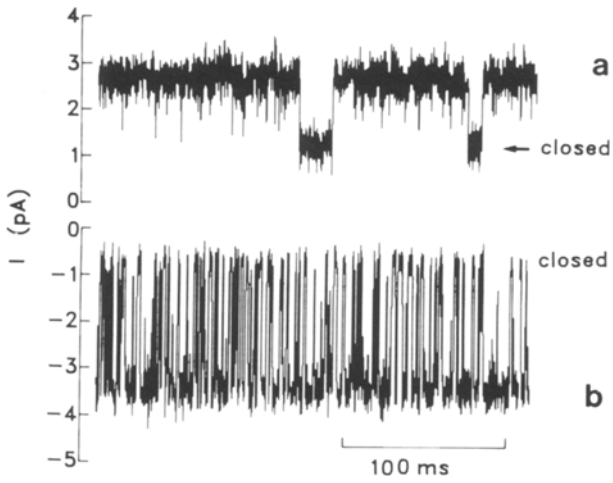

Fig. 5. Single-channel recordings of the outward rectifier K^+ channel in a single cell-attached patch for potentials of 50 mV (top trace, channel activated; $V_p = -50$ mV) and −65 mV (bottom trace, channel in the process of deactivation; $V_p = +65$ mV). Pipette solution (in mM): 100 KCl, 2 EGTA, 10 KOH, and 5 HEPES, pH 7.8. Bath: 100 KCl, 1 $CaCl_2$, 3 KOH, and 5 MES, pH 6.0. V_m is unknown, but should be in the range 0 to −20 mV in 100 mM KCl bath solution. Current traces have been inverted to conform to convention

Single-Channel Records

Activity of the outward rectifying channels is most frequently seen in attached patches and in the outside-out configuration for detached patches. The channels rarely function in inside-out detached patches. Often there are two or three of the cation channels in a single attached patch, although for clarity Fig. 5 shows current traces for a single channel. At depolarized V_m (Fig. 5*a*) the mean open times are best described by two exponential components, one with a short time constant of about 0.5 msec, the second being much longer at about 15 msec. The distribution of closed times is similar. Single-channel activity does not deactivate over periods of several minutes at depolarized PDs, in agreement with observations at the whole-cell level. When the clamp potential is pulsed negative of the reversal potential (V_r), so that the direction of current flow through the channels reverses (Fig. 5*b*), the kinetics of opening and closing change dramatically. For the burst of activity which is always observed at the beginning of a pulse (e.g., Figs. 5*b* and 6*a*) open- and closed-time distributions fit to single exponentials; time constants for both distributions depend on the potential to which the channel is pulsed. Open times are in the range of several milliseconds and shorten as V_m becomes more negative. Closed times increase from about 0.4 to 1 msec with more negative pulses. Bursts of activity last for several tens of milliseconds, then the channels turn off. Once the channels have been deactivated by negative pulses they very rarely open again, especially in attached patches, for as long as the membrane potential remains below V_r. However, the channels cannot be said to have inactivated since they can occasionally open again at potentials close to, but negative of V_r. Figure 6*a* shows the successive deactivation of three cation channels following two separate depolarizing pulses to the same potential, given to a single outside-out patch. A series of such pulses added together generate a deactivation curve (Fig. 6*b*) similar to those obtained from whole-cell recordings. Time constants calculated for single-channel curves fall within the same range of those found for whole membranes, which strengthens the argument that outward currents recorded from whole-cell preparations are due to the activity of the single channels described here.

Another Channel at Negative V_m?

Once the outward rectifiers have closed in attached patches or whole-cell preparations they do not open again until V_m is made more positive than V_r. Never-

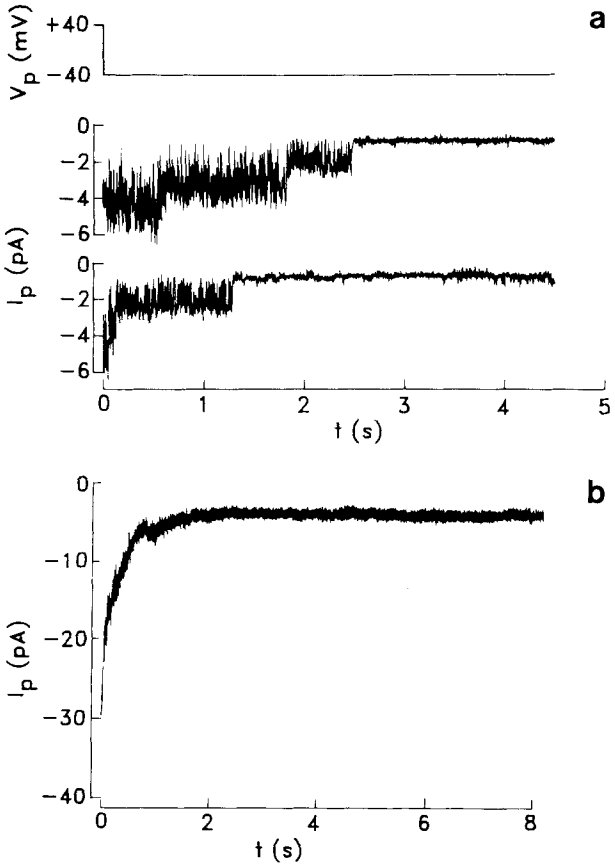

Fig. 6. Deactivation of the K^+ channel in an outside-out patch when the membrane potential was pulsed from +40 to −40 mV. (*a*) Two current *versus* time traces are shown with three channels initially on. Note the presence of small (<0.5-pA) channel openings after the K^+ channel has turned off. (*b*) Summation of 30 of the above pulses. Note that the minor fluctuations and openings have summed to a relatively flat "leak." Cyt. (in mM): 100 KCl, 0.01 $CaCl_2$, 4.8 KOH, and 5 HEPES, pH 7.8. Ext. (in mM): 100 KCl, 1 $CaCl_2$, 3.1 KOH, and 5 MES, pH 6.0

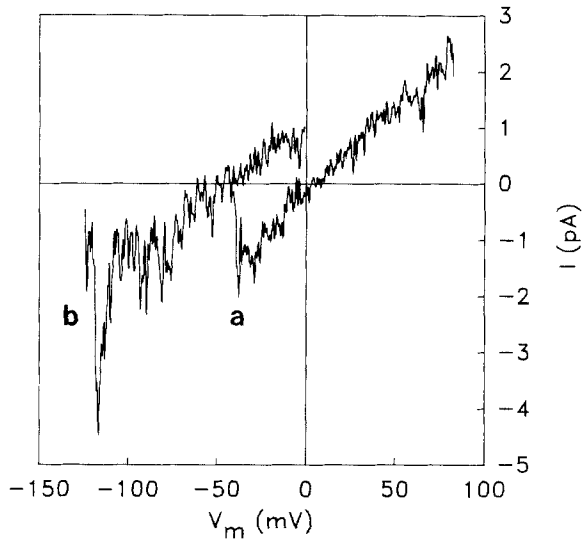

Fig. 7. Single-channel *I/V* curves from ramps on the outward rectifier in an outside-out patch. (*a*) With symmetrical K^+ concentrations (Ext. 103 mM KCl) the *I/V* curve reverses near zero (slope conductance 31 pS). (*b*) Substituting Na^+ for K^+ in the external solution (100 mM NaCl, 3 mM KCl) shifted the reversal potential to −50 mV (slope conductance 28 pS). Reversal potentials and slope conductance at the reversals were calculated from cubic polynomials fitted to the data by the least-squares method. Cyt. (in mM): 100 KCl, 0.01 $CaCl_2$, 4.8 KOH, and 5 HEPES, pH 7.8

theless, there is evidence for some continuing intermittent channel activity at negative pulse potentials, especially in detached outside-out patches (bottom trace, Fig. 6*a*). The nature of this small conductance has not yet been fully characterized. Activity of the channel is not at all obvious in the summed pulse set (Fig. 6*b*), and similarly the channel does not appear to make any significant contribution to whole-cell currents.

Characteristics of the Single Cation Channel

Single-channel *I/V* curves can be obtained by measuring the amplitude of the channel currents at different clamp voltages, but a more efficient way is to apply a short linear ramp of voltage to the channel as it opens. The same ramp is repeated when the channel is closed, and the difference between the two current traces represents the instantaneous *I/V* curve of the channel molecule in its open configuration (Fig. 7). The *I/V* curve reversing near zero in Fig. 7 is a typical *I/V* curve for the cation channel in symmetrical 100 mM K^+ and indicates a unit conductance of approximately 30 pS. Over the 120-mV range of the ramp (recorded at 10 kHz sampling, 1 kHz filter) the conductance is reasonably constant. The second *I/V* curve in Fig. 7*b* gives the reversal potential for the channel with Na^+ substituting for K^+ on the external face of the membrane. Using *I/V* curves such as those shown in Fig. 7 the permeability ratios have been calculated from the Goldman-Hodgkin-Katz equation; the channel is very selective for K^+ over Cl^- (PK^+/PCl^- was 32 and 37 for two cells, averaged for more than three *I/V* curves each), but not apparently very selective against Na^+ ions (PK^+/PNa^+ was 6.3). However, the Na^+ current entering the cell is appreciably lower than for K^+, since the channel enters a flickery mode in the presence of Na^+ (Fig. 7), which reduces the time-averaged current.

Anion Channel

The anion channel was first seen and characterized for detached inside-out patches, because it is under such conditions that it most clearly shows itself. In

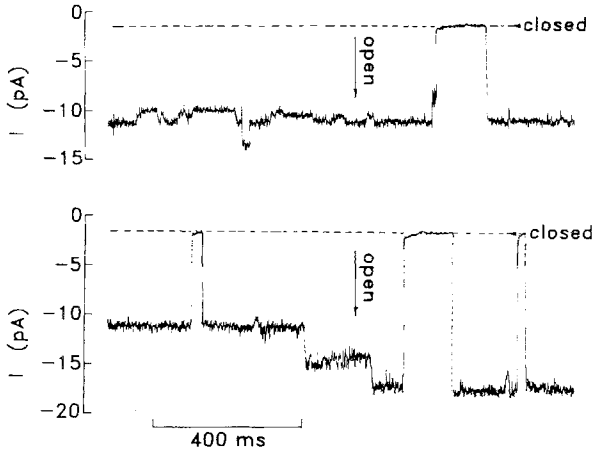

Fig. 8. Single-channel recording of the multistate anion channel in an inside-out patch with V_m at − 100 mV. Note the large transitions which occur through many of the substates. The full level corresponds to a conductance of about 165 pS. Cyt. and Ext. (in mM): 100 KCl, 1 $CaCl_2$, 3 KOH, and 5 MES, pH 6.0

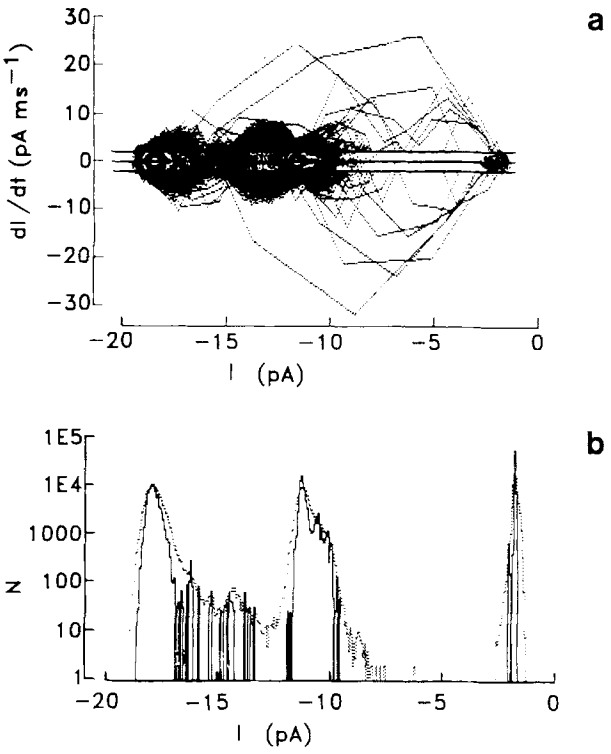

Fig. 9. Transition/amplitude analysis for part of the record shown in Fig. 8. The rate of change in current (dI/dt) is plotted against I to give a phase diagram which indicates the current levels to which the channel opens, as well as, the directions and magnitudes of the transitions from other levels. A threshold in dI/dt is set (horizontal lines) depending on the noise such that points occurring outside of this window are considered to be part of a transition to another level. The contours within the window indicate the density of points at a particular level of I. (*b*) Amplitude histograms obtained from the transition/amplitude analysis. The dotted line is the total amplitude histogram excluding transition points. The histogram is further sharpened to reveal the substates by plotting a weighted mean of the current whenever it remains within the dI/dt window longer than a certain period, in this case 3 msec (solid line)

standard whole-cell preparations (no added Ca^{2+}, 2 EGTA and 2 MgATP in the pipette solution) extreme hyperpolarizing potentials, say 150 mV negative of E_K, elicit only very brief spikes of inward current which have not yet been identified further (*data not shown*). Beyond these potentials the membrane resistance often appears to break down, although the reversibility of the effect might suggest a sudden activation of many large-conductance channels. Brief spikes of inward current have also been recorded for attached patches, their amplitudes suggesting activity of channels with conductance levels similar to those of the anion channel (*data not shown*). Interestingly, even in inside-out patches, and in appropriate conditions, the anion channel may not activate for several minutes following detachment.

A Multistate Channel

The current record shown in Fig. 8 illustrates the range of transitions made by the anion channel at high levels of cytoplasmic calcium and negative V_m. Much of the open time is spent at just a few preferred levels, with oscillations to and from sublevels that are apparently associated with them. Such activity could be the result of a collection of independently gated channels, but the large transitions that are seen make such an interpretation unlikely, as does the fact that the substates are never seen in isolation. This anion channel, able to pass from closed to fully open through all subconductance levels in one transition, or vice versa, must be considered a true multistate channel.

Identification of Subconductance Levels

Subconductance levels, and the relative amounts of time spent in each, can be extracted from total amplitude plots of the current data. It is necessary, however, to remove some of the noise from the amplitude plots to reveal the peaks more clearly. One way of doing this is described in Tyerman, Findlay and Terry (1989). Briefly the rate of change of membrane current (dI/dt) at each point in the record is plotted against the amplitude of the current (I) at that point; the result is a full description of the channel behavior in time which includes the magnitude and direction of all transitions made. Limits can then be superimposed on dI/dt to window those points which represent data sampled only at steady amplitude levels; points sampled while the current was in

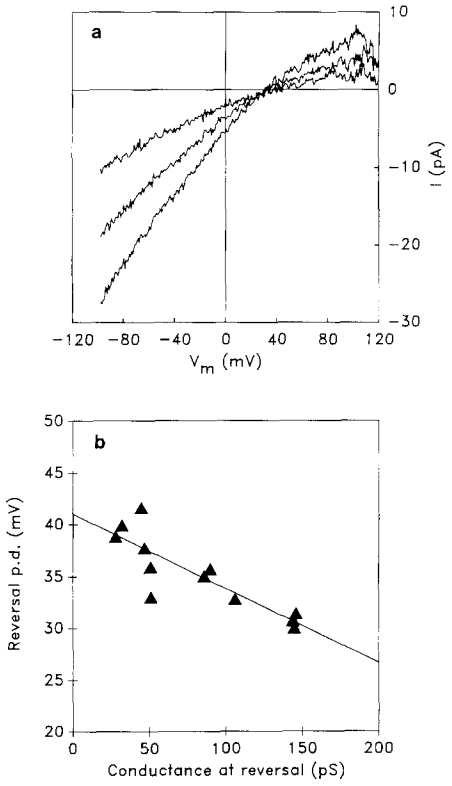

Fig. 10. (*a*) *I/V* curves of different substates of the anion channel obtained from ramps on an outside-out patch. There was about a 10-fold lower concentration of KCl in the external medium compared to the cytoplasmic side giving the positive reversal potential expected for an anion channel. The slope conductances at reversal were 47, 90 and 146 pS. (*b*) A plot of reversal potential *versus* conductance shows that the channel became more selective for Cl^- at lower conductances. Within the range of conductances the permeability ratios for Cl^-/K^+ ranged from 10.6 to 5.7. Reversal potentials and slope conductances determined as described for Fig. 7. Cyt. (in mM): 100 KCl, 2.1 $CaCl_2$, 2 EGTA (pCa 4), 13.2 KOH, and 5 HEPES, pH 7.3. Ext. (in mM): 10 KCl, 1 $CaCl_2$, 3 KOH, and 5 MES, pH 6.0

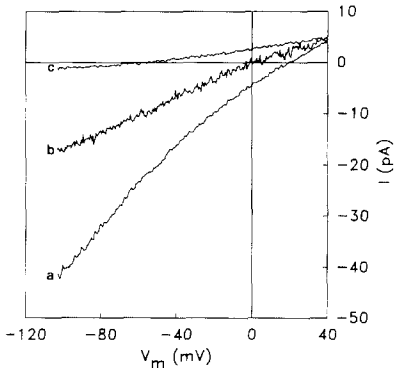

Fig. 11. Single-channel *I/V* curves for the anion channel with various ion gradients across inside-out patches. *a*. Cyt., 100 mM NO_3^-; Ext., 101 mM Cl^-. Slope conductance 209 pS at reversal PD +19 mV. *b*. Cyt., 100 mM NaCl; Ext., 100 mM KCl. Slope conductance 144 pS at reversal PD +2 mV. *c*. Cyt., 100 mM aspartate; Ext., 102 mM Cl^-. Slope conductance 36 pS at reversal PD −57 mV. Reversal potentials and slope conductances determined as described for Fig. 7

transition between levels can then be excluded from the total amplitude plot. The peaks in the amplitude plot can be made sharper by averaging those points in each sequence of the record which enters within the window of dI/dt. The minimum duration of such a sequence can be specified, to exclude very short events from the mean amplitude plot. Figure 9*a* is a transition/amplitude plot for approximately 20 sec of the record illustrated in Fig. 8. Figure 9*b* shows the total amplitude plot extracted from this diagram (minus transition points) superimposed on the mean amplitude plot. The transitions are complex, but for this part of the record were centered around a current level of 11 pA from which repeated transits were made to other levels just above and below. Transitions are possible between any of the levels, but by far the most frequent are to those levels which are closest. The closed state (at about 1.5 pA) has a small level associated with it, but a large gap, only rarely and briefly visited, lies between it and the first preferred level. This is not always the pattern; the current levels do not seem to be entirely predictable, either in number or position.

Conductance Levels and Permeability Characteristics

Voltage ramps applied to some of the various open levels of the channel unambiguously identify them as being selective for anions over cations (Fig. 10*a*). It is clearly difficult to assign a particular value for the unit conductance, and also rather meaningless. However, in symmetrical 100 mM Cl^-, the maximum conductance does not often exceed 200 pS; the value is usually around 120 to 150 pS. The reversal potentials for the different levels measured in Fig. 10*a* are not identical, and give a relative permeability of Cl^- : K^+ of between 10.6 to 5.7, the lower conductance level being somewhat more selective for Cl^- than the higher ones (Fig. 10*b*). Nitrate is approximately twice as permeable as Cl^-, but the relative aspartate permeability is low; Na^+ ions have approximately the same permeability as K^+ (all Fig. 11).

Conditions for Activation of Anion Channels

$[pCa\ 10,\ 2\ MgATP,\ pH\ 7.2]_{cyt}$. The anion channel can be activated in the presence of standard intracellular solution, but only in outside-out

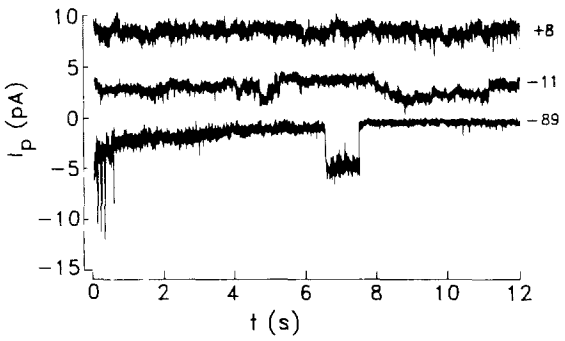

Fig. 12. Current recording on a large outside-out patch showing the outward rectifying K^- and inward rectifying anion channels. The membrane potential was pulsed from +30 mV to the millivolt values adjacent to the corresponding current traces. At −89 mV, negative of E_K, a number of K^- channels initially deactivate followed by the activation of anion channels (spikes of current and a single 1-sec opening to about 50 pS). Cyt.: Standard intracellular solution. Ext. (in mM): 10 KCl, 45 $CaCl_2$, 4.1 KOH, and 5 MES, pH 6.5

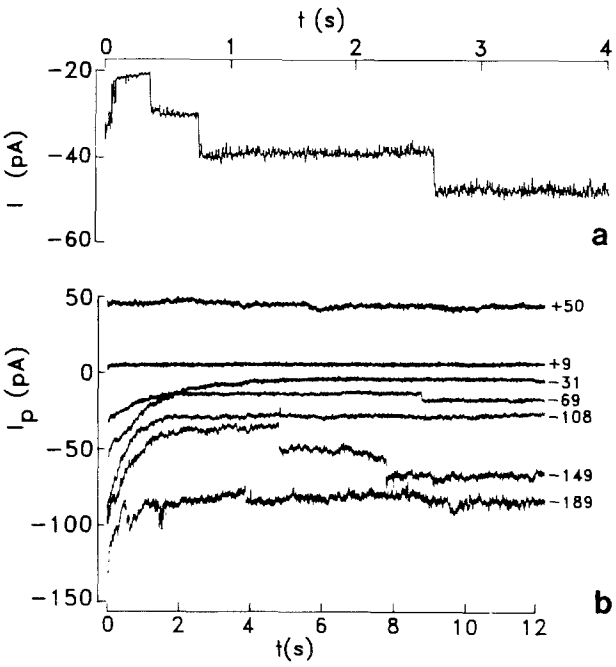

Fig. 13. (*a*) An inside-out patch where V_m was pulsed to −66 mV shows three anion channels which turned on after the K^- channels rapidly deactivated. There was no Mg-ATP in the solution bathing the cytoplasmic face of the membrane patch. The main conductance steps of the anion channels are each about 150 pS. Cyt. (in mM): 100 KCl, 0.01 $CaCl_2$, 4.6 KOH, and 5 HEPES, pH 7.8. Ext. (in mM): 100 KCl, 1 $CaCl_2$, 3 KOH, and 5 MES, pH 6.0. (*b*) Also without cytoplasmic Mg-ATP, a recording for a small whole cell pulsed to negative V_m shows current steps characteristic of the anion channel. Cyt.: as in *a*. Ext. (in mM): 100 KCl, 10 $CaCl_2$, 3 KOH, and 5 MES, pH 6.0

detached patches. Figure 12 shows an example of this. At −89 mV, large inward current spikes and (relatively) short openings to various conductance levels can be seen superimposed upon the deactivation of outward rectifiers. These currents indicate conductances of 50 to 100 pS (E_{Cl} = +2 mV).

[pCa 5, 0 MgATP, pH 7.8]$_{cyt}$. At increased levels of cytoplasmic Ca^{2+} and with no ATP present, the current record for negative voltage pulses looks very different to those shown in Fig. 3, as Fig. 13*a* and *b* shows. As the cation channels deactivate, the anion channels start to open, displaying time and voltage dependency. The anion channels quickly turn off with return of the clamp voltage to positive values (*not shown*). Such behavior is seen in both inside-out detached patches (Fig. 13*a*) and whole-cell preparations (Fig. 13*b*) if the appropriate solution is used to bathe the cytoplasmic face of the membrane. A single anion channel in these conditions can remain open for long periods, often many minutes, although during that time the anion channel will usually make frequent transitions to various conductance levels. Occasionally the anion channel will lose its voltage sensitivity, for reasons which are not as yet clear.

[pCa 10, 0 MgATP, pH 7.2]$_{cyt}$. Figure 14 is an example of a whole-cell record with a clear inward current for hyperpolarizing membrane potentials, similar to Fig. 13, but with much reduced $[Ca^{2+}]_{cyt}$. The inward current is seen superimposed on the deactivation of the outward rectifying cation channels. Detached outside-out patches in the same solutions show that this current develops in conductance steps characteristic of the anion channel (i.e., similar to the traces in Fig. 12). Given the low concentration of free Ca^{2+} in the pipette solution, it would seem that Ca^{2+} is not required for activation of the anion channel, although this presupposes perfect buffering over the entire inner face of the plasma membrane, which is perhaps unrealistic. It must be stressed that, in the presence of standard intracellular solutions which contain ATP, our whole-cell preparations show no activation of inward current in response to negative voltage steps of the amplitude shown here (*compare* Figs. 14 with 3).

From the evidence we have, it would appear that ATP can effectively inactivate the anion channel, possibly by shifting its activation potential to very negative values, or by reducing the open times (at any potential) to virtually zero. Removal of ATP from the cytoplasmic face seems to be sufficient to allow the channel to activate in conditions where it would otherwise not be seen.

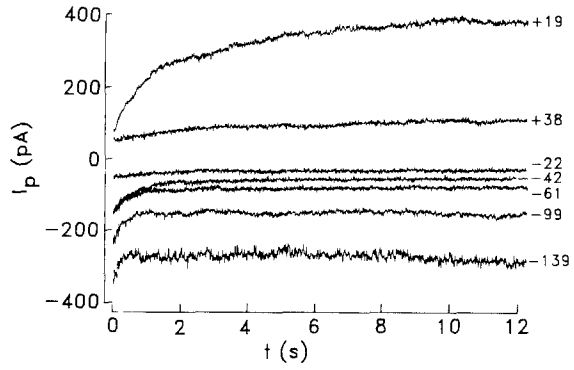

Fig. 14. A series of current recordings during voltage-clamp pulses on a whole-cell without Mg-ATP in the intracellular solution. A substantial and long-lasting inward current occurs. Outside-out patches in similar solutions also show anion channels which activate instantly at negative membrane potentials and which remain open almost continuously. Cyt. (in mM): 100 KCl, 2 $MgCl_2$, 8.4 KOH, 2 EGTA, and 10 HEPES, pH 7.2. Ext. (in mM): 100 KCl, 1 $CaCl_2$, 2 KOH, and 5 MES, pH 6.5

Discussion

Outward Currents and the Cation Channels

Depolarizing voltage pulses lead to outward current that activates in a time- and voltage-dependent way: such a description could apply to a whole cell preparation of practically any plant cell so far studied. The outward current is carried by outward rectifying cation channels which deactivate if the membrane voltage is made negative. Once deactivated they will not usually open from a closed state to conduct inward current. Activation potential is at or very close to the reversal potential of the channels, and the reversal potential is usually close to the equilibrium potential for potassium (the most permeable cation present in most circmstances). This much is true for outward rectifiers studied in plasma membranes of giant algae by standard electrophysiological means (e.g., Findlay & Coleman 1983; Bisson, 1984; Beilby, 1985) or those observed in patch-clamp preparations of plasma membranes of higher plant cells (*see* review by Tester, 1990).

Relative permeabilities of the plant plasma membrane outward rectifiers to different cations usually follows the sequence $K^+ > Rb^+ > Na^+ > Li^+ \gg Cs^+$, whatever the origin of the channels (e.g., Sokolik & Yurin, 1986; Schroeder, 1988; Tester, 1990) although Stoeckel and Takeda (1989) report a type of cation outward rectifier which apparently discriminates only poorly between the cations listed above. The *Amaranthus* cation channel has a moderately high permeability to sodium, as judged by reversal potentials, but the actual conductance to sodium is lower than suggested because the channel enters a flicker mode, characteristic of a partial block (Hille, 1984). The specific membrane conductances of our whole-cell preparations, with the outward rectifiers fully activated, is in the range 1 to 5 $S \cdot m^{-2}$. These values are similar to those found for a range of plant cells by other techniques, which encourages us to believe that the way in which we make protoplasts and form whole-cell preparations does not adversely affect the complement or activity of ion channels in the plasma membrane. Single-channel conductance of about 30 to 40 pS (in symmetrical 100 mM K^+) is in the middle of the range reported for other outward cation channels (20–65 pS, Tester, 1990).

Kinetics of Activation and Deactivation

Activation of the *Amaranthus* outward rectifiers follows a sigmoidal course similar to that seen in many other plant plasma membranes. We observe rates of activation that are faster than seen in suspension cultured corn cells (Ketchum et al., 1989; Fairley, Laver & Walker, 1991) and somewhat slower than those reported for guard cells (Schroeder et al., 1987; Schroeder, 1988). As with guard cells (Schroeder et al., 1987) we find considerable variation in the time constants for both activation and deactivation between different whole-cell preparations of *Amaranthus*. As Fig. 4 demonstrates, considerable variation is also possible for one cell when tested at different times, but in identical ionic conditions, during the course of a single experiment.

The changes in the kinetics of the cation channel upon reversal of current are striking. A similar behavior has been observed for one of the cation channels found in the membrane surrounding *Chara* drops. From Fig. 6*a* alone it is clear that a channel enters at least two different closed states during deactivation; one is a short-lived state from which the channel may reopen, the second a more permanent trap from which it cannot escape until V_m is depolarized once again. In outside-out patches the permanence of this second closed state is sometimes lost.

The Anion Channel

The *Amaranthus* anion channel has some characteristics in common with bacterial porins, channels normally found in the outer membranes of bacteria, and shed by some pathogenic strains in the course of infection (*see* Lynch et al., 1984). Concerned that

some stage or solution used in the course of experiments might lead to incorporation of an artifactual anion channel, we have tested the various solutions on a model membrane system, the membrane surrounding cytoplasmic drops from *Chara*. The normal channel complement of this system is already familiar to us (Tyerman & Findlay, 1989), and the membrane can be freshly and easily prepared by simple mechanical means. So far we have no evidence to suggest that the anion channel is anything but a natural component of the *Amaranthus* plasma membrane. Further tests are planned with artificial lipid bilayers formed on pipette tips (Hanke et al., 1984).

Inward Current and Anion Channels

Amaranthus protoplasts do not show inward current carried by cation-specific inward rectifiers in whole-cell preparations. Inward rectifiers identified for other cells have various conductances, from 4 to 8 pS for guard cells (Schroeder & Hagiwara, 1989) to about 25 to 35 pS for motor cells and aleurone cells (Bush et al., 1988; Moran & Satter, 1989); selectivity for K^+ over Na^+ is greater than measured in outward rectifiers (Bush et al., 1988; Schroeder, 1988). Cation inward rectifiers are numerous in aleurone and guard cell plasma membranes, where they apparently outnumber the outward rectifiers by at least 5 to 1 in guard cells (from conductance values reported in Schroeder and Hagiwara, 1989), but they are not active, or perhaps present, in all the cells of certain preparations in which they have been reported (Ketchum et al., 1989; Moran & Satter, 1989). Elevated $[Ca^{2+}]_{cyt}$ effectively inactivates cation inward rectifiers in guard cells (Schroeder & Hagiwara, 1989), but even at 10^{-10} M $[Ca^{2+}]_{cyt}$ negative pulses of V_m fail to activate measurable inward cation currents in whole-cell preparations of *Amaranthus* protoplasts. Small channels, not yet characterized, which have been seen in some detached patches (Fig. 6*a*) may represent an inward rectifying cation-selective conductance. However, the lack of a measurable inward cation current in whole-cell preparations suggests that either these channels have very small time-averaged conductances, or are relatively rare in *Amaranthus* plasma membrane.

Anion channels of *Amaranthus* have significant permeability to Na^+ and K^+. At low concentrations of permeant anions on either side of the membrane, the normal physiological condition, K^+ could constitute a significant proportion of the current carried by open anion channels. Guard cell anion channels conduct malate ions (Keller et al., 1989); the permeability of *Amaranthus* anion channels to malate has yet to be determined, but aspartate has a fairly low permeability. A high permeability of NO_3^- ions relative to Cl^- ions is a usual feature of anion channels (e.g., Keller et al., 1989; Tyerman & Findlay, 1989), and the *Amaranthus* channel is no exception.

Activation of Anion Channels

At physiological concentrations of $[Ca^{2+}]_{cyt}$ the *Amaranthus* anion channel, when not inactivated, behaves as an inward rectifier, opening only at hyperpolarized values of V_m. Similarly, hyperpolarizing voltage pulses open anion channels in *Asclepias* suspension cultured cells (Schauf & Wilson, 1987) and *Chara* plasma membrane (Coleman, 1986), both types of channel acting as inward rectifiers. Guard cell preparations are different; prepared one way the protoplasts can display a calcium-activated anion current which would depolarize the cell under normal conditions, but these currents are ephemeral in whole-cell preparations and do not rectify (Schroeder & Hagiwara, 1989). Prepared another way, the calcium-activated instantaneous current is not seen, but in its place is a much smaller anion inward current which is activated by depolarization of V_m (Keller et al., 1989). The open probability of this latter anion channel decreases for potentials both positive and negative of the point of maximum whole-cell anion conductance (V_m, -45 mV) (*ibid.*).

The *Amaranthus* anion channel is seen to be much less active in the presence of intracellular ATP. Excision of the membrane patch can sometimes partly release the anion channel from inactivation even in the presence of ATP, which suggests that the mechanism responsible for inactivation is not simply ATP itself, but some other agent reliant on ATP, and susceptible to disruption by other factors such as excision of the patch from the membrane. Many ion channels in animal cells have their activity modulated by phosphorylation, which is effected by various types of protein kinases (*see*, for example: Levitan, 1985; Lacerda, Rampe & Brown, 1988; Kume et al., 1989; Ribalet, Ciani & Eddlestone, 1989; Shearman, Sekiguchi & Nishizuka, 1989). ATP is usually required as the phosphorylating agent. ATP-dependent channels, found in many types of animal cells, are inactivated by elevated levels of cytoplasmic ATP (Noma, 1983; Cook & Hales, 1984; Ribalet et al., 1989; Weik & Neumcke, 1989). The mechanism probably involves an ATP-sensitive protein kinase attached to, or fixed close by the channel molecule (Ribalet et al., 1989). Wil-

liams et al. (1988) describe a Ca^{2+}-activated K^+ channel that is directly modulated by the binding of guanine nucleotides, guanosine 5′-monophosphate being the most effective activator of the channel; in this example phosphorylation of the channel or some regulatory subunit does not seem to be involved. The details of how ATP is involved in control of the activity of the *Amaranthus* anion channel have yet to be determined.

Single-Channel Data

Data for single anion channels in plants is sparse. All published reports for plasma membrane anion channels show multiple conductance levels (Coleman, 1986; Schauf & Wilson, 1987; Keller et al., 1989), though with variable values of maximum conductance and rather short open times (less than 100 msec is usual). The *Amaranthus* anion channel can be active under physiological conditions and within a physiological range of membrane potentials. When fully active it can be open for many seconds, even minutes at a time. It is a true multistate channel; discrete subconductance states can occur when the channel is open, but transitions between closed and full conductance levels are also possible without the intermediate states being visited. The actual number of conductance states does not appear to be fixed, either between separate channels in the same conditions, or even one channel at different holding potentials. Under certain conditions a single channel can show transitions between what appear to be evenly spaced subconductance levels, in a manner reminiscent of the anion and cation channels reported by Geletyuk and Kazachenko (1985; 1989). Under more physiological conditions the subconductance levels are fewer and unevenly spaced, although one possible explanation is that there are actually many evenly spaced levels but some of them are not visited. Permeability ratios of the various levels are similar but not identical; the higher conductance levels are slightly less selective for anions over cations than the lower levels. Therefore at present the evidence is not good enough to indicate whether the channel is of a single or multi-barrelled form (*see* Fox, 1987). We are looking at the possibility that this channel's subconductance levels might best be described in terms of a chaotic system. We have observed anion channels similar to the *Amaranthus* channel in all other plant plasma membranes that we have patched to date (*Stylidium, Triticum, Cucumis, Hordeum*: B.R. Terry, S.D. Tyerman, D.P. Schachtman and S. Double, *unpublished results*); such anion channels would seem to be a common feature in the plasma membranes of plant cells.

Physiological Significance of the Two Channels

Under normal physiological conditions activity of the cation channels will bring V_m negative towards the potassium equilibrium potential (E_K). Conversely, the anion channel will tend to produce a positive shift of V_m towards a point somewhere between E_K and E_{Cl}. In its inactivated state, the anion channel has a very low time-averaged conductance and will open only for values of V_m more negative than -150 mV. In this state the channels could work together as an efficient means of controlling the membrane potential, keeping it near some preferred value. This is important since the many electrogenic transport processes, primary and secondary, constantly perturb V_m, yet require constant electrochemical fields for their efficient operation. It is possible that such a voltage-stat arrangement might also be pH sensitive, serving to control the proton-motive force across the plasma membrane at different external pH. Such a mechanism has been proposed for the plasma membrane of *Chara inflata* (Tyerman et al., 1986*b*).

In its activated state the anion channel opens for relatively moderate negative potentials, at or positive to E_K. The large conductance and very long open times mean that, in conjunction with the outward rectifiers, considerable solute efflux will occur under these conditions. Clearly close regulation of one or other of these channels is necessary under normal conditions because of their complementary voltage sensitivity; left to run free they would quickly deplete solute concentrations within the cell.

We are very grateful to Maggi Boult who prepared the protoplasts for this study and for Dr. Daphne Elliott's stimulus to work on *Amaranthus*. This project was funded by the Australian Research Council.

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Received 20 June 1990; revised 10 September 1990