Malate-Permeable Channels and Cation Channels Activated by Aluminum in the Apical Cells of Wheat Roots[1]

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Aluminum (Al^{3+})-dependent efflux of malate from root apices is a mechanism for Al^{3+} tolerance in wheat (*Triticum aestivum*). The malate anions protect the sensitive root tips by chelating the toxic Al^{3+} cations in the rhizosphere to form non-toxic complexes. Activation of malate-permeable channels in the plasma membrane could be critical in regulating this malate efflux. We examined this by investigating Al^{3+}-activated channels in protoplasts from root apices of near-isogenic wheat differing in Al^{3+} tolerance at a single locus. Using whole-cell patch clamp we found that Al^{3+} stimulated an electrical current carried by anion efflux across the plasma membrane in the Al^{3+}-tolerant (ET8) and Al^{3+}-sensitive (ES8) genotypes. This current occurred more frequently, had a greater current density, and remained active for longer in ET8 protoplasts than for ES8 protoplasts. The Al^{3+}-activated current exhibited higher permeability to $malate^{2-}$ than to Cl^- ($P_{mal}/P_{Cl} \geq 2.6$) and was inhibited by anion channel antagonists, niflumate and diphenylamine-2-carboxylic acid. In ET8, but not ES8, protoplasts an outward-rectifying K^+ current was activated in the presence of Al^{3+} when cAMP was included in the pipette solution. These findings provide evidence that the difference in Al^{3+}-induced malate efflux between Al^{3+}-tolerant and Al^{3+}-sensitive genotypes lies in the differing capacity for Al^{3+} to activate malate permeable channels and cation channels for sustained malate release.

When aluminum (Al) is solubilized in acid soils to the phytotoxic species Al^{3+} it becomes a major factor limiting crop growth and yield (Foy et al., 1978; Kochian, 1995). A number of plant species and genotypes within species exhibit an inheritable tolerance to Al^{3+}. Two strategies that have been identified that allow plants to tolerate Al^{3+} are the exclusion of toxic Al^{3+} from the root apex by releasing Al^{3+}-chelating ligands such as organic acids and phosphate, or by releasing OH^- to increase external pH; and the detoxification of Al^{3+} once it has entered the cytoplasm by chelation and/or sequestration to less Al^{3+}-sensitive compartments (Taylor, 1991; Delhaize and Ryan, 1995; Kochian, 1995; Ma, 2000). Several Al^{3+}-tolerant plant species and genotypes exhibit Al^{3+}-dependent exudation of organic acids from their roots. For instance, the efflux of malate is stimulated from wheat (*Triticum aestivum*; Delhaize et al., 1993b; Basu et al., 1994; Pellet et al., 1996), citrate from maize, snapbean, and *Cassia tora* (Miyasaka et al., 1991; Pellet et al., 1995; Ma et al., 1997), and oxalate from buckwheat and taro (Ma and Miyasaka, 1998; Zheng et al., 1998). Organic acid release is restricted to the root apices, which is the critical zone for Al^{3+} stress (Ryan et al., 1993).

Using near-isogenic lines of wheat that differ in Al^{3+} tolerance at a single locus, Delhaize et al. (1993a, 1993b) showed that 10 times more malate was released from the root apices of an Al^{3+}-tolerant line than from an Al^{3+}-sensitive line when exposed to toxic levels of Al^{3+}. A similar Al^{3+}-activated efflux of malate has been found in other Al^{3+}-tolerant wheat genotypes (Basu et al., 1994; Ryan et al., 1995b; Pellet et al., 1996). Addition of malate to a nutrient solution containing a toxic concentration of Al^{3+} significantly improves the growth of Al^{3+}-sensitive wheat genotypes (Delhaize et al., 1993b; Ryan et al., 1995b). Taken together, these results suggest that one mechanism for Al^{3+} tolerance in wheat relies on the Al^{3+}-activated exudation of malate from the root apices. The organic anions protect the plants by chelating the toxic Al^{3+} cations in the rhizosphere to form non-toxic complexes.

Malate exists predominantly as the divalent anion in the cytoplasm, and movement of malate out of the root cells is an energetically passive process because of the large negative electrical potential difference across the plasma membrane. Thus, Al^{3+}-stimulated malate efflux is likely to be mediated by activation of an anion channel permeable to malate in the plasma membrane of root apical cells. The observations that Al^{3+}-activated efflux of malate from wheat roots (Ryan et al., 1995a) and oxalate from buckwheat roots (Zheng et al., 1998) is sensitive to several anion-channel blockers are in line with this proposition.

[1] This study was funded by the Australian Research Council.

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Malate content in root tissues and the activities of enzymes involved in malate synthesis (phospho*enol*-pyruvate carboxylase and malate dehydrogenase) are not significantly different between wheat genotypes that differ in Al^{3+}-induced malate efflux (Ryan et al., 1995a). Thus, it appears that the capacity of efflux rather than synthesis of malate accounts for the difference in malate efflux between the Al^{3+}-tolerant and -sensitive genotypes.

Anion channels have been characterized in the plasma membranes of a range of different plant cells where they are known to be involved in several important cellular functions. These include turgor regulation, stomatal movement, nutrient acquisition, and control of membrane potential (Tyerman, 1992; Schroeder, 1995; Ward et al., 1995; Barbier-Brygoo et al., 2000). Two types of anion channels in the plasma membrane have been identified in protoplasts derived from wheat roots. One is the outwardly rectifying anion channel that is activated at membrane potentials more positive than the equilibrium potential for the permeant anion (Skerrett and Tyerman, 1994). This channel has been suggested to mediate uptake of Cl^- and NO_3^- into the root cells in the presence of high concentrations of external Cl^- and NO_3^- (Skerrett and Tyerman, 1994). The second anion channel is activated by Al^{3+} (Ryan et al., 1997) and is observed in the plasma membrane of protoplasts isolated from root tips of Al^{3+}-tolerant genotypes of wheat. This channel is activated specifically by Al^{3+}, inhibited by the anion channel blockers niflumic acid and 5-nitro-2-(3-phenylpropyl amino)-benzoic acid, and remains active for long periods provided Al^{3+} is present in the bathing solution (Ryan et al., 1997). These characteristics are comparable with the Al^{3+}-induced malate efflux from intact roots (Delhaize et al., 1993b; Ryan et al., 1995a), leading Ryan et al. (1997) to propose that the anion channel they characterized mediates the malate efflux stimulated by Al^{3+}. However, the question as to whether the Al^{3+}-activated anion channel was actually permeable to malate remained unanswered. Moreover, if the Al^{3+}-activated anion channel is responsible for malate efflux, some differences would be expected in channel activity, selectivity, or gating properties between the Al^{3+}-tolerant and -sensitive wheat genotypes. This information is important for understanding the physiology of Al^{3+}-tolerance, as well as for identifying the genes that confer Al^{3+} tolerance in wheat.

In wheat the malate efflux stimulated by Al^{3+} is accompanied by enhanced K^+ efflux from the root apices (Ryan et al., 1995a). This allows a net flux of malate that does not decrease the external pH. Present models for the mechanism of stomatal closure suggest that the activation of an anion channel in the plasma membrane of the guard cell depolarizes the membrane past the equilibrium potential for potassium (E_K), and leads to a coordinated efflux of anions and K^+ (Schroeder, 1995). A similar mechanism may explain the Al^{3+}-activated efflux of K^+ and malate from Al^{3+}-tolerant wheat roots, but supportive electrophysiological data are lacking. Outward and inward rectifying K^+ channels have been characterized in the plasma membrane of wheat root cells (Schachtman et al., 1991; Findlay et al., 1994; Gassmann and Schroeder, 1994; Skerrett and Tyerman, 1994), but Al^{3+} has been shown to be a potent antagonist of both these channels (Gassmann and Schroeder, 1994; Ryan et al., 1997). Therefore, the mechanism underlying the Al^{3+}-dependent efflux of K^+ from Al^{3+}-tolerant wheat genotypes remains unclear. One possibility not examined previously is whether a freely diffusible molecule in the cytosol, which is required for K^+ channel activity in the presence of Al^{3+}, is lost from the protoplast during the whole-cell experiments by dilution into the pipette solution. There are several candidate molecules of a size that would readily be perfused from the cytoplasm and that may be required for channel activation, but one that has been shown to activate K^+-outward channels in mesophyll cells of broad bean is cAMP (Li et al., 1994).

In the present study we used the whole-cell patch-clamp technique and near-isogenic lines of wheat differing in Al^{3+}-tolerance at a single locus (Delhaize et al., 1993a) to determine whether malate anions could carry currents through Al^{3+}-activated anion channels. We looked for differences in activation of the anion channel currents between the near isogenic wheat lines, and we investigated the effect of cAMP on the activity of K^+ currents.

RESULTS

To investigate whether the Al^{3+}-activated anion channel identified previously (Ryan et al., 1997) is permeable to malate, similar experiments to those reported by Ryan et al. (1997) were performed with a pipette solution that contained malate as the main anion. To suppress the initially large background K^+ currents, tetraethylammonium (TEA^+) was used as the main cation in the pipette. Several types of currents measured in the initial sealing solution were present in protoplasts isolated from ES8 and ET8 wheat root apices. These include a time-dependent and instantaneous inward current and a small instantaneous outward current. A time-dependent outward current was often observed when K^+ was used as a main cation in the pipette solution. These currents resembled those time-dependent inward and outward K^+ and non-selective cation currents that have been characterized previously (Findlay et al., 1994; Skerrett and Tyerman, 1994; Tyerman et al., 1997) and were not examined in the present study.

Al^{3+}-Activated Inward Currents in ET8 and ES8 Protoplasts

For ET8 and ES8 protoplasts positive and negative voltage-pulses activated small inward and outward currents. The inward currents did not display any appreciable time dependence in the control bathing solution (Fig. 1, A and D). The average magnitude of the inward current was similar for both lines (−Al in Fig. 2B). For some of the ET8 and ES8 protoplasts, addition of 50 μM $AlCl_3$ (free Al^{3+} activity, $\{Al^{3+}\}$ = 18 μM) to the bathing solution elicited (after a delay) a time-dependent inward current upon negative voltage pulses (example responses shown in Fig. 1, B and E). During the delay and in protoplasts that did not show a response, the inward current was often inhib-

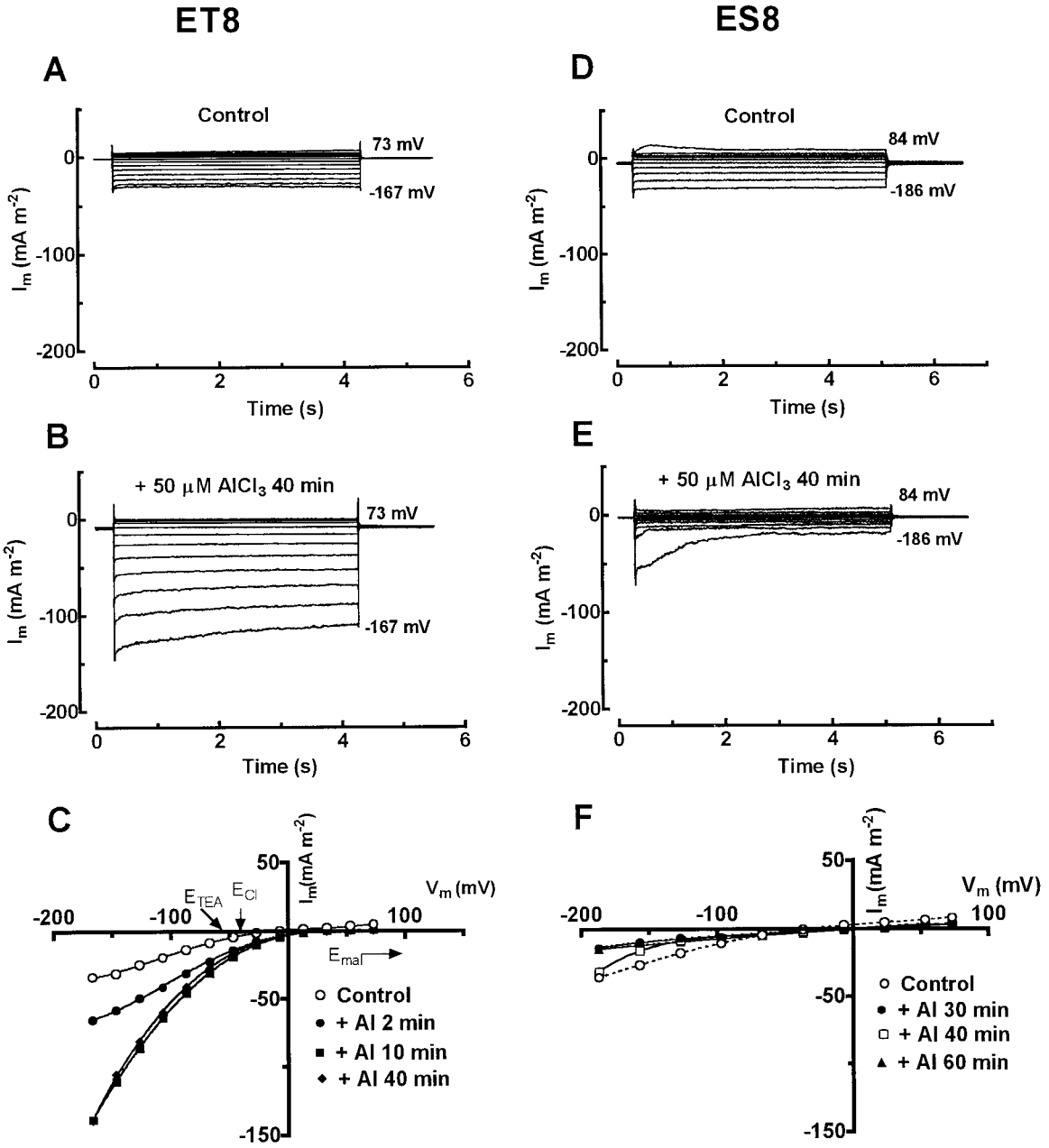

Figure 1. Al^{3+}-activated inward currents recorded for whole-cell patches of an ET8 (A–C), and ES8 (D–F), protoplast. Superimposed current traces in response to voltage-pulses ranging from −167 to +73 mV (ET8) or from −186 to +84 mV (ES8) before (A and D), and 40 min after (B and D) the addition of 50 μM $AlCl_3$. C and D, The initial current was used to construct current-voltage curves for the protoplasts in control solution (○) and after various times of exposure to 50 μM $AlCl_3$. The pipette solution contained 40 mM malate, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 2 mM Na_2ATP, 10 mM EGTA, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid), pH 7.2 with 110 TEAOH.

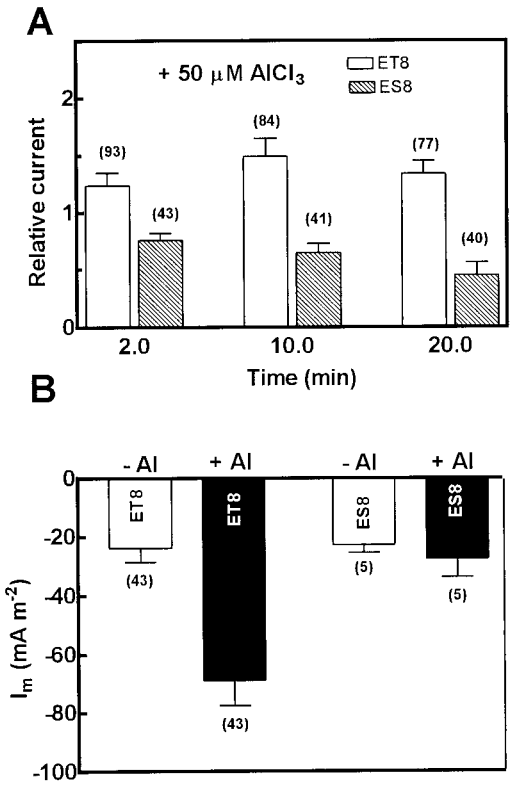

Figure 2. Effects of Al^{3+} on the inward current in ET8 and ES8 protoplasts. A, Changes in relative inward current following addition of 50 μM $AlCl_3$. The currents were collected from all protoplasts examined and normalized to the initial current for a voltage pulse to −180 mV measured before addition of $AlCl_3$. The mean initial current density in the control solution at −180 mV was −22.6 ± 4.4 mA m^{-2} (n = 84) for ET8 protoplasts and −23.1 ± 5.2 mA m^{-2} (n = 38) for ES8 protoplasts. B, Mean initial current density from only those protoplasts exhibiting an Al^{3+}-activated inward current. Values represent the mean of the maximum currents measured at −180 mV after addition of 50 μM $AlCl_3$. Data are mean ± SE (number of protoplasts).

ited. Figure 2A shows the mean inward currents relative to those before addition of $AlCl_3$ for all ET8 and ES8 protoplasts tested at 2, 10, and 20 min after the addition of 50 μM $AlCl_3$. On average, ET8 protoplasts increased inward current, whereas ES8 decreased inward current after the addition of $AlCl_3$. These changes in current are not due to non-specific time-dependent changes because the average inward currents measured in control solution decreased insignificantly over 60 min (P = 0.07) for ET8 and ES8 protoplasts. The difference between the two lines is a result of a greater proportion of ET8 protoplasts showing an Al^{3+}-activated inward current (occurrence rate 39% in ET8, 11% in ES8) combined with the activated current being larger and occurring within a shorter delay time for ET8 protoplasts than for ES8 protoplasts (Table I).

The Al^{3+}-activated current in ET8 protoplasts showed a strong inward rectification (Fig. 1, B and C) and remained active for as long as $AlCl_3$ was present in the bathing solution. The current versus voltage (I-V) curves show that activation of the inward current was accompanied by a positive-going shift in reversal potential (from −18 to approximately +60 mV in Fig. 1C). About 70% of ET8 protoplasts that showed the Al^{3+}-activated inward current responded within 10 min of exposure to $AlCl_3$. The mean delay time for activation of the inward current was 9.1 min for ET8 (Table I).

In contrast to ET8 protoplasts, addition of Al^{3+} to ES8 protoplasts generally inhibited the background inward currents. Inward currents such as that shown in Figure 1E were observed in only 11% of protoplasts after prolonged exposure to Al^{3+} (mean delay was 36 min, Table I). The average maximum amplitude of the Al^{3+}-induced inward current in ES8 protoplasts was only slightly larger than that measured prior to addition of Al^{3+} (Fig. 2B). The reversal potential of the Al^{3+}-activated inward current for ES8 protoplasts was often less positive than that for ET8 protoplasts (Fig. 1F; Table I), but this was not statistically significant. Another difference to ET8 protoplasts is that the Al^{3+}-activated inward current in ES8 protoplasts was not sustained in the presence of constant Al^{3+} in the bath solution (Fig. 1F).

The Al^{3+}-Activated Inward Current Was Inhibited by Niflumate and Diphenylamine-2-Carboxylic Acid (DPC)

It has previously been shown that malate efflux from intact ET8 roots was inhibited by the anion channel inhibitors niflumate (Ryan et al., 1995a) and DPC (T. Kataoka, A. Stekelenbury, E. Delhaize, and P.R. Ryan, unpublished data). The Al^{3+}-activated inward current was inhibited by 100 μM niflumate (Fig.

Table I. *Comparison of the Al^{3+}-activated inward current in the ES8 and ET8 genotypes*

Occurrence rate is the percentage of total cells measured in which Al^{3+} activated an inward current. Delay refers to the average time elapsed between the addition of Al^{3+} and the activation of an inward current. Current density (I_m) refers to the maximum current after Al^{3+} addition measured at −180 mV. The reversal potential for the Al^{3+}-activated inward current is also shown (E_{rev}). The values are the mean and SEM with the no. of protoplasts examined in the brackets.

Genotype	Occurrence Rate	Delay	I_m	E_{rev}
	%	min	mA m^{-2}	mV
ET8	39 (109)	9.1 ± 1.0 (43)	−68.8 ± 7.6 (43)	35.4 ± 3.9 (43)
ES8	11 (44)	36.0 ± 6.8 (5)	−27.5 ± 5.7 (5)	24.2 ± 9.1 (5)

3, A and B) and by 10 μM DPC (Fig. 3, D and E). The current-voltage curves shown in Figure 3C (niflumate) and Figure 3F (DPC) show the average response to the inhibitors and indicate that inhibition is not voltage dependent. At −186 mV, 100 μM niflumate inhibited the initial current by 61% ± 13% (n = 4), and 10 μM DPC inhibited the current by 51% ± 14% (n = 3). A relatively large "spiky" inward current was often observed at the most negative voltage levels when the DPC concentration was greater than 20 μM and/or the protoplasts were exposed to DPC longer than 10 min (data not shown). This observation suggests that DPC may have other effects on the plasma membranes under these conditions.

Al^{3+}-Activated Inward Current Carried by Malate Efflux

The pipette solution in most of the experiments contained 40 mM malate and a small amount of Cl^- (2–4 mM). Inclusion of Cl^- was necessary because the Ag/AgCl half-cell requires a stable Cl^- concentration

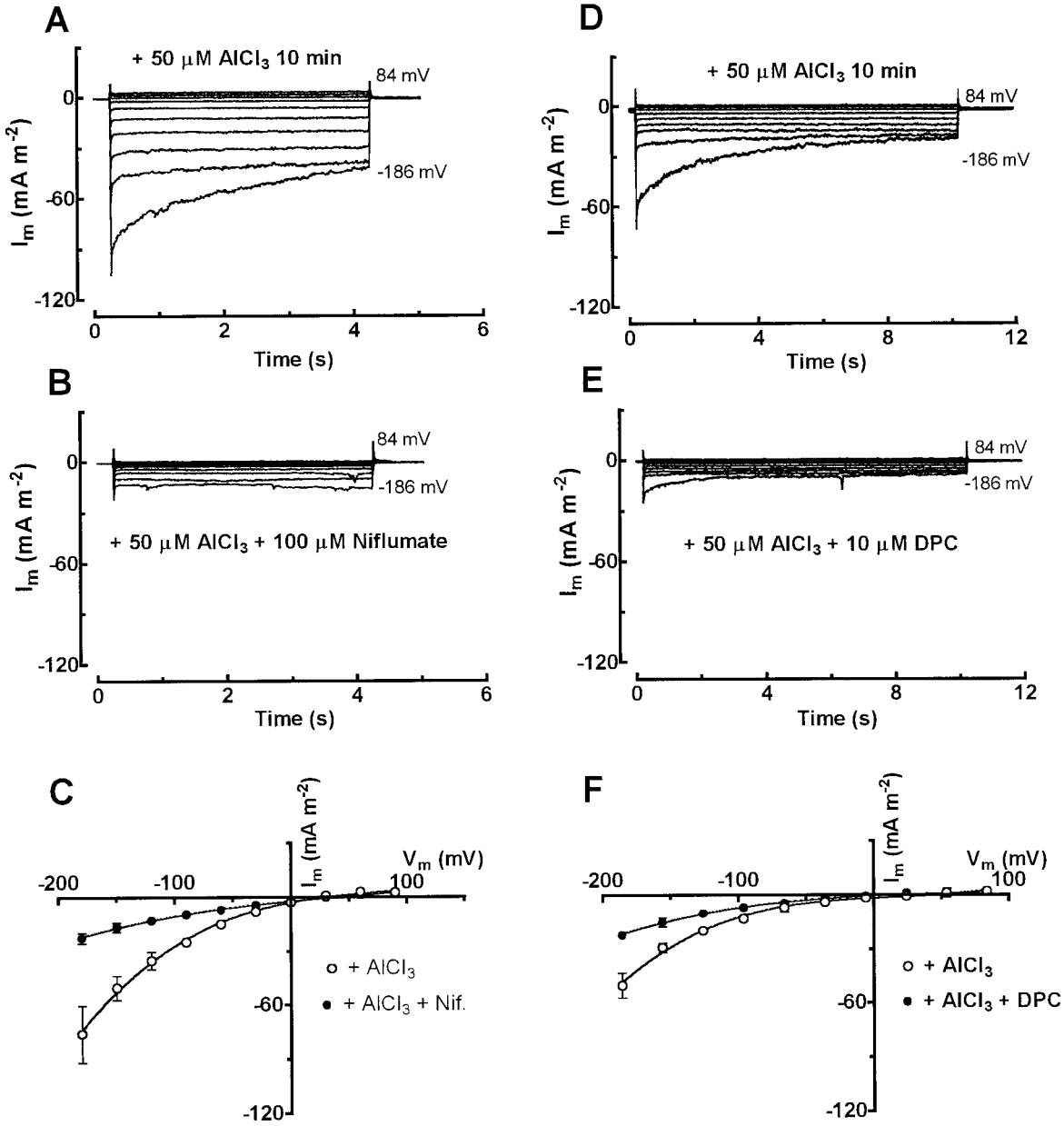

Figure 3. Effects of anion channel antagonists on the Al^{3+}-activated inward current in ET8 protoplasts. A and D, Al^{3+}-activated current caused by addition of 50 μM $AlCl_3$. Superimposed current traces in response to voltage pulses ranging from −186 to +84 mV at 30-mV intervals from a holding potential of 24 mV. Addition of 100 μM niflumate (B) or 10 μM DPC (E) in the presence of 50 μM $AlCl_3$. Current-voltage curves using initial currents in the absence and presence of 100 μM niflumate (C) or 10 μM DPC (F). Data are mean ± SEM from four protoplasts (niflumate) and three protoplasts (DPC).

to reduce the junction potentials. The reversal potential of the Al^{3+}-activated inward current under bi-ionic conditions could normally be used to determine the relative permeability of the underlying anion channels to malate^{2-} and Cl^-. However, malate cannot be added to the bathing solution in these experiments because the continued activation of the current requires the presence of Al^{3+} in the bathing solution (Ryan et al., 1997). The addition of malate also inhibits the Al^{3+}-activated inward current by chelating external Al^{3+} (data not shown). Therefore, three methods were used to determine whether the Al^{3+}-activated anion channel was permeable to malate. The first method involved changing the external Cl^- concentration after the inward current was activated by Al^{3+}, and following the shift in reversal potential (the Al^{3+} concentration was altered to maintain $\{Al^{3+}\}$ constant). Figure 4 shows the response of an ET8 protoplast where the addition of Al^{3+} to the bath activated an inward current (Fig. 4, A and B) and shifted the reversal potential from +8 mV to more positive than +70 mV (Fig. 4D). The magnitude and time-dependence of the inward current was relatively unaffected by increasing the external Cl^- concentration to 50 mM (Fig. 4C). A shift in reversal potential could not be discerned even though E_{Cl} was shifted from −40 to −81 mV by increasing the external Cl^- concentration (Fig. 4D). Identical results were observed for four protoplasts and the mean reversal potentials in 10 and 50 mM external Cl^- were 39.6 ± 6.6 and 37.8 ± 9.1 mV, respectively.

In a second method to test malate permeability the Ag/AgCl half-cell was replaced with a platinum electrode and Cl^- was omitted from the pipette solution altogether. Under these experimental conditions, addition of Al^{3+} still activated an inward current that was comparable with those measured previously (Fig. 5). Only a single result was obtained using this method.

To measure the relative malate^{2-} to Cl^- permeability ratio, accurate reversal potentials needed to be obtained and background currents not contributing to the Al^{3+}-activated anion currents need to be subtracted. Subtracting the I-V curve measured in the control solution from the I-V curve measured after addition of Al^{3+} is one way to remove the background currents from the Al^{3+}-activated cur-

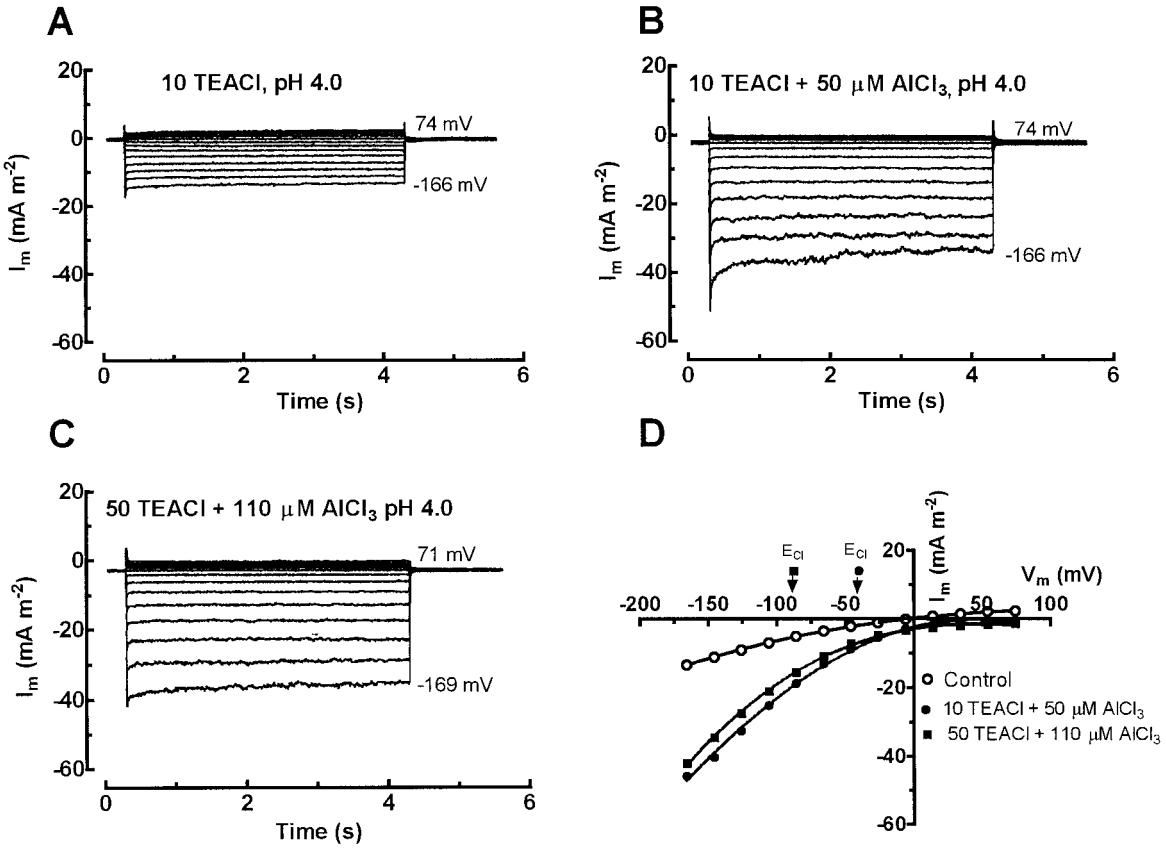

Figure 4. Effect of external TEACl concentration on the Al^{3+}-activated inward current in an ET8 protoplast. Current traces evoked by voltage pulses between +74 and −166 mV in 20-mV intervals from a holding potential of −26 mV before (A) and 8 min after (B) exposure to 50 μM $AlCl_3$. C, The bath solution was changed from 10 mM TEACl, 0.2 mM $CaCl_2$, and 50 μM $AlCl_3$ ($\{Al^{3+}\}$ = 18 μM, pH 4.0) to 50 mM TEACl, 0.2 mM $CaCl_2$, and 110 μM $AlCl_3$ ($\{Al^{3+}\}$ = 17 μM, pH 4.0). D, Current-voltage curves using the initial currents from A through C. Pipette solution contained 40 mM malic acid, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 2 mM Na_2ATP, 10 mM EGTA, 10 mM HEPES, and 110 mM TEAOH, pH 7.2.

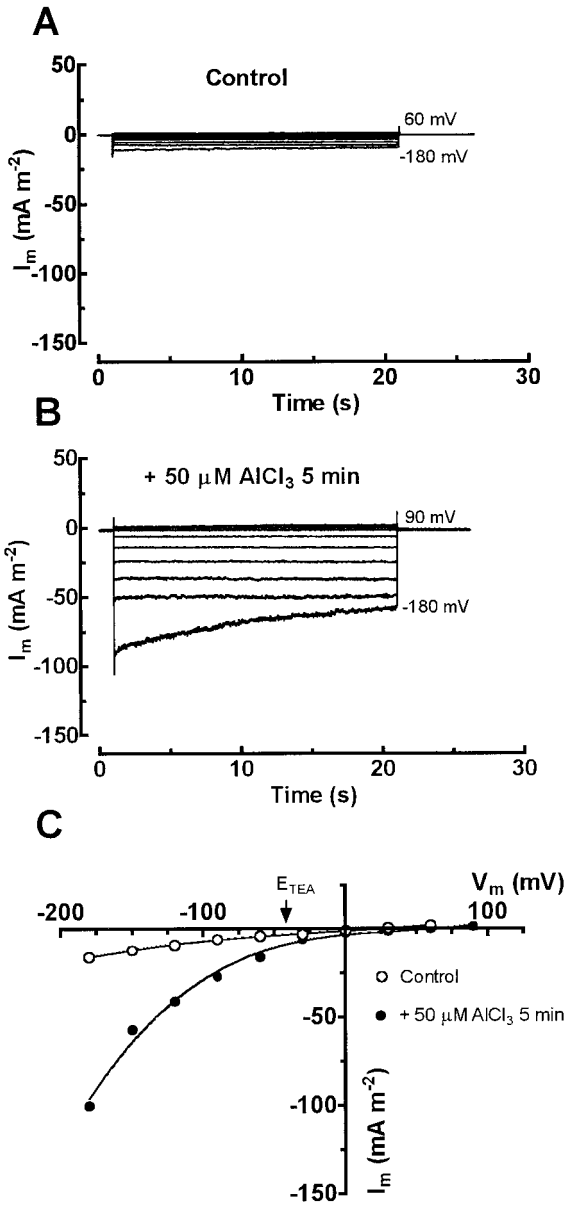

Figure 5. Al^{3+}-activated inward current for a whole-cell patch of an ET8 protoplast with the pipette solution containing no Cl^-. Current traces elicited by voltage pulses ranging from −180 to 60 mV in control solution (A) and −180 to 90 mV after 5 min from addition of 50 μM $AlCl_3$ to the bath solution. C, Current-voltage curves of initial currents shown in A and B. Pipette solution contained 40 mM malic acid, 2 mM $CaSO_4$, 2 mM $MgSO_4$, 2 mM Na_2ATP, 10 mM EGTA, 10 mM HEPES, and 110 mM TEAOH, pH 7.2.

rent, assuming that only the anion channels are activated and no other electrogenic transport is affected by Al^{3+}. The reversal potential of this net current was used in the modified Goldman-Hodgkin-Katz equation (Lewis, 1979) to estimate $P_{mal}{}^{2-}/P_{Cl}{}^{-}$. The calculated $P_{mal}{}^{2-}/P_{Cl}{}^{-}$ from the experiment without Cl^- present in the pipette solution was 8.4.

Reversal potentials were also obtained by subtracting the I-V curves before and after addition of niflumate or DPC in 10 mM external TEACl and assuming that the anion current was the only electrogenic transport inhibited by these compounds. Outward K^+ channels are blocked by niflumate (Garrill et al., 1996), but in the present experiments TEA^+ was the only cation in the pipette solution. When the average reversal potential of the net currents (45 ± 12 mV, n = 6) was used the $P_{mal}{}^{2-}/P_{Cl}{}^{-}$ is 2.6. With this permeability ratio it would be expected that a 5-fold increase in external Cl^- would shift the reversal potential more negative by 23 mV. The finding above (Fig. 4) that the reversal potential did not change significantly under these conditions may indicate that the permeability ratio increased with increasing external Cl^- concentration. In an alternate manner, it may suggest that the value for $P_{mal^{2-}}/P_{Cl^-}$ of 2.6 underestimates the real ratio.

Although Al^{3+} is a potent antagonist of Ca^{2+} channels in wheat and Arabidopsis root cells (Piñeros and Tester, 1995; Huang et al., 1996; Kiegle et al., 2000), the possibility that the Al^{3+}-activated inward current corresponded in part to an increase in Ca^{2+} influx was also tested. The inward current was first activated by Al^{3+} and then the external Ca^{2+} concentration was increased from 0.2 to 20 mM (adjusting $[Al^{3+}]$ to maintain $\{Al^{3+}\}$ constant). Under these conditions the inward current was reduced slightly, and was not increased, indicating that Ca^{2+} influx was probably not contributing to the current (data not shown). These separate lines of evidence argue that the Al^{3+}-activated inward current largely corresponds to malate efflux.

Al^{3+} Activation of a K^+ Outward Current in the Presence of cAMP

Ryan et al. (1995a) have shown that the Al^{3+}-activated malate efflux from root apices of ET8 wheat was accompanied by a simultaneous efflux of K^+ ions. Time-dependent K^+ outward currents are prominent in ET8 and ES8 protoplasts at depolarized membrane voltages when K^+ was included in the pipette solution (Fig. 6A). As reported previously (Ryan et al., 1997), the time-dependent K^+ outward current in ET8 and ES8 protoplasts was markedly inhibited when the protoplasts were exposed to Al^{3+} (Fig. 6, B–D). However, when 0.5 mM cAMP was included in the pipette solution, ET8 protoplasts showed the following response sequence as shown by the example in Figure 7. To begin with, Al^{3+} inhibited a K^+ outward current (Fig. 7, A and B). Then after about 10 min, depending on the protoplast, a K^+ outward current returned. This outward current could be observed by positive-going voltage pulses for at least 60 min in different protoplasts (Fig. 8A). The voltage dependence of the steady-state outward current that re-activated was similar to the initial current in the control solution as indicated by the I-V curves (Fig. 7D). However, there

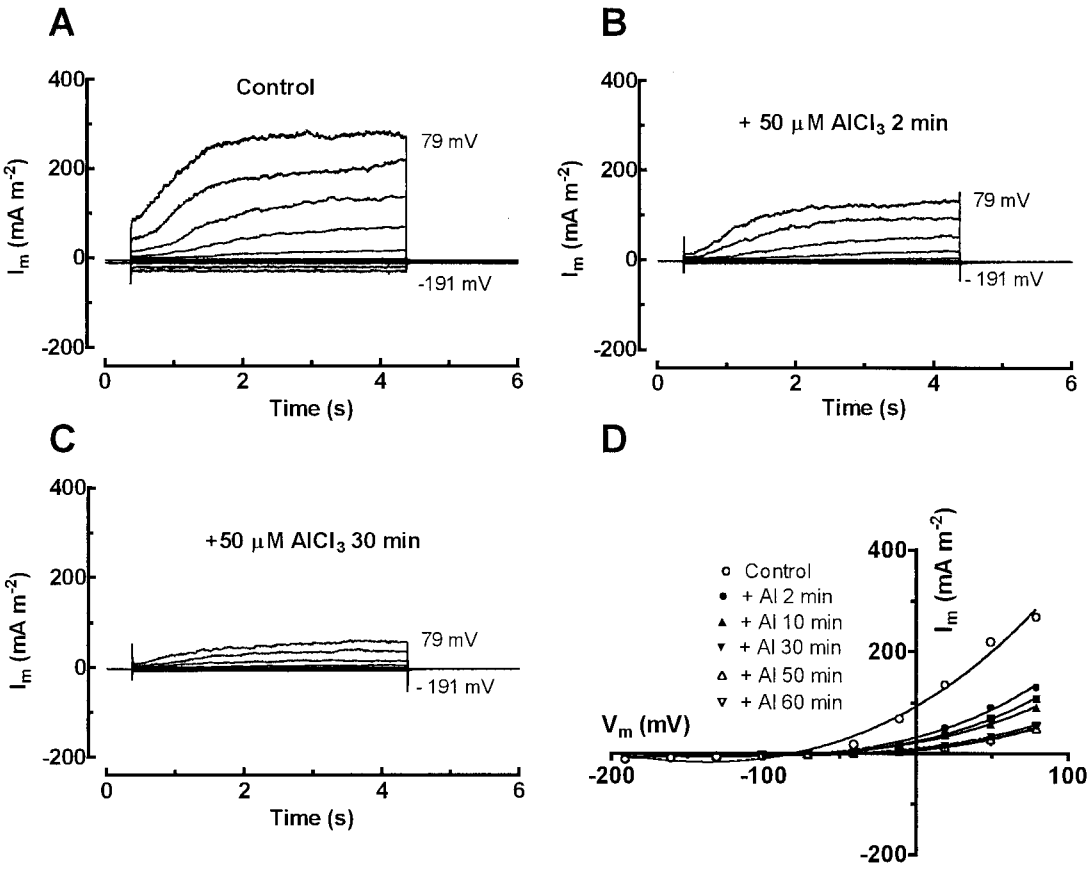

Figure 6. Effects of Al^{3+} on K^+ outward current in a whole-cell patch of an ET8 protoplast. A, Superimposed current traces activated by pulses ranging from −191 to +79 mV at 20-mV intervals from a holding potential of −111 mV in the absence of Al^{3+} (A), and after 2 (B) and 30 min (C) of exposure to 50 μM $AlCl_3$ (pH 4.0). D, Steady-state current-voltage curves obtained before and after addition of 50 μM $AlCl_3$ to the bath. Pipette solution contained 40 mM malic acid, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP, 10 mM EGTA, 10 mM HEPES, and 90 mM KOH, pH 7.2. Control solution contained 1 mM KCl and 0.2 mM $CaCl_2$, pH 4.0.

appeared to be a greater proportion of time dependent current compared with initial current after the re-activation (compare with Fig. 7, A and B). For ES8 protoplasts there was a sustained inhibition of steady-state outward current when challenged with external Al^{3+}, regardless of the presence or absence of cAMP in the pipette solution (Fig. 8B). Although the examples shown in Figures 7 and 8 do not show any activation of inward current, we did observe Al^{3+}-activated inward current in some protoplasts that also displayed K^+ outward current. However, this was difficult to quantify because of similarities in the kinetics of K^+ outward rectifier deactivation and inactivation of Al^{3+}-activated inward current. By using TEA^+ instead of K^+ in the pipette solution we could examine the effects of cAMP on the inward current. The amplitude and kinetics of the Al^{3+}-activated inward current did not appear to be changed significantly by the presence of cAMP in the pipette solution for ES8 or ET8 (data not shown).

DISCUSSION

Al^{3+}-Activated Inward Current Is Carried by Malate

Ryan et al. (1997) had previously shown that Al^{3+} activates a Cl^--permeable anion channel in the plasma membrane of protoplasts derived from root apices, but not from mature roots, of an Al^{3+}-tolerant wheat genotype. In the present study the permeability of the Al^{3+}-activated anion channels to malate was examined using protoplasts derived from root apex of the same Al^{3+}-tolerant genotype (ET8) as the previous study. An inward current was activated after addition of 50 μM $AlCl_3$ (pH 4.0) in approximately 40% of the protoplasts examined when 40 mM malate was used as the main anion in the pipette solution. This is comparable with the previous study in which approximately one-half the protoplasts exhibited an Al^{3+}-activated inward current when Cl^- was the main permeant anion (Ryan et al., 1997). Several characteristics of the Al^{3+}-activated inward current found in this study are comparable with the Al^{3+}-activated Cl^- permeable channel, including fast activation by hy-

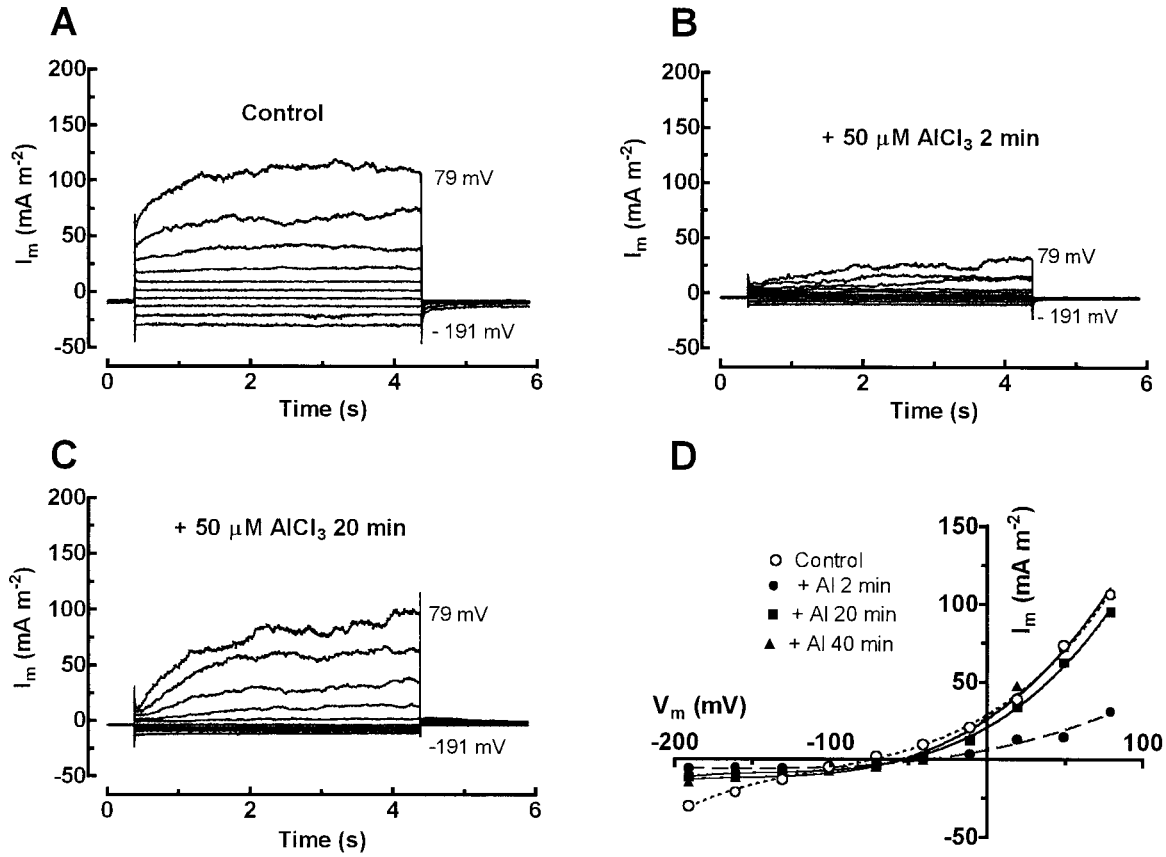

Figure 7. Effect of cAMP present in the pipette solution on the response of K^+ outward current in a whole-cell patch of an ET8 protoplast. Superimposed current traces activated by voltage-pulses between −191 and +79 mV at 30-mV intervals from a holding potential of −111 mV in control solution (A), and 2 (B) and 20 min (C) after exposure of the protoplast to 50 μM $AlCl_3$ (pH 4.0). D, Steady-state current-voltage curves obtained before and after exposure to 50 μM $AlCl_3$ (pH 4.0). Pipette solution contained 0.5 mM cAMP, 40 mM malic acid, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2ATP, 10 mM EGTA, 10 mM HEPES, and 90 mM KOH, pH 7.2. Control solution contained 1 mM KCl and 0.2 mM $CaCl_2$, pH 4.0.

perpolarizing voltage-pulses, long-lasting activation in the presence of external Al^{3+} (Fig. 1C), and inhibition by the anion channel antagonist niflumate (Fig. 3). However, the Al^{3+}-activated Cl^- current in the whole-cell patch clamp configuration shown by Ryan et al. (1997) was much noisier than we observed in the present study. Because the noise variance increases in proportion with the number of channels activated, with the square of the single channel current, and as the channel open probability approaches 0.5, any combination of differences in these characteristics caused by different experimental conditions could lead to the difference in noise characteristics. A higher single channel current for Cl^- than for malate may result from the higher concentration of Cl^- (i.e. 100 mM) used in the pipette solution of the previous study compared with the $malate^{2-}$ concentration (i.e. 40 mM) used in the present study. There may also be a difference in the permeation properties of the two anions such that malate permeates more slowly than Cl^- under an equivalent electrochemical gradient despite the channel showing the opposite in terms of the relative permeability determined from reversal potentials. This can occur if the channel pore has binding sites with a higher affinity for malate than Cl^-.

Unlike the Al^{3+}-activated current with Cl^- as the main anion (Ryan et al., 1997), the Al^{3+}-activated current with malate often exhibited slow inactivation at the most negative membrane voltages (Figs. 3 and 5). The more negative-voltage pulses used to evoke the Al^{3+}-activated inward current in this study compared with those used by Ryan et al. (1997) may account for the difference. Alternatively, this difference could be due to differences in channel gating properties caused by the presence of malate. The presence of malate in the external medium can alter anion channel gating characteristics in guard cells (Hedrich and Marten, 1993). It is also possible that the flux of malate induced by the more negative membrane voltages is sufficient to allow significant malate accumulation in the unstirred layer around the protoplast such that the effective Al^{3+} concentration adjacent to the cell membrane is reduced. This would then reduce the degree of channel activation during a pulse if the Al^{3+} concentration goes below

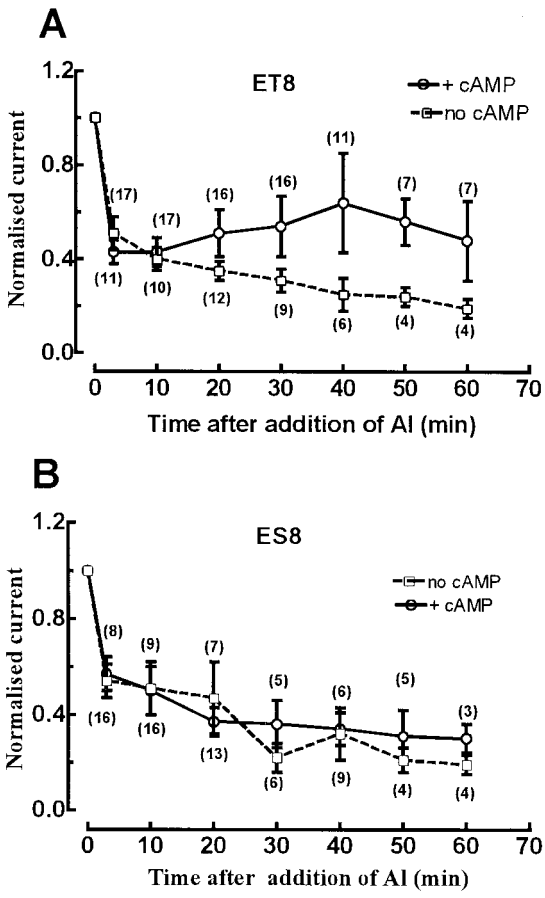

Figure 8. Time-dependent changes in outward current for whole cell patches of ET8 (A) and ES8 (B) protoplasts in the presence and absence of cytoplasmic cAMP. The current was normalized to the initial current value at +79 mV measured in control solution. The data are mean ± SEM (number of protoplasts). Current density at +79 mV in control solution was 197.1 ± 29.8 mA m^{-2} (n = 17) and 184.6 ± 19.7 mA^{-2} (n = 11) for ES8 and ET8 protoplasts, respectively.

the saturation concentration of the "receptor." Chloride efflux would not be expected to do this because of the minimal effect that Cl^- has on the Al^{3+} activity. It is interesting in this respect to note that Al^{3+} induced an inactivating Cl^- current in Al^{3+}-tolerant maize root tips (Piñeros and Kochian, 2001). Similar kinetics have been observed for anion channels in guard cells (Linder and Raschke, 1992; Schroeder and Keller, 1992), tobacco suspension cells (Zimmermann et al., 1994), xylem parenchyma cells (Köhler and Raschke, 2000), and Arabidopsis hypocotyl cells (Frachisse et al., 2000).

Several approaches were used to identify the ions contributing to the Al^{3+}-activated inward current. The reversal potential of the Al^{3+}-activated current was always much more positive than E_{Cl} and surprisingly did not shift when E_{Cl} was varied (Fig. 4). Moreover, no increases in the current amplitude were observed when the external concentrations of TEA^+ or Ca^{2+} were raised, indicating that the Al^{3+}-activated inward current is unlikely to result from the stimulation of cation influx. Furthermore, in one experiment Al^{3+} was shown to activate an inward current of similar characteristics when the pipette solution contained malate, but no Cl^- (Fig. 5). These findings show that the Al^{3+}-activated inward current is mainly carried by malate anions flowing out of the cell.

Anion channels characterized in the plasma membrane of other cell types and from other species exhibit a P_{mal}/P_{Cl} of less than 1. For example, the guard cell slow anion channel has a P_{mal}/P_{Cl} value of 0.24 (Schmidt and Schroeder, 1994), which is similar to the quick activating anion channel identified in barley xylem parenchyma cells (Köhler and Raschke, 2000). In contrast, the channels that account for the Al^{3+}-activated inward current in wheat roots are more permeable to $malate^{2-}$ than to Cl^- with a P_{mal}/P_{Cl} of 2.6. The large variability of these measurements, and the estimated P_{mal}/P_{Cl} of 8.4 from one experiment, indicate that the permeability ratio could even be higher than 2.6. Because we have measured only whole cell currents, we cannot dismiss the possibility that more than one type of anion channel was activated by Al^{3+} and perhaps with different relative permeabilities. Nevertheless, at least one type of the anion channels present must have very high malate permeability, which is novel for plasma membrane anion channels. Malate-permeable anion channels have been identified in the tonoplast of *Kalanchoe digremontiana* (Cheffings et al., 1997) and Arabidopsis and the estimated P_{mal}/P_{Cl} for the latter example is 3.5 (Cerana et al., 1995). An anion channel in the plasma membrane of Arabidopsis hypocotyl cells was recently shown to be more permeable to divalent SO_4^{2-} than to Cl^- (P_{SO4}/P_{Cl} = 2.0), but the permeability to $malate^{2-}$ was much lower (P_{mal}/P_{Cl} = 0.03; Frachisse et al., 1999). The Al^{3+}-activated inward current exhibited strong inward rectification and virtually no outward current could be detected at voltages as positive as 90 mV (compare with Figs. 1 and 3). This rectification could be explained, in part, by the absence of malate in the bath solution and the corresponding very positive equilibrium potential for $malate^{2-}$ anions.

Activation of the Inward Current Is Different in ET8 and ES8

Consistent differences were observed between the ET8 and ES8 genotypes in the activation of the malate current by Al^{3+}. The response was observed about four times more frequently in ET8 protoplasts than for ES8 protoplasts and the maximum current density was 2.5 times greater in ET8 protoplasts (Table I). Activation of the inward current in ES8 protoplasts occurred after a longer exposure to Al^{3+} and, when activation did occur, the inward current was relatively short-lived compared with ET8 (Fig. 1, C and F). Since these two genotypes are virtually identical

except for a single locus difference that controls their sensitivity to Al^{3+} stress, we can be confident that the differences mentioned above are not caused by genotypic variation. We can speculate that a single locus is likely to be involved with the transport of malate.

Activation of malate channels by Al^{3+} could involve a direct interaction between $Al^{3+}$and the channel protein or an indirect interaction via intermediate steps such as a secondary messenger cascade. Failure of previous attempts to activate the anion channel by Al^{3+} using the outside-out patch configuration (Ryan et al., 1997) could be viewed as evidence that soluble intermediates are involved. In reality, many more replicate experiments would be required to be confident that a negative result was meaningful. The observation that a lag occurred between the addition of Al^{3+} and the activation of the current (Table I; Ryan et al., 1997) is different to the activation of malate efflux from intact roots, which occurs immediately. This lag could be an artifact of protoplast preparation or it could indicate that intermediate steps are involved in the activation. Any involvement of soluble molecules would be less efficient in the whole-cell configuration because the cytoplasm in the protoplasts is perfused by the pipette solution. Anion channels in plant cells have been shown to be modulated by a range of intracellular factors, including cytoplasmic Ca^{2+}, nucleotides, pH, and kinases (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Schmidt et al., 1995; Pei et al., 1997; Frachisse et al., 2000). Al^{3+} has been shown to change cytoplasmic Ca^{2+} activities (Zhang and Rengel, 1999) and to interfere with the phosphoinositide signaling pathway in wheat root cells (Jones and Kochian, 1995). However, preliminary studies suggest that IP_3, guanosine 5′-[γ-thio]triphosphate, and Ca^{2+} levels in the pipette do not effect the Al^{3+}-activated anion channels in ET8, but this requires verification (Ryan et al., 1997). Therefore, the single locus difference between the two genotypes, ES8 and ET8, could encode the channel protein itself or a component of the signaling pathway that modulates channel activity. The finding that cAMP was required for activation of a K^+ outward rectifier during Al^{3+} treatment (see below) supports a role for intermediate steps in activation. The regulation of the anion channel and cation channel are likely to be coordinated in some way. In maize root protoplasts, Piñeros and Kochian (2001) recently demonstrated that Al^{3+} can activate Cl^- channels in excised patches, suggesting that Al^{3+} acts directly on the anion channel or that a receptor for Al^{3+} and associated signaling cascade is present in the plasma membrane.

The Al^{3+}-activated inward current shares a number of similarities with the guard cell slow anion channels that are proposed to also mediate a sustained release of anions (Schroeder et al., 1993; Leonhardt et al., 1999). The similarities include slow inactivation (Schroeder et al., 1993; Leonhardt et al., 1999) and sensitivity to the anion channel blockers niflumate and DPC (Leonhardt et al., 1999). Recent studies have suggested that the guard cell slow anion channel could be an ATP-binding cassette (ABC) transporter or tightly controlled by an ABC protein (Schmidt et al., 1995; Leonhardt et al., 1999). One of the known antagonists of ABC proteins, DPC, has been shown to inhibit the guard cell slow anion channel activity and stomatal closure (Leonhardt et al., 1999). DPC also inhibited the Al^{3+}-stimulated malate efflux from intact roots of Al^{3+}-tolerant wheat genotypes (T. Kataoka, A. Stekelenburg, E. Delhaize, and P.R. Ryan, unpublished data) and also inhibited the Al^{3+}-activated inward current (Fig. 3). These similarities between the Al^{3+}-activated anion channel and ABC proteins flag an interesting direction for future work. It is interesting that another ABC protein in yeast called Pdr12 is probably involved in the efflux of mono-carboxylic acids from cells of *Saccharomyces cerevisiae* (Piper et al., 1998).

Involvement of cAMP in Activation of a K^+ Outward Current in ET8 Protoplasts

Al^{3+} has been shown to be a potent antagonist of the K^+ inward (Gassmann and Schroeder, 1994) and K^+ outward rectifying channels in wheat root cells (Ryan et al., 1997). The finding here that Al^{3+} strongly inhibited the K^+ outward channels in ET8 and ES8 protoplasts confirms the previous observation (Fig. 8). However, in ET8 protoplasts Al^{3+} activated an outward K^+ current when 0.5 mM cAMP was present in the pipette solution (Figs. 7 and 8A). This re-activation of outward current was not observed in protoplasts of ES8 (Fig. 8B). These results suggest that activation of a cation outward channel in the plasma membrane of the ET8 root cells is modulated by cytoplasmic cAMP. We cannot yet determine if the re-activated outward current in ET8 protoplasts is the same current as that initially present before the addition of Al^{3+}. Modulation of K^+ channel activity through direct interaction between cAMP and channel proteins or cAMP-dependent protein kinase have been identified in many plant and animal cells (Labarca et al., 1996 and refs. therein). In mesophyll cells of broad bean, cAMP stimulates K^+ outward channel activities through a cAMP-regulated protein kinase (Li et al., 1994). Furthermore, a cyclic nucleotide-gated non-selective inward cation channel in Arabidopsis has recently been cloned and characterized (Leng et al., 1999). Concentrations of cAMP do change in response to some stresses (Reggiani, 1997) and growing evidence suggests that cAMP could be an important secondary messenger involved in transducing environmental signals into alterations of cellular metabolism (Assmann, 1995).

In conclusion, Al^{3+} activates inward currents across the plasma membrane of wheat root cells that

correspond to malate efflux. More importantly, we have shown that the Al^{3+}-activated malate current in the Al^{3+}-tolerant ET8 genotype occurs more frequently after a shorter delay, has a greater current density, and remains active for longer compared with the Al^{3+}-sensitive ES8 genotype. Our data also indicate that Al^{3+} markedly inhibits a time-dependent K^+ outward rectifier in ET8 and ES8 root apical cells. However, in ET8 protoplasts the same, or another type of outward K^+ current is reactivated when the nucleotide cAMP was present in the patch-pipette. These differences between the ET8 and ES8 protoplasts match with the observation of a sustained release of malate from the roots of the ET8 genotype and a small, but measurable efflux from ES8 roots (Delhaize et al., 1993b; Ryan et al., 1995a). These findings provide strong evidence in support of a model where the sustained efflux of malate and K^+ from the root apices of Al^{3+}-tolerant wheat genotypes is mediated by an Al^{3+}-activated anion channel, and an outward K^+ channel in the plasma membranes of the root cells. These findings also indicate that the Alt1 locus, which controls Al^{3+} tolerance in these near-isogenic lines of wheat (Delhaize et al., 1993a), may encode the anion channel itself or a protein that regulates the activity of the anion channel.

MATERIALS AND METHODS

Plant Materials and Protoplasts Preparation

The near-isogenic wheat (*Triticum aestivum*) lines, ET8 (Al-tolerant) and ES8 (Al-sensitive), differing in Al tolerance at the Alt1 loci (Delhaize et al., 1993) were used in the present study. Seeds of ET8 and ES8 were surface sterilized with 0.5% (w/v) sodium hypochlorite and grown for 4 to 5 d in 0.2 mM $CaCl_2$ solution (pH 4.5) as described previously (Ryan et al., 1997). Protoplasts of the terminal 2 to 3 mm of roots were isolated using the procedure of Schachtman et al. (1991).

Electrophysiology

Patch pipettes pulled from borosilicate glass blanks (Clark Electromedical, Reading, UK) were coated with Sylgard (184 silicone elastomer kit, Dow Corning Co., Midland, MI). Pipettes filled with the pipette solutions (see below) had resistances ranging from 10 to 18 MΩ in sealing solution (i.e. 10 mM KCl and 10 mM $CaCl_2$). The voltage across the patch was controlled and current was measured using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Series resistance was compensated to approximate 50% and capacitance was compensated. Pulse protocols with sampling frequencies of 2 kHz and corresponding filtering frequencies of 0.5 kHz were used to elicit the current from a holding potential that was close to the resting membrane potential. Sufficient time between voltage pulses was given to allow currents to settle to the steady clamp current for the particular holding potential before a new pulse was applied. Records were stored and analyzed using pClamp 6.0 (Axon Instruments). Junction potentials for each change in bathing solution were calculated and corrected for using the program JPCalc (P.H. Barry, University of New South Wales, Kensington, NSW, Australia). All the experiments were carried out at room temperature (20°C–22°C). The I-V curves were constructed using initial or steady current values that were measured after approximately 50 ms and at the end of voltage-pulses respectively. The I-V curves were fitted with third order polynomials using Prism program (Graph Pad Software, San Diego).

Experimental Solutions

All pipette solutions were composed of 40 mM malic acid, 1 or 2 mM $CaCl_2$, 2 mM $MgSO_4$, 2 mM Na_2ATP, 10 mM EGTA, and 10 mM HEPES, pH 7.2, adjusted with either tetraethylammonium hydroxyl (TEAOH; TEA-based) or KOH (K-based). Osmolality of the pipette solution was adjusted to 720 mOsmol kg^{-1} with sorbitol. Details of pipette solutions were given in the appropriate figure legends. The protoplasts were first placed in a chamber filled with a "sealing solution" composed of 10 mM KCl, 10 mM $CaCl_2$, 5 mM MES [2-(*N*-morpholino)-ethanesulfonic acid], pH 6.0, and an osmolality of 700 mOsm kg^{-1} adjusted with Tris and sorbitol, respectively. After the whole-cell patch configuration was achieved, the bath solution was replaced by control solution (0.2 mM $CaCl_2$, 10–50 mM tetraethylammonium chloride, TEACl, or KCl, pH 4.0). An identical solution containing 50 to 110 μM $AlCl_3$ was used to examine the response of whole-cell conductance to Al^{3+}. Bath solutions containing $AlCl_3$ were prepared from 10 mM stock in 0.1 mM HCl. To prevent the formation of triskaidekaaluminum, the pH of solutions was raised slightly prior to the addition of Al^{3+} and then adjusted down with HCl if required (Ryan et al., 1997). All solutions were kept at 4°C until used and were filtered through a 0.2-μm filter (Millipore, Bedford, MA) before use. When required the chemical speciation program GEOCHEM (Parker et al., 1987) was used to compute the free activities of ions. All chemicals used in the present study were purchased from Sigma (St. Louis), and DPC, cAMP, niflumate were dissolved in dimethylsulphoxide, whose final concentration was less than 0.2% (v/v).

ACKNOWLEDGMENT

We thank Wendy Sullivan for expert technical assistance.

Received August 7, 2000; returned for revision October 1, 2000; accepted November 15, 2000.

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