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Inhibition of Electrogenesis by Aluminum in Characean Cells

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The effects of aluminum (Al) on electrogenesis at the plasma membrane were examined in internodal cells of *Chara corallina*. After treating cells with 0.1 mM $AlCl_3$ (pH 4.5), we measured both the membrane potential and the membrane resistance in artificial pond water (pH 5.6). Electrogenesis at the membrane was significantly inhibited by the treatment with $AlCl_3$. A decrease in the pump current of the electrogenic proton pump and/or a decrease in the electrical resistance (an increase in conductance) of the passive diffusion channel were considered to be responsible for the inhibition of electrogenesis. $CaCl_2$ had a partial ameliorating effect. Both malic acid and citric acid were very effective in reversing the effects of $AlCl_3$. In addition, these organic acids restored electrogenesis in cells that had been treated with $AlCl_3$. It is suggested that Al affects electrogenesis from the exterior of the membrane, at least during the initial stages of treatment (4–24 h).

Key words: Aluminum — *Chara* — Electrogenesis — Membrane potential — Membrane resistance.

Aluminum toxicity is one of the factors that seriously limits plant growth in acid soils (Foy et al. 1978, Wright 1989). When soil is acidified, aluminum (Al), one of the most abundant elements in soil, is solubilized to toxic ions. Inhibition of root growth is the principal and most easily recognizable symptom of Al toxicity (Bennet and Breen 1991, Kochian 1995, Ryan et al. 1993, Clarkson 1965). It has been reported that both cell division and the elongation growth of roots are inhibited by Al within 2 to 6 h after the start of exposure to Al (Clarkson 1965). The plasma membrane has been suggested to be a site of primary lesions due to Al (Bohm-Tuchy 1960, Hofler 1958). Analysis of membrane-related phenomena seems, thus, to be a fruitful approach to a full elucidation of the mechanism of Al toxicity.

Analysis of electrical properties provides a direct and quantitative method for the functional characterization of a membrane. Electrical analysis of the effects of Al has been performed in some plants (Kinraide 1988, Miyasaka et al. 1989, Lindberg et al. 1991, Olivetti et al. 1995, Reid et al. 1995, Lindberg and Strid 1997). Characean cells have proved to be the most suitable material for analyzing the electrical characteristics of membranes of plant cells (Shimmen et al. 1994). Thus, characean cells appear suitable for analysis of the effects of Al on the electrical characteristics of the cell membrane. In the present study, we analyzed the effects of Al on electrogenesis at the cell membranes of internodal cells of *Chara corallina*.

Materials and Methods

Chara corallina was cultured as described previously (Mimura and Shimmen 1994). Internodal cells were isolated from neighboring cells and kept in artificial pond water (APW) that contained 0.1 mM KCl, 1 mM NaCl and 0.1 mM $CaCl_2$ (pH 5.6). The membrane potential (E_m) was measured as reported previously (Shimmen and Tazawa 1977). Membrane resistance (R_m) was measured by applying small pulses of current across the membrane.

Cells were incubated in APW supplemented with 0.1 mM $AlCl_3$ for the indicated periods of time. Addition of 0.1 mM $AlCl_3$ caused a decrease in the pH of APW from 5.6 to about 4.5. Hereafter, APW supplemented with 0.1 mM $AlCl_3$ is simply referred to as Al medium. In most experiments, the pH of the Al medium was 4.5. In the experiments for which results are shown in Table 1, the pH of the Al medium was increased to 5.0 with NaOH. The pH values of the various Al media are indicated in the text. After incubation of cells in Al medium, E_m was measured in APW (pH 5.6). Thirty min after insertion of the microelectrode, we measured both E_m and R_m. Five cells were used in each experiment and average values of E_m and R_m are shown with S.E. To modify the pH of APW in experiments designed to analyze the dependency on pH of E_m, we used DMGA (pH 4.7, 5.7), MES (pH 6.7) and HEPES (pH 7.7) buffers at 0.5 mM, with adjustment of the pH with NaOH in each case. Experiments were performed at room temperature (23–26°C).

Results

Some cells died during incubation in Al medium (pH 4.5). E_m of five surviving cells was measured in APW (pH 5.6) at intervals of 24 h (Fig. 1A). Most of the control cells that were incubated in APW (pH 5.6) survived during the incubation. Before treatment with Al, the average value of E_m was −197 mV. After a one day incubation in Al medium (pH 4.5), E_m fell to −104 mV, and similar values were maintained until the fourth day. On the fifth day, E_m changed further in the positive direction. In control cells, E_m remained almost constant for 3 d and then changed slightly in the negative direction. R_m before treatment was

Abbreviations: APW, artificial pond water; DMGA, 3-,3-dimethylglutaric acid; E_m, membrane potential; R_m, membrane resistance.

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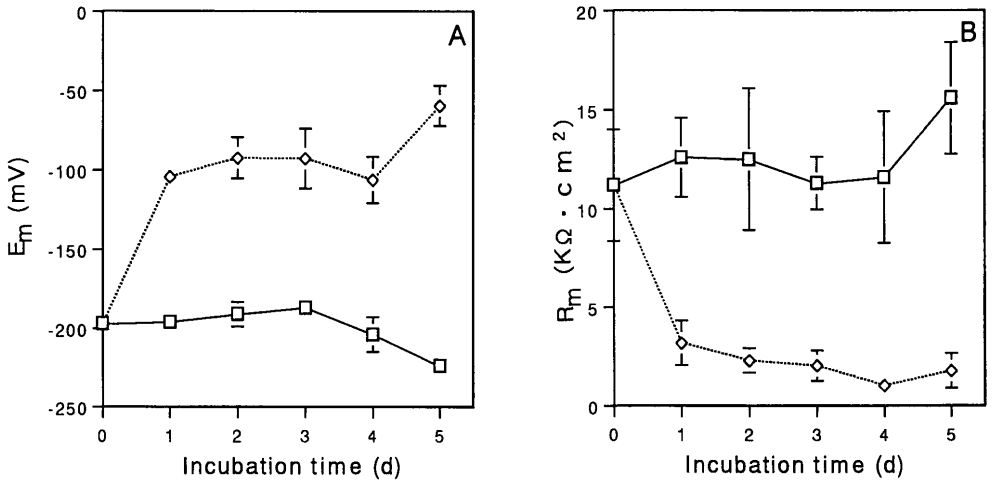

Fig. 1 Long-term effects of Al on E_m (A) and R_m (B). Cells were incubated in APW (pH 5.6; □) or in Al medium (pH 4.5; ◇). At 24-h intervals, E_m and R_m were measured in APW (pH 5.6). In all figures, mean results are shown with S.E. (bars).

11.2 KΩ cm². During the treatment with Al, it decreased dramatically. In control cells, R_m remained almost constant for 4 d and then increased slightly on the fifth day.

It was clear that Al caused extensive depolarization of the membrane within 24 h. The time courses of changes in E_m and R_m at the early stages of treatment were analyzed in further detail. E_m changed gradually in the positive direction and reached −125 mV after 3 h of treatment (Fig. 2A). The pattern of changes in R_m was different. For the first hour of treatment, R_m remained unchanged and then it decreased gradually (Fig. 2B).

In the above experiments, the pH of the Al medium was 4.5. Since we suspected that low pH per se might affect E_m and R_m, we examined the effects of low pH (Table 1). Cells were incubated in APW, the pH of which had been adjusted to 4.5 with HCl, for one day. After this treatment, we measured E_m in APW (pH 5.6) and found that it was −144 mV (Exp. 3). This value was significantly more positive than the E_m of cells incubated at pH 5.6 (Exp. 1; −183 mV). R_m was also decreased by the treatment with acid APW. Thus, the results in Fig. 1 and 2 were due to the effects of both Al and low pH. The E_m (−183 mV) of cells incubated in APW of pH 5.6 in this experiment was slightly more positive than that (−197 mV) of cells for which results are shown in Fig. 1A. This difference was caused by the difference between cultures.

When cells were incubated for 24 h in APW, the pH of which had been adjusted at 5.0 with HCl (Table 1, Exp. 2), E_m was found to be similar to that of cells incubated at pH 5.6 (Exp. 1). R_m was increased to some extent by the incubation in APW (pH 5.0). When cells were incubated in Al medium at pH 5.0 for 24 h (Exp. 4), an E_m of −142 mV was re-

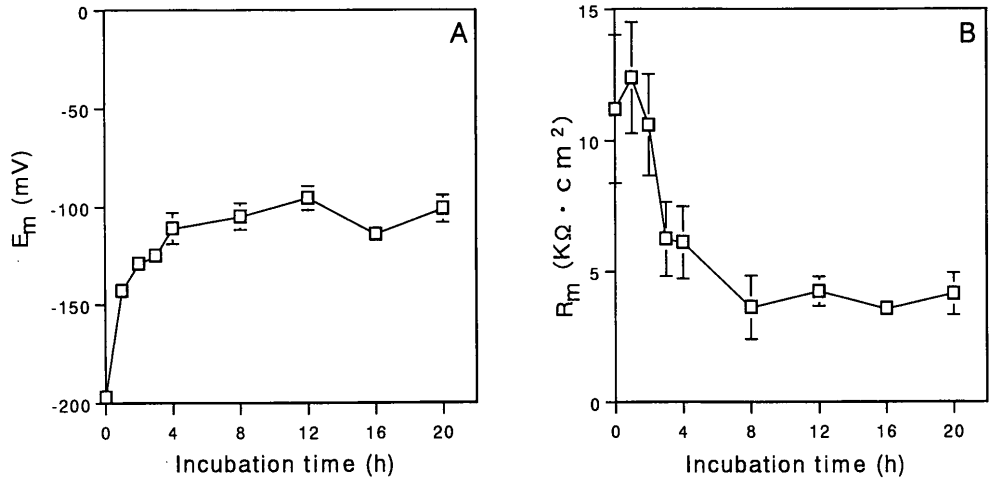

Fig. 2 Short-term effects of Al on E_m (A) and R_m (B). Cells were incubated in Al medium (pH 4.5). E_m and R_m were measured in APW (pH 5.6).

Table 1 Effects of pH and aluminum on E_m and R_m

Exp.	$AlCl_3$ (mM)	pH	E_m (mV)	R_m (KΩ cm^2)
1	0	5.6	−183±5	12±1
2	0	5.0	−186±3	19±3
3	0	4.5	−144±2	6±1
4	0.1	5.0	−142±9	20±5

Cells were incubated for 24 h. E_m and R_m were measured in APW (pH 5.6).

corded. Comparing this value with the E_m of cells that had been incubated in APW of pH 5.0 (Exp. 2, −186 mV), we can conclude that Al itself had a depolarizing effect on the membrane at pH 5. R_m was not affected within the initial 24-h period.

It has been reported that, in higher plants, cations can have an ameliorating effect on Al toxicity (Kinraide and Parker 1987, Kinraide et al. 1994). We examined the effects on Al toxicity of $CaCl_2$ and KCl. Cells were incubated in Al medium (pH 4.5) supplemented with $CaCl_2$ at 0, 1 and 10 mM. E_m was then measured every 4 h in APW (pH 5.6); (Fig. 3A). $CaCl_2$ at 1 mM did not have an ameliorating effect on the membrane depolarization caused by the treatment with Al. $CaCl_2$ at 10 mM partially reversed the depolarizing effect of Al. The addition of 10 mM $CaCl_2$ suppressed the decrease in R_m caused by Al treatment (Fig. 3B). When cells were incubated in Al medium (pH 4.5) supplemented with 10 mM KCl, both E_m and R_m changed in almost the same manner as they did in cells incubated in Al medium (pH 4.5) without KCl (data not shown).

In the next experiments, we examined the reversibility of the effects of Al (Fig. 4). After incubation in Al medium (pH 4.5) for 4 h, cells were separated in two groups. One group was kept in Al medium (pH 4.5) and the other group was transferred to APW (pH 5.6). E_m and R_m were then measured every 4 h in APW (pH 5.6). After transfer of Al-treated cells to APW, E_m changed slightly in the negative direction but did not return to the original value (Fig. 4A). R_m decreased slightly after the transfer to APW (Fig. 4B).

It has been reported that Al-tolerant higher plants excrete organic acids for the detoxification of Al ions (Delhaize et al. 1993, Basu et al. 1994, Pellet et al. 1995, Ryan et al. 1995). We examined whether malic acid and citric acid might have an ameliorating effect on damage due to Al in characean cells. Cells were divided into three groups and the groups were incubated in Al medium, in Al medium supplemented with 1 mM malic acid (sodium salt) and in Al medium supplemented with 1 mM citric acid (sodium salt), respectively. The pH of all Al media was adjusted to 4.5 with HCl. After treatment of cells for 4 h, we measured E_m and R_m in APW (pH 5.6) (Table 2). The E_m of cells incubated in Al medium was −112 mV, a value that was significantly more positive than that of untreated cells, as shown in Fig. 2A. However, the E_m of cells incubated in Al medium supplemented with either malic acid or citric acid was significantly more negative than that of cells incu-

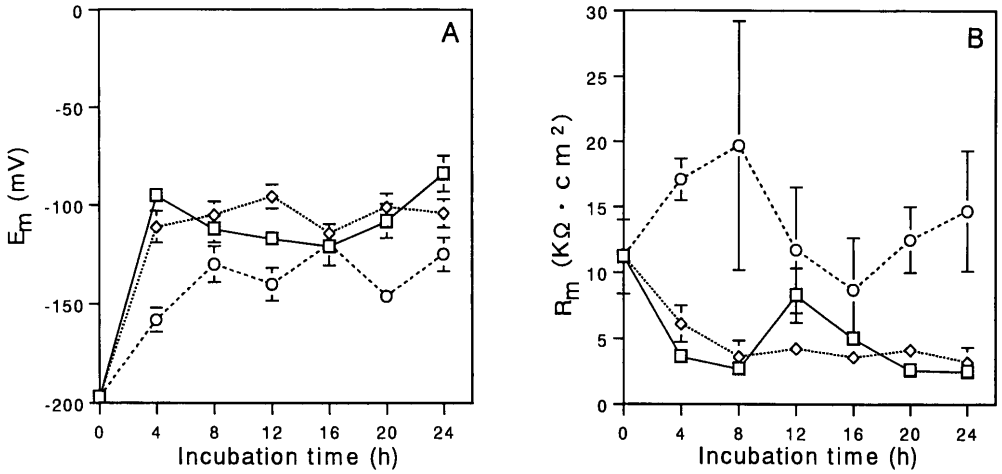

Fig. 3 Ameliorating effects of $CaCl_2$. Cells were incubated in Al medium (pH 4.5) supplemented with $CaCl_2$ at various concentrations as follows: ◇, 0 mM; □, 1 mM; ○, 10 mM. At 4-h intervals, E_m (A) and R_m (B) were measured in APW (pH 5.6).

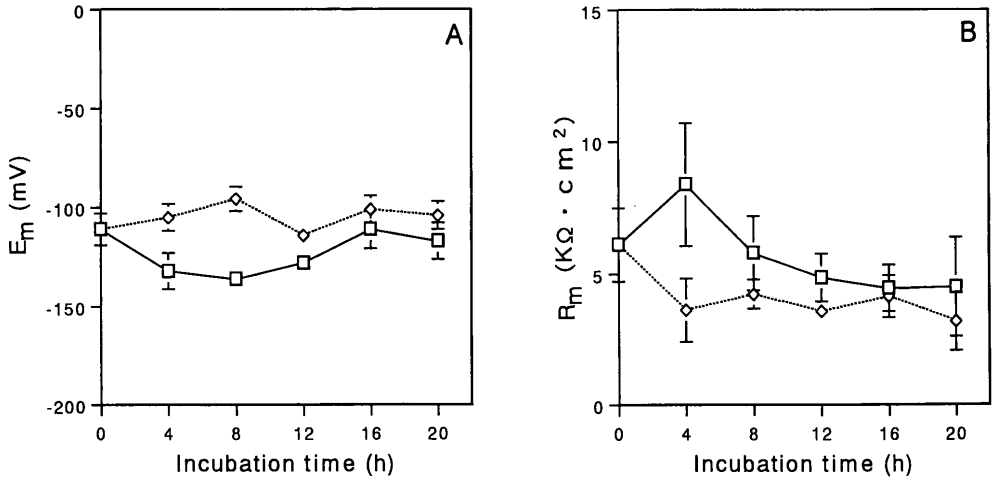

Fig. 4 Irreversible effects of Al on E_m (A) and R_m (B). Cells were incubated in Al medium (pH 4.5) for 4 h and were separated into two groups. One group was incubated in Al medium (pH 4.5; ◇) and the other group in APW (pH 5.6; □). At 4-h intervals, E_m and R_m were measured in APW (pH 5.6).

bated in Al medium without an organic acid.

We next examined the effects of organic acids on the recovery from damage by Al. Cells were treated with Al medium (pH 4.5) for 4 h. They were then separated into three groups and incubated in APW (pH 5.6), APW supplemented with 1 mM malic acid (pH 5.6) and APW supplemented with 1 mM citric acid (pH 5.6), respectively. After about 4 h, we measured E_m in APW (pH 5.6); (Table 3). As already shown in Fig. 4, E_m did not recover during incubation in APW (pH 5.6). However, E_m recovered almost completely as a result of treatment with either organic acid.

We next examined the effect of citric acid on the recovery of cells after prolonged treatment with Al (Table 4). Cells were incubated in Al medium (pH 4.5) for 24 h and then transferred to APW supplemented with 1 mM citric acid (pH 5.6). Then, after 4, 24 and 72 h, we measured E_m in APW (pH 5.6). After the prolonged treatment with Al medium, cells had to be incubated in medium that contained the organic acid for an extended period for the complete recovery of E_m.

We investigated the effects of Al on the dependence on pH of E_m and R_m. Cells were incubated either in APW (pH 5.6) or in Al medium (pH 4.5) for 24 h. In preliminary experiments, we found that E_m recovered slightly upon incubation with a pH buffer (DMGA), if the duration of treatment with Al was relatively limited. Since the restorative effect of the buffer rendered our initial results ambiguous, we extended the incubation time in Al medium to 24 h. After the prolonged treatment with Al, E_m did not recover within a short period of treatment with the buffer (at least 1 h). After treatment of cells with Al (pH 4.5), we inserted a microelectrode into individual cells in unbuffered APW (pH 5.6). After E_m had stabilized, the external pH was changed in steps from 4.7 to 5.7 to 6.7 to 7.7 with appropriate buffers (Fig. 5). The E_m of cells that had been incubated in APW (pH 5.6) for 24 h exhibited strong dependence on the external pH. After incubation in Al medium (pH 4.5) for 24 h, the dependence on pH of E_m became less significant. The R_m of the cells used in this experiment was larger than that of cells used in experiments for which results are shown in Fig. 1 and 2. This difference in R_m was due to

Table 2 Ameliorating effects of organic acids on E_m

Medium	E_m (mV)
Al medium	−112±11
Al medium+1 mM malic acid	−173± 5
Al medium+1 mM citric acid	−164± 2

Cells were incubated in the respective media (pH 4.5) for 4 h and E_m was measured in APW (pH 5.6).

Table 3 Effects of organic acids on recovery after a 4-h treatment with aluminum

Treatment	E_m (mV)
APW	−120±14
APW+1 mM malic acid	−187± 9
APW+1 mM citric acid	−207± 2

Cells were incubated in Al medium (pH 4.5) for 4 h and then transferred to APW (pH 5.6), either to APW supplemented with 1 mM malic acid (pH 5.6) or to APW supplemented with 1 mM citric acid (pH 5.6). After a 4-h incubation, E_m was measured in APW (pH 5.6).

Table 4 Effects of citric acid on recovery after a 24-h treatment with aluminum

Recovery time (h)	E_m (mV)
4	−144± 9
24	−179± 5
72	−204±10

Cells were incubated in Al medium (pH 4.5) for 24 h and then allowed to recover in APW (5.6) supplemented with 1 mM citrate for the indicated periods of time.

differences between cultures. The R_m of cells incubated in APW ranged from 14–18 KΩ cm^2 at all pH values tested. In cells incubated in Al medium, R_m was about 5 KΩ cm^2 at all pH values tested (Fig. 5B).

We examined the effects of Al on the survival of cells using six different treatments. Ten cells were subjected to each treatment. Death of cell was recognized either by loss of turgor pressure or by decolorization. All treatments were repeated for five times and average values are shown in Fig. 6. We obtained the following results. (1) When cells were kept in APW (pH 5.6), almost all cells survived for 5 d at least. (2) Upon incubation in Al medium (pH 4.5), only 24% of cells survived. (3) When cells were pre-incubated in Al medium (pH 4.5) for 4 h and then incubated in APW (pH 5.6), 44% of cells survived. (4) Upon incubation in Al medium supplemented with 10 mM $CaCl_2$ (pH 4.5), 70% of cells survived for 5 d at least. (5) When cells were incubated in Al medium supplemented with 1 mM malic acid (pH 4.5), 96% of cells survived. (6) Upon incubation in Al medium supplemented with 1 mM citric acid (pH 4.5), 86% of cells survived for 5 d at least. We also studied the effect of low pH on the survival of cells. Ten cells were incubated in APW, the pH of which had been adjusted to 4.5 with HCl.

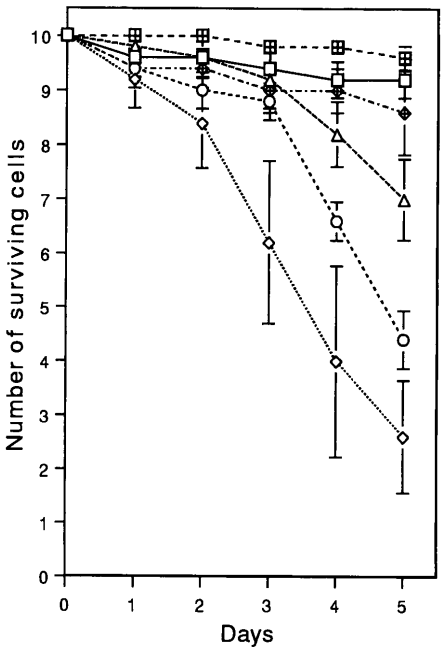

Fig. 6 Effects of $CaCl_2$ and organic acids on the survival of cells in Al medium. Ten cells were subjected to each treatment and all treatments were repeated five times. Average values are shown with S.E. □, Cells were incubated in APW (pH 5.6); ◇, cells were incubated in Al medium (pH 4.5); ○, cells were pre-treated by incubation in Al medium (pH 4.5) for 4 h and then they were incubated in APW (pH 5.6); △, cells were incubated in Al medium (pH 4.5) supplemented with 10 mM $CaCl_2$; ⊞, cells were incubated in Al medium supplemented with 1 mM malic acid (pH 4.5); ◈, cells were incubated in Al medium supplemented with 1 mM citric acid (pH 4.5).

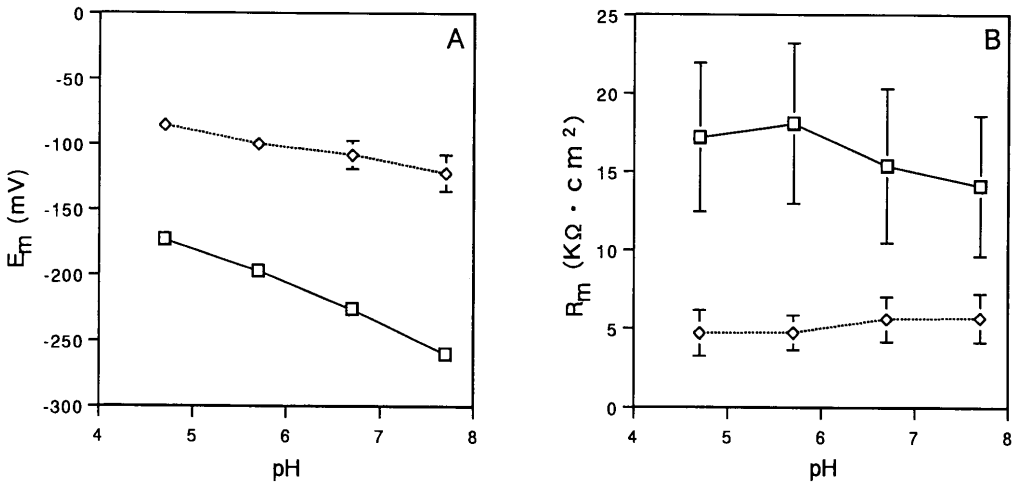

Fig. 5 Effects of Al on the dependence on pH of E_m (A) and R_m (B). Cells were incubated in APW (pH 5.6; □) or in Al medium (pH 4.5; ◇) for 24 h. After incubation, a microelectrode was inserted into cells in APW (pH 5.6). After E_m had stabilized, the pH of the external medium was changed in steps from 4.7 to 5.7 to 6,7 and finally to 7.7.

After 5 d, all cells were still alive.

Discussion

The technique of electrophysiology can provide very detailed insights into the functional integrity of cell membranes. Reid et al. (1995) reported that the cell membrane of *Chara* was slightly hyperpolarized by addition of Al to the external medium. Our results are apparently inconsistent with their results. However, the two sets of experiments differ in some respects. (1) Reid et al. (1995) measured E_m 30 min after the addition of $AlCl_3$, while we started measurements at least 60 min after the start of treatment. (2) Although Reid et al. (1995) measured E_m in the presence of Al, we measured it in APW without Al. (3) The most important difference relates to the pH of the external medium during measurements of E_m. Reid et al. (1995) measured E_m at pH 4.4, while we measured E_m in APW at pH 5.6. Kawamura et al. (1980) reported that the E_m of cells that contained ATP and that of cells that lacked ATP was almost the same at low pH in tonoplast-free cells. Their results indicates that the component of E_m generated by the electrogenic proton pump (active component of E_m) is masked at low pH. Therefore, the effect of Al on the electrogenic activity of the proton pump is overlooked if E_m is measured at low pH. The hyperpolarization of E_m by treatment with Al at low pH is probably caused by a change in ion (K^+) permeability, as suggested by Reid et al. (1995).

At higher pH, the active component of E_m is more significant (Kawamura et al. 1980). Therefore, a higher pH during measurements of E_m is recommended when one examines the effect of Al on electrogenesis. When APW is prepared at a high pH, a buffer is generally used. We found that, at the beginning of the treatment with Al, E_m recovered slightly upon addition of buffer to APW. The restorative effect of buffer, therefore, rendered the results ambiguous. However, after prolonged treatment with Al medium, this restorative effect of buffer was not observed within a short period of time (at least 1 h). To avoid even limited recovery due to the buffer, we measured E_m in unbuffered APW (pH 5.6). Since the active component of E_m is significant at pH 5.6 (Kawamura et al. 1980), we were able to examine the effect of Al on the electrogenic activity of the proton pump by measuring E_m in unbuffered APW (pH 5.6).

It has been reported that Al induces various changes in plant membranes, such as a decrease in membrane fluidity (Vierstra and Haug 1978), an increase in permeability to nonelectrolytes, a decrease in membranes partiality for lipid permeators and a decrease in permeability to water (Zhao et al. 1987). The present electrophysiological study showed that electrogenesis at the membrane was inhibited by treatment with $AlCl_3$. Since E_m was rapidly affected by $AlCl_3$ (Fig. 2), we suggest that Al might attack the membrane from the outside, at least at the initial stages of treatment. This suggestion is supported by the fact that the membrane recovered from Al damage when organic acids were applied externally (Table 3, 4). Since values of pK_1 of citric acid and malic acid are 3.1 and 3.4, respectively, both organic acids are negatively charged at pH 5.6. Therefore, it is suggested that these organic acids can not freely permeate the plasma membrane and exert their restorative effects by removing tightly attached aluminum ions from the cell surface. When cells were transferred to APW that lacked an organic acid (pH 5.6) after a 4-h incubation in Al medium, E_m and R_m did not recover (Fig. 4), an indication that the association of aluminum ions with the membrane was strong. Jones and Kochian (1997) reported that Al does not inhibit the plasma membrane H^+-ATPase and that Al binds to liposomes. Binding of Al to liposomes was prevented by citric acid and malic acid. It is suggested that Al might inhibit electrogenesis by binding to phospholipids of the plasma membrane in *Chara*.

The ability of Al in the medium to depolarize the membrane decreased when the pH was increased from 4.5 to 5.0 (compare Fig. 1A and Exp. 4 in Table 1). A decrease in the depolarizing effect of the Al medium caused by an increase in pH might have two explanations. The depolarizing effect of low pH was removed (compare Exp. 2 and 3 in Table 1) and the concentration of the toxic ionic species (Al^{3+}) was decreased by the increase in pH (Kinraide 1991).

E_m after treatment with Al (pH 4.5) was about -100 mV at pH 5.6 (Fig. 1, 2). When the activity of the electrogenic proton pump is inhibited by depletion of either ATP or Mg^{2+} ions in tonoplast-free cells, E_m depolarizes to about -100 mV (Shimmen and Tazawa 1977). We postulated that the active component of E_m generated by the electrogenic proton pump might be lost upon treatment with Al. We examined this possibility by analyzing the dependence of E_m on the external pH (Fig. 5). Kawamura et al. (1980) reported that the active component of E_m, generated by the electrogenic proton pump, is sensitive to the external pH. As seen in Fig. 5, E_m of cells incubated in APW (pH 5.6) was strongly dependent on the external pH. Incubation of cells in Al medium (pH 4.5) resulted in a decrease in the dependence of E_m on pH, an indication that the active component of E_m had become smaller. Two possibilities can be suggested to explain the decrease in the active component of E_m by the treatment with Al. The pump current might have been inhibited by Al, and/or the pump current might have been short-circuited by an increase in the conductance of the passive diffusion channel. This second possibility is supported by the fact that R_m was decreased by the treatment with Al (Fig. 1B). R_m might be decreased by an increase in permeability to K^+ ion as suggested by Reid et al. (1995). Activation of anion channels by Al has been reported in wheat roots (Ryan et al. 1997).

After a 1-h treatment of cells with Al medium (pH

4.5), E_m changed significantly in the positive direction but R_m was unchanged (Fig. 2). This result might be explained as follows. Upon treatment with Al (pH 4.5) for 1 h, E_m changed in the positive direction as a result of partial inhibition of the pump current. Prolonged treatment resulted in further depolarization due to an increase in the conductance of the passive diffusion channel, in addition to inhibition of the pump current. Upon treatment with Al at pH 5.0 for 24 h (compare results for Exp. 2 and 4 in Table 1), E_m changed in the positive direction to some extent but R_m was unchanged even after 24 h. The pump current might have been partially inhibited but conductance of diffusion channels was unaffected. In Al medium (pH 4.5), R_m decreased dramatically after 24 h (Fig. 1B). The decrease in R_m in Al medium (pH 4.5) might have been caused by low pH, at least at the beginning of the treatment (24 h), since R_m decreased dramatically after a 24-h incubation of cells in APW (pH 4.5) that lack Al (compare results for Exp. 1 and Exp. 3 in Table 1).

In Al-tolerant wheat, Al stimulates the efflux of malic acid from roots (Delhaize et al. 1993, Basu et al. 1994, Ryan et al. 1995). In Al-tolerant maize, the efflux of citric acid is stimulated by Al (Pellet et al. 1995). In both cases, it has been suggested that organic acids excreted from roots detoxify Al, with the result that these plants are able to tolerate exposure to Al. In the present study, both organic acids had significant ameliorating effects (Table 2). In addition, organic acids appeared to act by removing tightly attached Al ions from the surface of cells (Table 3, 4). It is unknown whether characean cells excrete organic acids upon treatment with Al. However, characean cells appear to be a suitable material for analyzing the ameliorating effects of organic acids.

The death of cells and the inhibition of electrogenesis at the membrane upon various treatments were clearly correlated (Fig. 6). Simple removal of $AlCl_3$ from the external medium after a 4-h incubation in Al medium failed to restore electrogenic activity (Fig. 4). Reflecting this result, more than 50% of cells died after 5 d (Fig. 6). Organic acids had a strong ameliorating effect on the damage to electrogenicity caused by Al. Thus, most cells survived in Al medium supplemented with an organic acid. In summary, therefore, we conclude that the plasma membrane is a primary target of Al, at least at the initial stages of treatment with Al. The ameliorating effect of malic acid was stronger than that of citric acid, and this result is inconsistent with the report that citric acid is a stronger chelator of Al than malic acid and has stronger ameliorating effect in higher plants (Ownby and Popham 1989). The reason for this discrepancy remains to be resolved.

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