Effects of Aluminum on Plasma Membrane as Revealed by Analysis of Alkaline Band Formation in Internodal Cells of *Chara corallina*

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ABSTRACT. To study the mechanism of aluminum toxicity in plant cells, the effects of aluminum on alkaline band formation were analyzed in the internodal cells of *Chara*. After cells were treated with AlCl_3 , they were examined for their capacity to develop alkaline bands. Treating cells with AlCl_3 medium at pH 4.5 completely inhibited alkaline band formation. When either CaCl_2 or malic acid was added to the AlCl_3 medium (pH 4.5), it did not produce an ameliorative effect, whereas addition of both CaCl_2 and malic acid induced a significant ameliorative effect. It was found that treatment at pH 4.5 in the absence of AlCl_3 strongly inhibited alkaline band formation. This inhibition by the low pH (4.5) treatment was effectively ameliorated by CaCl_2 . At higher pH (5.0), malic acid alone produced a significant ameliorative effect on aluminum inhibition of alkaline band formation, but CaCl_2 did not. Recovery from aluminum inhibition was also studied. When cells treated with AlCl_3 at pH 4.5 were incubated in artificial pond water, they could not recover the capacity to develop alkaline band. When either malic acid or CaCl_2 was added to artificial pond water, cells recovered their alkaline band formation. It was concluded that one of the primary targets of aluminum is the plasma membrane and that aluminum affects the plasma membrane from the cell exterior at the beginning of the treatment (within 24 h). It was also suggested that the aluminum treatment impairs the HCO_3^- influx mechanism but not the OH^- efflux mechanism.

Key words: Alkaline band/Aluminum/ Ca^{2+} /*Chara*/Malic acid/ OH^-

Aluminum is one of the most abundant elements in the soil and is not toxic in its insoluble form. However, it has a strong toxic effect on plants when solubilized in acidic soil (8). The most prominent effect of aluminum first appears in the roots, which are directly exposed to this form of soluble aluminum (2, 4, 17, 35). Attention has been focused on the plasma membrane as the primary target, where aluminum appears to significantly affect the ion flux across the plasma membrane (7, 10, 11, 12, 13, 25, 27, 32, 33, 43). Aluminum also affects the permeability of the plasma membrane to nonelectrolytes and water (45) and appears to decrease the fluidity of the membrane (42).

These results strongly support the hypothesis that the primary target of aluminum is the plasma membrane.

Some plants show tolerance against aluminum. Based on an analysis of plasma membrane vesicles isolated from aluminum-sensitive and -tolerant wheat, Yermiyahu *et al.* (44) suggested that the greater part of aluminum tolerance is based on a mechanism reducing the amount of aluminum that can reach the plasma membrane. For this tolerance mechanism, the binding of aluminum by organic acids at the outer surface of the plasma membrane has been suggested, and the exudation of organic acids upon aluminum treatment has been reported in many aluminum tolerant plants (1, 6, 24, 28, 30, 34). Citric acid applied exogenously protects intact plants (29) and membrane vesicles (39) from the perturbative effects of aluminum, and transgenic plants overproducing citric acid had been shown to have an increased aluminum tolerance (5).

Characean cells have been widely used for studying membrane phenomena of plant cells (9, 37), and may provide a model system for the analysis of the alumi-

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Abbreviations: APW, artificial pond water; BFS, band formation solution; TAPS, N-tris(hydroxymethyl) methyl-3-aminopropane-sulfonic acid.

num syndrome. However, analysis of the aluminum effect in Characeae is sparse (31, 40). In our previous paper, we analyzed the effects of aluminum using the electrophysiological method (40), which is the most sensitive way to quantitatively evaluate ionic fluxes. In aquatic plants, photosynthesis induces dramatic changes of ion transport at the plasma membrane. Since most of the carbon dioxide takes the form of HCO_3^- at the pH region of habitat of Characeae, cells must use HCO_3^- as a carbon source. Since CO_2 is used as a substrate of photosynthesis in the cell, OH^- must be extruded as a waste to the cell exterior, otherwise the cytoplasmic pH would have to increase considerably. Since cells extrude OH^- at specialized regions of the cell surface, so-called alkaline bands are formed on the cell surface (19). Two membrane transport phenomena are involved in this alkaline band formation, HCO_3^- influx and OH^- efflux (20). The change of pH at the cell surface can be visualized using a pH indicator dye, phenol red (38). In the present study, we analyzed the effect of aluminum on alkaline band formation in order to study the effects of aluminum on the transport process occurring across the Characean plasma membrane.

Materials and Methods

Chara corallina was cultured as reported previously (26). Internodal cells were isolated from neighboring cells and kept in artificial pond water (APW) containing 0.1 mM KCl, 1 mM NaCl and 0.1 mM CaCl_2 (pH about 5.6).

To examine the capacity of the cell to form alkaline bands, a band formation solution (BFS) was used, which was prepared by adding 0.5 mM NaHCO_3 and 5 mg/100 ml phenol red to APW (pH 6.5–7). Just after preparation, the pH of BFS was about 6.5 and gradually increased with time. BFS was freshly prepared just prior to use. A polyacrylate block with channels was prepared; the width, depth and length of the channel were about 3 mm, 3 mm and 80 mm, respectively. An internodal cell was rinsed with BFS and then placed in this channel, after which it was gently washed with BFS two or three times. After the channel was filled with BFS, it was overlaid with a thin sheet of polyacrylate (about 2 mm). The polyacrylate block was placed in a transparent polyacrylate box, the bottom of which was filled with a thin layer of pure water. The lid was put on the box to avoid evaporation of solution in the channel. Cells were illuminated with fluorescent lamps ($200 \mu\text{E}/\text{m}^2/\text{sec}$) for 20 min at 24–27°C.

Before aluminum treatment, internodal cells were examined to ascertain the extent of alkaline band formation. Only cells having a strong and stable banding pattern were used for analysis. Aluminum treatment was carried out by incubating internodal cells in APW supplemented with 0.1 mM AlCl_3 . When 0.1 mM AlCl_3 was added to APW, the pH value of the medium was about 4.5. In some experiments, either 10 mM

CaCl_2 or 1 mM malic acid (2 Na) was added to the aluminum medium and the pH was then adjusted to 4.5 with HCl. In a parallel series of experiments, 0.2 mM AlCl_3 was added to APW and the pH value was adjusted to 5.0 with NaOH. To examine the effect of acidic pH treatment on alkaline band formation, cells were treated in APW in which the pH value had been adjusted to either 4.5 or 5.0 with HCl. After the treatment with aluminum or low pH, cells were thoroughly washed with BFS and then examined in terms of their ability to develop alkaline bands. When a cell formed even a faint alkaline band after 20 min, it was scored as being capable of developing alkaline band(s). When incubation time in BFS was prolonged, the number of cells showing alkaline bands increased in some experiments. However, only cells showing band(s) after 20 min incubation were scored as being capable of developing alkaline band(s). The results were recorded by taking photographs.

To examine whether the mechanism of OH^- efflux is damaged by the aluminum treatment, an alkaline-loading experiment was carried out. Cells were incubated in APW supplemented with 1 mM NH_4Cl and 1 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) (pH 8.7 adjusted with NaOH) for 30 min at room temperature. The cytoplasmic pH increases due to the protonation of NH_3 passively flowing into the cytoplasm (3, 18). After cells were rinsed with BFS two or three times, they were placed in channels prepared in polyacrylate blocks (see above) and then incubated in darkness for 15–30 min.

Results

After confirming that *Chara* cells had the potential to form alkaline bands (Fig. 1a), they were treated in 0.1 mM AlCl_3 (pH 4.5) for 4 or 24 h and then examined for their capacity to develop alkaline bands. In all cells examined, alkaline band formation was completely inhibited (Table I, Exps. 1 and 2). Figure 1b shows a cell treated with 0.1 mM AlCl_3 (pH 4.5) for 24 h, indicating the absence of alkaline bands. Since it has been reported that Ca^{2+} has an ameliorating effect on aluminum toxicity in higher plants (15, 16), we next examined the effect of Ca^{2+} on aluminum-induced inhibition of alkaline band formation. Although 10 mM CaCl_2 was supplemented to the aluminum medium (pH 4.5), alkaline band formation was inhibited in most cells (Table I, Exp. 3). Strong ameliorative effects of organic acid have also been reported in various plants as summarized in the *Introduction*. We also reported that malic acid had a strong ameliorative effect on the aluminum inhibition of electrogenic transport in *Chara* (40). Cells were incubated in 0.1 mM AlCl_3 (pH 4.5) supplemented with 1 mM malic acid for 4 h and then examined for alkaline band formation. Unexpectedly, none of cells formed alkaline bands (Table I, Exp. 4). The combined effects of Ca^{2+} and malic acid in amelio-

Fig. 1. Inhibition of alkaline band formation by treatment with aluminum. A cell showed significant alkaline bands before treatment with AlCl_3 (a). By treatment in 0.1 mM AlCl_3 (pH 4.5) for 24 h, alkaline band formation was completely inhibited (b). When the cell was treated in APW supplemented with 1 mM NH_4Cl and 1 mM TAPS (pH 8.7) for 30 min, it strongly alkalized the external medium (c). Bar represents 5 mm.

rating the aluminum inhibition were next examined. Cells were incubated in 0.1 mM AlCl_3 (pH 4.5) supplemented with 10 mM CaCl_2 and 1 mM malic acid for 4 h and then examined for alkaline band formation. All cells examined formed alkaline bands, indicating that Ca^{2+} and malic acid exert a strong combined effect (Exp. 5).

Takabatake and Shimmen (40) reported that treatment of *Chara* cells at low pH in the absence of AlCl_3 caused partial inhibition of electrogenesis and drastic increase in electric conductance of the plasma membrane. Therefore, the effect of low pH treatment on alkaline band formation was also examined. When cells were incubated in APW (pH 4.5) for 4 h, alkaline band formation was inhibited in half of the cells examined (Exp. 6). The ameliorative effect of Ca^{2+} on inhibition of alkaline band formation by low pH treatment was

examined. Cells were incubated for 4 h in APW (pH 4.5) supplemented with 10 mM CaCl_2 . In all cells examined, alkaline band formation was observed (Exp. 7). Next, the ameliorating effect of malic acid on low pH damage was investigated. Cells were incubated in APW (pH 4.5) supplemented with 1 mM malic acid for 4 h. Alkaline band formation was observed only in 3 out of 8 cells examined (Exp. 8).

Collectively, these experiments established that either low pH (4.5) or AlCl_3 treatment inhibits alkaline band formation. Ca^{2+} has an ameliorative effect on the inhibition by low pH and malic acid on the inhibition by aluminum. Therefore, both malic acid and Ca^{2+} had to be added to ameliorate the aluminum inhibition at pH 4.5. To confirm this finding, the aluminum effect was examined at higher pH values. However, it is impossible to significantly increase the pH, as aluminum

Table I. EFFECTS OF VARIOUS TREATMENT ON ALKALINE BAND FORMATION

Exp.	Treatments					Results (a/b) [#]
	AlCl_3 (mM)	pH	Malic acid (mM)	CaCl_2 (mM)	Time (h)	
1	0.1	4.5	0	0.1	4	0/21
2	0.1	4.5	0	0.1	24	0/10
3	0.1	4.5	0	10.1	4	2/10
4	0.1	4.5	1	0.1	4	0/11
5	0.1	4.5	1	10.1	4	11/11
6	0	4.5	0	0.1	4	6/12
7	0	4.5	0	10.1	4	8/8
8	0	4.5	1	0.1	4	3/8
9	0	5.0	0	0.1	4	11/13
10	0.2	5.0	0	0.1	4	0/13
11	0.2	5.0	1	0.1	4	11/14
12	0.2	5.0	1	0.1	24	6/7
13	0.2	5.0	0	10.1	4	1/13
14	0.2	5.0	0	10.1	24	1/13

After treatment by aluminum or low pH, cells were incubated in BFS to examine alkaline band formation.

[#] a: number of cells developed alkaline bands, b: number of cells examined.

in solution changes its form depending on the pH. Al^{3+} , which is supposed to be most toxic, is abundant at lower pH values and decreases dramatically as the pH value increases (14). In addition, aluminum solution forms a precipitate at higher pH values, and so, pH 5 was chosen for the following experiments. When cells were treated in APW (pH 5.0) for 4 h, alkaline band formation was observed in 11 out of 13 cells examined (Exp. 9). Since the concentration of toxic Al^{3+} at pH 5 is about 1/2 of that at pH 4.5 (14), the concentration of AlCl_3 was increased to 0.2 mM. When cells were incubated in 0.2 mM AlCl_3 (pH 5) for 4 h, alkaline band formation was completely inhibited in all cells examined (Exp. 10). Next, the ameliorative effects of malic acid and Ca^{2+} on the aluminum inhibition were examined at pH 5.0. Cells were incubated in 0.2 mM AlCl_3 (pH 5.0) supplemented with 1 mM malic acid for 4 h and then examined for alkaline band formation. Alkaline band formation was observed in 11 out of 14 cells examined (Exp. 11). When the treatment time in 0.2 mM AlCl_3 (pH 5.0) supplemented with malic acid was increased to 24 h, a similar result was obtained (Exp. 12). Cells were incubated in 0.2 mM AlCl_3 (pH 5.0) supplemented with 10 mM CaCl_2 for 4 or 24 h. In both cases, alkaline band formation was observed only in one out of 13 cells examined (Exps. 13 and 14). These results strongly suggest that malic acid has a strong ameliorative effect on aluminum inhibition but that Ca^{2+} does not.

The reversibility of aluminum inhibition was examined (Table II). After cells were treated in 0.1 mM AlCl_3 (pH 4.5) for 24 h, they were given a recovery treatment in APW (pH 5.6) for 24 h. However, after this incubation in APW, alkaline band formation did not recover in any of the cells (Exp. 1). When 1 mM malic acid was added to APW (pH 5.6), all cells showed alkaline bands (Exp. 2). In another experiment, addition of 10 mM CaCl_2 resulted in 71% recovery (Exp. 3). The reversibility of inhibition by low pH treatment was also examined. After 24 hr incubation in APW (pH 4.5) in the absence of AlCl_3 , only one cell

formed alkaline band out of 10 cells examined. Cells were subjected to recovery treatment in APW (pH 5.6) for 24 hr. Nine cells formed alkaline bands, and one cell showed weak and uniform alkalization along the whole surface.

It is suggested that at least two membrane transport mechanisms are involved in the formation of alkaline bands, influx of HCO_3^- and efflux of OH^- . Lucas *et al.* (22) suggested co-transport with H^+ for HCO_3^- uptake. In the following experiments, we examined whether the mechanism of OH^- efflux was inhibited by the aluminum treatment. After cells were incubated in 0.1 mM AlCl_3 (pH 4.5) for 24 h, they were incubated in APW supplemented with 1 mM NH_4Cl and 1 mM TAPS (pH 8.7) for 30 min. Cells were thoroughly washed with BFS and were examined for OH^- efflux in the dark. As seen in Figure 1c, cells extruded OH^- , although bands were not formed. Alkaline-loading experiment was also carried out using aluminum-untreated cells. Although alkalization of the external medium occurred, again there was no band formation (data not shown). It was suspected that release of OH^- bound to the cell wall may have resulted in alkalization of the medium. To test this possibility, the same experiment was carried out using isolated cell walls. Both ends of an internodal cell were removed by cutting and the cellular contents were squeezed out. Cell walls thus prepared were incubated in NH_4Cl solution for 30 min. After washing with BFS, the cell wall preparations did not induce alkalization of the bathing medium, indicating that cell wall is not concerned with OH^- efflux from alkaline-loaded cells.

The effect of aluminum treatment and alkaline loading on cytoplasmic streaming was studied (Table III). In control cells, the velocity of cytoplasmic streaming was 100 $\mu\text{m/s}$ (Exp. 1). After treatment in 1 mM NH_4Cl for 30 or 60 min, the velocity was reduced to 59 $\mu\text{m/s}$ (Exp. 2), but treatment of cells in 0.1 mM AlCl_3 (pH 4.5) for 24 h did not inhibit cytoplasmic streaming (Exp. 4). When cells treated with AlCl_3 for 24 h were incubated in 1 mM NH_4Cl for 30 min, the ve-

Table II. REVERSIBILITY OF ALUMINUM INHIBITION OF ALKALINE BAND FORMATION

Exp.	Recovery condition		Results (a/b) [#]
	CaCl_2 (mM)	malic acid (mM)	
1	0.1	0	0/7
2	0.1	1	8/8
3	10.1	0	6/8

After treatment in 0.1 mM AlCl_3 (pH 4.5) for 24 h, cells were recovered in various conditions for 24 h. Alkaline band formation was then examined.

[#] a: number of cells developed alkaline bands, b: number of cells examined.

Table III. EFFECTS OF ALKALINE-LOADING ON CYTOPLASMIC STREAMING

Exp.	Aluminum treatment	Alkaline loading (min)	Velocity ($\mu\text{m/s}$)
1	—	0	100 ± 2
2	—	30	59 ± 3
3	—	60	59 ± 5
4	+	0	102 ± 2
5	+	30	53 ± 12
6	+	60	42 ± 13

In Exps. 4–6, cells were treated in 0.1 mM AlCl_3 (pH 4.5) for 24 h before alkaline loading.

locity decreased to 53 $\mu\text{m/s}$ (Exp. 5). Further incubation in NH_4Cl resulted in further inhibition of cytoplasmic streaming (Exp. 6). The extent of inhibition of cytoplasmic streaming by alkaline-loading was quite variable in aluminum-treated cells. Although the extent of inhibition in some aluminum-treated cells was similar to that in aluminum-untreated cells, it was often very great in other aluminum-treated cells, resulting in the large standard error in Experiments 5 and 6.

Discussion

The effects of aluminum on plasma membrane were studied by analyzing its influence on alkaline band formation. Treatment of cells with AlCl_3 at pH 4.5 or 5.0 resulted in the complete inhibition of alkaline band formation. Inhibition by AlCl_3 at pH 4.5 was ameliorated only in the presence of both CaCl_2 and malic acid. However, at pH 5, malic acid alone showed a significant ameliorative effect against aluminum inhibition. It was found that treatment at low pH (4.5) in the absence of AlCl_3 strongly inhibited formation of alkaline bands and this inhibition could be ameliorated by Ca^{2+} . Based on the results obtained in the present study, it would appear that the effect of low pH *per se* must be considered in studying the aluminum syndrome in plants. Characeae species living in the habitat of neutral or alkaline pH may be especially sensitive to low pH treatment. In our culture condition, the pH of the culture medium ranged between 7.3–8.4. We have reported that treatment of cells at low pH induced partial inhibition of electrogenesis and drastic increase in the membrane conductance (40).

In formation of alkaline bands, at least three processes are involved, influx of HCO_3^- across the plasma membrane, fixation of CO_2 in the cell and efflux of OH^- across the plasma membrane (20). The recovery experiments after the aluminum treatment suggested the site of aluminum action to cells. After the aluminum treatment, alkaline band formation was not recovered when cells were incubated in APW. Addition of malic acid to APW was very effective in recovery from an aluminum inhibition. As discussed in our previous paper (40), it seems reasonable to assume that malic acid rendered the recovery via an effect on the outer surface of the plasma membrane. Judging from the pKa values of malic acid (3.40, 5.05), almost all molecules would be present in the dissociated form, which is impermeable to the membrane. Thus, the exogenously added malic acid may remove the aluminum bound to the outside of the plasma membrane. Cytoplasmic streaming was not affected by the aluminum treatment (Table III, Exps. 1 and 4). This suggested that the intracellular pH and ATP concentration, both of which sensitively affect the rate of cytoplasmic streaming (36, 41),

were not significantly changed by the aluminum treatment. This finding also supports the hypothesis that aluminum exerted its effect at the cell exterior. Although Ca^{2+} did not show an ameliorative effect against AlCl_3 inhibition (Table I, Exps. 13 and 14), it was effective in mediating recovery after the AlCl_3 treatment (Table II, Exp. 3). The possibility is suggested that aluminum bound to the plasma membrane is easily dissociated by Ca^{2+} in the absence of AlCl_3 .

When aluminum-treated cells were subjected to an alkaline-loading protocol, they showed significant efflux of OH^- (Fig. 1C). This result unequivocally indicates that the mechanism of OH^- efflux was not impaired by the aluminum treatment *per se*. Inhibition of photosynthesis by the aluminum treatment is unlikely as aluminum appears to render its effects from the cell exterior, as discussed above. Therefore, we tentatively concluded that aluminum damaged the mechanism of HCO_3^- influx. Although strong alkalization of the external medium occurred after alkaline-loading, discrete bands were not formed. The importance of cytoplasmic streaming in alkaline band formation has been previously suggested (21, 23). It is suspected that cytoplasmic streaming was stopped by alkaline-loading protocol. Cytoplasmic streaming was inhibited but not completely stopped by the alkaline-loading (Table III). Thus, an unknown factor(s) responsible for the formation of bands may have been damaged by the aluminum treatment. However, aluminum-untreated cells also did not form bands after alkaline-loading treatment. This result suggested that the banding mechanism does not work upon uncontrolled alkalization of the cytoplasm. Partial inhibition of cytoplasmic streaming might also cause a reduction in alkaline banding capacity after such an alkaline-loading treatment.

The present study showed that aluminum strongly affects ion transport across the *Chara* plasma membrane and that the effect is not always indiscriminate. Aluminum seems to inhibit HCO_3^- but not OH^- transport. Thus, aluminum can be a useful tool for analysis of alkaline band formation in the giant cells of the Characeae.

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