Involvement of Membrane Potential in Alkaline Band Formation by Internodal Cells of Chara corallina

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Internodal cells of Chara corallina form alkaline bands on their surface upon illumination via photosynthesis. In the present study, the effect of KCl on alkaline band formation was analyzed. When the extracellular KCl concentration was increased, alkaline band formation was extensively inhibited. Electrophysiological analysis unequivocally showed the need for inner negative membrane potential for alkaline band formation.

**Keywords:** Alkaline band — Chara — Depolarization — K⁺ — Membrane potential — Photosynthesis.

Abbreviations: APW, artificial pond water; BFS, band formation solution; E_m, membrane potential.

**Introduction**

In aquatic habitats where plants and/or algae are growing thickly, the water pH changes extensively. Although the pH value is about 7 at night, it increases to about 10 in the daytime (e.g. Van et al. 1976). This pH change is closely related to the photosynthetic fixation of inorganic carbon.

Characean cells have been a suitable material for studies on plant cell biology, especially cytoplasmic streaming (Kamiya 1981) and ion transport (Tazawa and Shimmen 2001). One of the most significant advantages is the large size of the internodal and leaflet cells. In Chara corallina, alkaline bands are formed on the surface of these cells (Spear et al. 1969, Lucas and Smith 1973). Lucas and Smith (1973) suggested that alkalization of the external medium results from HCO₃⁻ uptake, CO₂ fixation and subsequent localized OH⁻ efflux. However, the incorporation mechanism of inorganic carbon is not fully understood (Walker et al. 1975, Price et al. 1985, Shiraiwa and Kikuyama 1989). Extracellular alkalization can be induced by either efflux of OH⁻ or influx of H⁺. Lucas and Ferrier (1980) suggested that alkaline bands are formed by efflux of OH⁻ but not influx of H⁺. Hereafter, we tentatively postulate OH⁻ efflux for simplification of the discussion.

Systematic analyses of alkaline band formation in Chara have been carried out by Lucas and his co-workers (cited in Lucas 1983). Chloroplasts are anchored to the stagnant gel layer over the whole area of the cell. Therefore, photosynthesis is carried out over the whole area, although distribution of the photosynthetic activity is not uniform (Plieth et al. 1994, Bulychev et al. 2001). However, OH⁻ is extruded at limited areas. The situation inevitably suggests that cytoplasmic streaming is necessary for delivering OH⁻ to the alkaline bands.

It seems reasonable simply to assume that the OH⁻ carrier of the plasma membrane is localized in the alkaline bands. However, OH⁻ is extruded over the whole surface of the internodal cells, when cytoplasmic streaming is inhibited by cytochalasin B (Lucas and Dainty 1977). This result suggested that the OH⁻ carrier is distributed over the whole surface of the plasma membrane. By intracellular perfusion, the tonoplast can be removed, producing a tonoplast-free cell (Williamson 1975, Tazawa et al. 1976). In tonoplast-free cells, the chemical composition of the cytoplasmic space can be freely controlled (Tazawa et al. 1987). Therefore, the pH value of the original cytoplasmic space can also be modified (Tazawa and Shimmen 1982). When the intracellular pH was increased in tonoplast-free cells, OH⁻ was extruded over the whole surface (Lucas and Shimmen 1981). After disintegration of the tonoplast, most cytoplasm detaches from actin bundles and only a small amount of cytoplasm slides along actin bundles, resulting in severe inhibition of intracellular transport. The result in tonoplast-free cells also indicates that the OH⁻ extrusion system is distributed over the whole surface of the plasma membrane.

Lucas (1976) reported that an increase in the KCl concentration of the external medium inhibited the incorporation of inorganic carbon when internodal cells were bathed in an alkaline solution but not in an acidic solution. In some cells, however, carbon fixation in the alkaline solution was not inhibited in the presence of KCl (Lucas 1976). It is speculated that a decrease in the incorporation of inorganic carbon has significant effects on formation of the alkaline band. In the present study, we examined the effect of KCl on alkaline band formation in combination with electrophysiology.

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Results

Effects of 10 mM KCl

Alkaline bands were formed in band formation solution (BFS) by 20 min illumination, and photographs were taken. After the external medium was replaced with BFS supplemented with 10 mM KCl, specimens were incubated for 60 min under illumination. The external medium was again exchanged with the same medium. After 20 min illumination, photographs were taken (Fig. 1). In most cells, the alkaline bands became very weak after the KCl treatment (Fig. 1, Cell 1), indicating significant inhibition of the \( \text{OH}^-/\text{CO}_2 \) efflux. However, strong alkaline bands were formed even after the KCl treatment in some cells (Fig. 1, Cell 2), indicating that the \( \text{OH}^- \) efflux was scarcely affected. Four among 22 cells formed strong bands after the 10 mM KCl treatment (Table 1, Experiment 1A).

The mode of inhibition was discrete, i.e. there was no intermediate inhibition. Therefore, the results are simply presented as either strong or weak in the following experiments.

Experiments were carried out in combination with electrophysiology. The microelectrode method was not suitable for analysis in the present study, where alkaline bands were visualized. An internodal cell was separated into pools A and B (Fig. 2). It was mounted so that the node in pool B was attached to the partition (P). Cell parts in pools A and B were electrically insulated by embedding the cell in the groove of P using white vaseline. Pools A and B were filled with BFS and 100 mM KCl, respectively. The major part of pool A was covered with a piece of glass in order to make the alkaline bands clearly visible. The distal end of pool A was opened in order to make a space for insertion of an electrode. After electrodes were inserted into both pools, the potential difference was measured. In the presence of 100 mM \( \text{K}^+ \), the membrane depolarizes to a level close to 0 mV. The node is very sensitive to \( \text{K}^+ \) and the membrane in pool B is promptly depolarized upon addition of 100 mM KCl (Shimmen 2008). The potential difference between the two pools represents the membrane potential (\( E_m \)) of the part of the cell in pool A (\( \text{K}^+ \)-anesthesia method, Shimmen et al. 1976). In the previous study, the medium in pool A had been made isotonic to 100 mM KCl by adding sorbitol (Shimmen et al. 1976). However, a decrease in the turgor pressure sometimes produces ill effects on the formation of alkaline bands (Lucas and Alexander 1981). Although pools A and B were not isotonic, a reasonable

Experiments 1–5 were carried out using internodal cells having native nodes. Experiment 6 was carried out using internodal cells lacking a native node. A: analysis without \( E_m \) measurement; the number of cells forming a strong alkaline band/number of cells examined. B: analysis using the \( \text{K}^+ \)-anesthesia method; the number of cells maintaining a strong alkaline band and negative \( E_m \)/number of cells examined.

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<th>Experiment No.</th>
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Experiments 1–5 were carried out using internodal cells having native nodes. Experiment 6 was carried out using internodal cells lacking a native node. A: analysis without \( E_m \) measurement; the number of cells forming a strong alkaline band/number of cells examined. B: analysis using the \( \text{K}^+ \)-anesthesia method; the number of cells maintaining a strong alkaline band and negative \( E_m \)/number of cells examined.

Fig. 1 Effect of 10 mM KCl on alkaline band formation. After 20 min illumination in BFS, photographs were taken (upper). The external medium was replaced with BFS supplemented with 10 mM KCl and kept for 60 min under illumination. The external medium was again replaced with the same medium. After 20 min illumination, photographs were taken (lower). In Cell 1, alkaline bands became weak after the KCl treatment. In Cell 2, however, strong alkaline bands were formed after the treatment. Scale bar = 5 mm.

Fig. 2 Simultaneous monitoring of alkaline band and membrane potential. An internodal cell was separated into pools A and B. It was mounted so that the node in pool B was attached to the partition (P). Cell parts in pools A and B were electrically insulated by embedding the cell into the groove of P using white vaseline. Pools A and B were filled with BFS and 100 mM KCl, respectively. The major part of pool A was covered with a sheet of glass (cov). The distal end of pool A was uncovered in order to make a space for insertion of an electrode. After electrodes were inserted into both pools (arrows), the potential difference was measured. After checking the occurrence of alkaline bands in pool A, analysis was started. The blue bar represents 5 mm. For further explanation, see the text. Scale bar = 5 mm.
membrane potential, \(-223 \pm 3\) mV \((n = 15)\), was measured in artificial pond water (APW) buffered with 5 mM HEPES-Tris (pH 7.0). This value was almost the same as that measured by the microelectrode method (Shimmen 2003). In addition, this highly negative \(E_m\) was stably maintained for \(>5\) h \((n = 5, \text{ data not shown})\).

After mounting on the chamber, cells were illuminated until \(E_m\) was stabilized. During the incubation, alkaline bands were formed. Occasionally, some cells failed to form strong alkaline bands. These cells were excluded from the following analysis. The \(E_m\) value in BFS under illumination was \(-173 \pm 6\) mV \((n = 14)\), which was more positive than that in APW buffered at pH 7.0. It is suggested that the membrane was depolarized due to activation of alkaline bands (Ogata 1983, Ogata et al. 1983, Ogata et al. 1987, Fisahn and Lucas 1992, Ogata 2000).

The following experiments were carried out using cells which formed strong alkaline bands in the chamber of the \(K^+\)-anesthesia method. Cells in BFS were illuminated for 20 min in order to check formation of strong alkaline bands. After confirming the occurrence of strong alkaline bands, the medium in pool A was replaced with BFS supplemented with 10 mM KCl. After the 60 min illumination, the external medium was again replaced with the same medium. After 20 min illumination, the alkaline band formation was checked. Two typical examples of electrical measurement are shown in Fig. 3. When 10 mM KCl was added to pool A, a small depolarization was induced (Cell 1). \(E_m\) stayed at the polarized level and strong alkaline bands were observed throughout the measurement. After the end of the electrical measurement, the external medium was replaced with BFS supplemented with 10 mM KCl. Upon illumination, strong bands were again formed (data not shown). In Cell 2, a sudden large depolarization was induced at about 20 min after addition of 10 mM KCl and then the \(E_m\) stayed at the depolarized level. Before the large depolarization, strong alkaline bands were formed in the presence of 10 mM KCl. After the large depolarization, however, all bands became faint, probably due to diffusion of \(OH^+\) from the areas of alkaline bands. At the time shown with an upward pointing arrow, the external medium was replaced with the same medium containing 10 mM KCl. Upon illumination, only weak bands were formed (data not shown). The time interval between addition of 10 mM KCl and the sudden depolarization was variable among specimens. The same experiments in combination with electrophysiology were carried out in 13 cells (Table 1, Experiment 1B). In five cells, the membrane remained polarized for 80 min and strong bands were formed in all cells. In eight cells, large depolarization was induced within 60 min after addition of 10 mM KCl. In these cells, strong bands were formed before the large depolarization. However, bands became very weak after the depolarization in all cells.

The effect of 10 mM NaCl was analyzed. After checking alkaline band formation in BFS, the external medium was replaced with BFS supplemented with 10 mM NaCl. After 80 min incubation under illumination, all cells formed strong alkaline bands. In addition, the membrane remained polarized \((n = 11, \text{ data not shown})\). Thus, \(K^+\) but not \(Cl^-\) is responsible for the weakening of alkaline bands and membrane depolarization.

Since formation of alkaline bands is strongly dependent on cytoplasmic streaming (Lucas and Dainty 1977), the effect of 10 mM KCl on cytoplasmic streaming was studied. Six cells were subjected to this analysis. Before the KCl treatment, the average speed was \(80 \pm 4\) \(\mu m s^{-1}\) \((n = 6)\). The external medium was replaced with BFS supplemented with 10 mM KCl and illuminated for 80 min. During this treatment, alkaline bands became weak in all cells, suggesting large depolarization. The speed of cytoplasmic streaming was measured to be \(77 \pm 4\) \(\mu m s^{-1}\) \((n = 6)\), which was close to the value before the KCl treatment.

BFS contained 0.1 mM CaCl\(_2\). In the following experiments, the effect of increasing the CaCl\(_2\) concentration was studied. After checking the band formation in BFS supplemented with 1 mM CaCl\(_2\), the external medium was replaced with fresh BFS supplemented with 1 mM CaCl\(_2\) and 10 mM KCl. Among eight cells examined, five cells formed strong bands after the 80 min treatment (Table 1, Experiment 2A). Analysis in combination with

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**Fig. 3** Effect of 10 mM KCl on membrane potential. After checking formation of strong alkaline bands in BFS, the external medium was replaced with BFS supplemented with 10 mM KCl (10 mM KCl). In Cell 1, the membrane remained polarized during the measurement. In Cell 2, \(E_m\) suddenly changed to the positive direction and remained at the depolarized level in the presence of 10 mM KCl. At the time shown with an upward pointing arrow, the external medium was again exchanged with BFS supplemented with 10 mM KCl.
Electrophysiology was also carried out in six cells. In four cells, both polarization and strong alkaline bands were maintained. In two cells, however, a large depolarization was induced during the incubation, resulting in weakening of the alkaline bands (Table 1, Experiment 2B).

The effect of 10 mM CaCl₂ was examined (Table 1, Experiment 3). Addition of 10 mM CaCl₂ per se does not inhibit the alkaline band formation (Shimmen et al. 2003). After checking the band formation in BFS supplemented with 10 mM CaCl₂, the external medium was replaced with BFS supplemented with 10 mM CaCl₂ and 10 mM KCl. In all cells, strong bands were maintained after the 80 min incubation in BFS supplemented with 10 mM KCl and 10 mM CaCl₂ (Table 1, Experiment 3A). Both membrane polarization and strong alkaline bands were maintained in all cells in BFS supplemented with 10 mM KCl and 10 mM CaCl₂ during the measurement (Table 1, Experiment 3B).

**Effect of 100 mM KCl**

After checking the formation of strong alkaline bands in BFS, the external medium was replaced with BFS supplemented with 100 mM KCl, and cells were illuminated. During the incubation in the presence of 100 mM KCl, all alkaline bands became diffuse. After 30 min, the external medium was again replaced with BFS supplemented with 100 mM KCl. After the 20 min illumination, only weak bands were formed in all cells (Table 1, Experiment 4A).

Experiments were carried out in the presence of 10 mM CaCl₂. After checking the band formation in BFS supplemented with 10 mM CaCl₂, the external medium was replaced with BFS supplemented with 10 mM CaCl₂ and 100 mM KCl. After the 30 min incubation, the external medium was again replaced with BFS supplemented with 10 mM CaCl₂ and 100 mM KCl. After the 20 min illumination, all cells showed only weak bands (Table 1, Experiment 5A). Analysis in combination with electrophysiology was carried out. In all cells examined, the membrane soon depolarized and only weak bands were formed (Table 1, Experiment 5B).

The effect of 100 mM KCl on cytoplasmic streaming was examined. Experiments were carried out using a conventional chamber. After measurement of the speed in BFS, the external medium was replaced with BFS supplemented with 100 mM KCl, and cells were illuminated. Just after addition of 100 mM KCl, cytoplasmic streaming was stopped (within 10 s). In one cell, recovery started after a delay (Fig. 4). In another cell, the speed recovered to a level close to that before addition of 100 mM KCl. Fundamentally the same results were obtained in another three cells.

**Analysis in internodal cells lacking a native node**

Both nodes were removed by ligation and following cutting of internodal cells (see Materials and Methods). After checking the band formation in BFS supplemented with 10 mM CaCl₂, the external medium was replaced with BFS supplemented with 10 mM CaCl₂ and 100 mM KCl. After the 30 min incubation, the external medium was again replaced with BFS supplemented with 10 mM CaCl₂ and 100 mM KCl. After the 20 min illumination, strong alkaline band were formed (Fig. 5). Among 30 cells examined, 29 cells formed strong alkaline bands (Table 1, Experiment 6A).
Electrophysiological analysis was carried out. In this case, one node was left attached. A specimen was mounted on the chamber so that the cell end with the native node was positioned in pool B (Fig. 2). Therefore, the part of the cell in pool B was easily depolarized by adding 100 mM KCl. Pool A was first filled with BFS supplemented with 10 mM CaCl₂. Eₘ was −149 ± 7 mV (n = 5) and strong bands were formed. After the external medium was replaced with BFS supplemented with 10 mM CaCl₂ and 100 mM KCl, specimens were illuminated for 30 min. The external medium was again replaced with the same medium and illuminated for 20 min. In all cells, membrane polarization (−163 ± 9 mV, n = 5) and strong alkaline bands were maintained after 60 min incubation in BFS supplemented with 100 mM KCl and 10 mM CaCl₂ (Table 1, Experiment 6B).

Discussion

The formation of strong alkaline bands was significantly affected by the KCl treatment and was intimately correlated with the membrane polarization. Even in the presence of 100 mM KCl, strong bands were formed in cells lacking native nodes, if the membrane remained polarized (Table 1, Fig. 5). It must be remembered that alkaline band formation is strongly dependent on cytoplasmic streaming (Lucas and Dainty 1977) and that cytoplasmic streaming is regulated by membrane potential. Upon generation of an action potential, cytoplasmic streaming stops and then recovers gradually within several minutes (Barry 1968, Hayama et al. 1979). Shimmen and Tazawa (1984) reported that the membrane depolarization and the low speed of cytoplasmic streaming were maintained in the presence of 100 mM KCl in Nitella axilliformis. On the other hand, in C. corallina (Fig. 4). However, strong alkaline bands were not formed. When formation of strong alkaline bands was inhibited in the presence of 10 mM KCl, the speed of cytoplasmic streaming was the same as that before addition of 10 mM KCl. Thus, it is concluded that inhibition of formation of strong alkaline bands was caused by membrane depolarization but not by inhibition of cytoplasmic streaming.

Alkaline band formation is transiently inhibited upon generation of an action potential (Bulychev et al. 2004, Eremina et al. 2007, Krupenina and Bulchey 2007, Krupenina et al. 2008). When an action potential is generated, cytoplasmic streaming stops transiently (Hayama et al. 1979). Thus, both membrane depolarization and cessation of cytoplasmic streaming may be involved in inhibition of the alkalization upon generation of an action potential.

The nodal end and flank are electrophysiologically differentiated with respect to their ion sensitivity (Shimmen 2001, Shimmen 2008). When the K⁺ concentration was increased to 100 mM, the node membrane promptly depolarizes. On the other hand, the flank membrane remains polarized for a longer period of time. By increasing the Ca²⁺ concentration, polarization of the flank membrane is maintained further (Shimmen 2001). On the other hand, the node membrane promptly depolarizes upon an increase in the KCl concentration to 100 mM even in the presence of 10 mM CaCl₂ (Shimmen 2008). Thus, it was suggested that the membrane polarization is maintained by removing the native nodes. In cells lacking a native node, strong alkaline bands and membrane polarization were maintained in BFS supplemented with 10 mM CaCl₂ and 100 mM KCl (Table 1, Fig. 5). Irrespective of the types of external medium, strong alkaline bands were formed in all cells which maintained membrane polarization. On the other hand, alkaline bands became significantly weak in all cells which induced large depolarization (Table 1). Thus, formation of strong alkaline bands and membrane polarization are well correlated. In the presence of 10 mM KCl, strong alkaline bands were formed before depolarization but they became weak after the depolarization (Fig. 3, Cell 2). It must be stressed that formation of strong bands was affected by the membrane polarization in the same cell and the same medium, APW supplemented with 10 mM KCl. This result unequivocally indicated that formation of strong alkaline bands is intimately linked to the membrane polarization.

Incorporation of inorganic carbon is inhibited by the KCl treatment (Lucas 1976). Since alkaline band formation is coupled with photosynthetic carbon assimilation, inhibition of alkaline band formation by the membrane depolarization might be caused by inhibition of incorporation of inorganic carbon. Thus, dependency of inorganic carbon incorporation on the membrane potential is an important subject for future research. It is expected that the result may open a way for elucidation of the mechanism of inorganic carbon incorporation in Characeae.

Materials and Methods

Chara corallina was cultured as reported previously (Mimura and Shimmen 1994). Internodal cells were isolated and kept in APW containing 0.1 mM KCl, 1 mM NaCl and 0.1 mM CaCl₂ (pH about 5.6) until use.

Alkaline bands were visualized using a pH indicator dye, phenol red (Spear et al. 1969, Takano and Shimmen 1999, Shimmen and Yamamoto 2002, Shimmen et al. 2003). The pKₐ value of phenol red is 8.0, and the color exhibits a gradual transition from yellow to red over the pH range 6.6–8.0. An internodal cell was placed in a channel (3 mm wide and 3 mm deep) of a transparent polyacrylate block. The channel was filled with BFS, which was prepared by adding 0.5 mM NaHCO₃ and 5 mg 100 ml⁻¹ phenol red to APW. Just after preparation, the pH of BFS was about 6.6. However, it increased slightly with time. In the present study, BFS was used just after preparation. The channel was
coupled with a thin transparent polyacrylate sheet. The polyacrylate block was transferred into a transparent polyacrylate box, covered with a thin transparent polyacrylate sheet. The polyacrylate box was placed on the bottom of which was filled with a thin layer of pure water, and the box was covered with a transparent lid. The cell was illuminated with fluorescent lamps (200 μmol m⁻² s⁻¹ at the upper surface of the polyacrylate box). After illumination in BFS, a photograph was taken to record the position and the strength of the alkaline bands.

Internodal cells lacking a native node were prepared (Fig. 6). After cell turgor pressure was decreased by adding 150 mM sorbitol to the external medium, the flanking region close to the nodal end was tightly ligated with polyester thread. The part of the cell between the ligature and native node was cut to remove the native node.

Electrical potential was amplified with an amplifier (Microelectrode Amplifier MEZ7101, Nihon Kohden, Tokyo Japan) and recorded using a pen-writing recorder (VP-6358A, National Panasonic, Tokyo Japan). Experiments were carried out at room temperature (23–27°C).

References


Plieth, C., Tabrizi, H. and Hansen, U. (1994) Relationship between banding and photosynthetic activity in Chara corallina as studied by the spatially different induction curves of chlorophyll fluorescence observed by an image analysis system. Physiol. Plant. 91: 205–211.


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