Mechanism of Inhibition of Cytoplasmic Streaming by Auxin in Root Hair Cells of Hydrocharis

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It has been reported that auxin accelerates cytoplasmic streaming at low concentrations and inhibits it at high concentrations in several plant cells. In the present study, the mechanism of inhibition of cytoplasmic streaming by naphthalene acetic acid (NAA) at high concentrations was analyzed in root hair cells of Hydrocharis. Because the effective concentration of NAA inhibiting cytoplasmic streaming decreased when the extracellular pH (pHo) was lowered, it was hypothesized that cytoplasmic streaming is inhibited by NAA via acidification of the cytoplasm. This possibility was supported by the fact that acetic acid, propionic acid and decanoic acid also inhibited cytoplasmic streaming at low pHo. Acidification of the cytoplasm disturbed the orientation of actin filaments (AFs) and disrupted cortical microtubules (MTs). The effects of NAA were reversible; both cytoplasmic streaming and organization of the cytoskeleton were recovered upon removal of NAA. During the recovery, tracks of cytoplasmic streaming in the subcortical region temporarily showed a helical pattern along the longitudinal direction of the cell. Fluorescence staining of cytoskeletons revealed that both AFs and MTs aligned obliquely to the longitudinal axis of the cell. The helical streaming returned to the original reverse fountain streaming after several hours. The simultaneous changes in the organization of both cytoskeletons supported our previous report that the organization of AFs is regulated by MTs.

Key words: Actin filament — Auxin — Cytoplasmic streaming — Hydrocharis — Microtubule — Root hair cell.

Cytoplasmic streaming is responsible for intra-cellular transport of organelles and chemical substances in plants. Physiological and morphological studies in Characeae have suggested involvement of the actin-myosin system in cytoplasmic streaming (cf. Kamiya 1981, 1986, Shimmen 1988). In a variety of plants, the presence of AFs and the inhibition of cytoplasmic streaming by cytochalasins were reported (Staiger and Schiwa 1987). Myosins which slide along AFs were purified from lily pollen tubes (Kohno et al. 1992, Yokota and Shimmen 1994) and from Chara (Yamamoto et al. 1994). AFs are thought to function as a track on which myosin bound to organelles moves (Williamson 1975, Nagai and Hayama 1979, Shimmen and Tazawa 1982, Kohno and Shimmen 1988). It has been reported that myosin moves along AFs from the pointed end to the barbed end in characean cells and pollen tubes (Kersey et al. 1976, Kohno et al. 1990). Thus, the polarity of the AFs determines the direction and pattern of cytoplasmic streaming. The patterns of cytoplasmic streaming should therefore reflect the organization of AFs. However, the molecular mechanism of AF organization in plant cells is still unclear.

Cytoplasmic streaming has been classified into several types based on patterns of streaming of the cytoplasm (Kamiya 1959). Circulation is the most commonly observed; streaming occurs at the subcortical region and in the transvacuolar strands. Organization of streaming tracks is very dynamic. In contrast, characean cells show rotational streaming; cytoplasm streams at the subcortical region with a constant velocity and a fixed direction. Because of these characteristics, characean cells are a most suitable material for elucidating the basic mechanism of motive force generation. However, these characteristics are rather unusual compared to the dynamic ones in many other plant cells. Although cytochalasin treatment disrupts the organization of AF in many plant cells (Staiger and Schiwa 1987), it stabilizes bundles of actin filaments against low ionic strength in Chara (Williamson 1978).

The root hair cell of Hydrocharis, an aquatic monocotyledon, is a suitable material for observing cytoplasmic streaming (Kamiya 1959). In young and shorter root hair cells, so-called reverse fountain streaming is observed. Cytoplasm streaming acropetally at the subcortical region and basipetally in a transvacuolar strand, longitudinally penetrating the central vacuole. After the root hair cells grow longer, both acropetal and basipetal streaming occur in the subcortical region.

Shimmen et al. (1995) showed that actin filaments are responsible for organization of the transvacuolar strand and the occurrence of cytoplasmic streaming. Reverse foun-
inhibit streaming is an intermediate between circulation and rotation (Kamiya 1959). Measurement of velocity is easy, because the cytoplasm streams with a constant velocity and a fixed direction in the subcortical region and the transvacuolar strand. The organization of the transvacuolar strand is very dynamic. It sometimes disappears by attaching to the subcortical layer and then reappears. However, it always locates at the tip region in the longitudinal direction of the root hair cell. Treatment with cytochalasin B (CB), reversibly destroys AFs and the transvacuolar strand (Shimmen et al. 1995). Thus, the root hair cell of Hydrocharis is suitable for analyzing the dynamic organization of AFs, as well as measuring the velocity. We previously reported that MTs regulated the organization of AFs in the subcortical region in root hair cells (Tominaga et al. 1997).

The effects of auxin on cytoplasmic streaming in plant cells have been well reported. At low concentrations, cytoplasmic streaming is accelerated in Avena coleoptile (Thimann and Sweeney 1937, Sweeney and Thimann 1937, Sweeney 1941, Sweeney and Thimann 1942), Avena root hairs (Sweeney 1944), staminal hair-cells of Tradescantia (Kelso and Turner 1955) and tomato root hairs (Aylng et al. 1994). At high concentrations, however, cytoplasmic streaming is inhibited in these four cell types (Thimann and Sweeney 1937, Sweeney and Thimann 1937, Sweeney 1941, 1944, Kelso and Turner 1955, Aylng et al. 1994). However the mechanism of auxin effects on cytoplasmic streaming is unknown. In the present study, the mechanism of inhibition of cytoplasmic streaming in root hair cells of Hydrocharis by NAA at high concentrations was analyzed. It was hypothesized that acidification of the cytoplasm due to influx of protonated NAA into the cells caused cessation of cytoplasmic streaming. Dynamic changes in tracks of the cytoplasmic streaming were observed upon removal of the higher concentration of NAA. We discussed the mechanism of organization of cytoskeletons based on this phenomenon.

Materials and Methods

Plant material—Hydrocharis dubia was cultured in plastic containers filled with tap water and with soil and mold at the bottom. The plastic containers were placed in an air-conditioned room (24°C) and illuminated with fluorescent lamps (2,300 lux). Young roots were isolated and kept in 5 mM CaSO4 solution before use.

Light microscopy—Cytoplasmic streaming was observed with a microscope (Olympus IMT-2) equipped with phase contrast optics. The velocity of cytoplasmic streaming were measured by chasing the movements of large cytoplasmic particles. Micrographs of the cell were taken with a microscope equipped with Nomarski optics (Zeiss Axioshot).

Treatment with chemicals—a-Naphthalene acetic acid (Sigma), acetic acid (Wako Pure Chemical Industries. LTD., Osaka, Japan), and sodium propionate (Wako Pure Chemical Industries. LTD., Osaka, Japan) were directly dissolved in 5 mM CaSO4 solution. Then the pH of the solutions was adjusted with KOH. Stock solutions of 3-indoleacetic acid (Wako Pure Chemical Industries. LTD., Osaka, Japan) (500 mM), decanoic acid (Wako Pure Chemical Industries. LTD., Osaka, Japan) (200 mM), β-naphthaldehyde (Sigma) (200 mM), α-naphthyl propionate (Sigma) (200 mM), β-naphthyl propionate (Sigma) (200 mM) and a-naphthaldehyde (Sigma) (2 M) were prepared by dissolving them in ethanol. Stock solutions of indoleacetic acid (IAA) and derivatives of NAA were added to 5 mM CaSO4 solution; then the pH was adjusted with either KOH or HCl. The final concentration of KCl was lower than 0.8 mM.

Fluorescence staining—We fluorescently visualized AFs and MTs as described previously (Tominaga et al. 1997). Ethylene glycol-bis-succinimide succinate (EGS) (Dojindo Laboratories Co., Kumamoto, Japan) was used for preservation of MTs (Abdella et al. 1979). EGS was dissolved in dimethylsulfoxide (DMSO) at 200 mM as a stock solution. Specimens were treated in EMP solution (10 mM ethylene glycol-bis (β-aminoethoxy ether) N,N,N,N'-tetracetate (EGTA), 2 mM MgCl2, 50 mM piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES), pH 7.0) supplemented with 1 mM EGS and 0.5% Triton X-100 for 1 h. After washing with EMP solution three times, specimens were fixed with 1% paraformaldehyde in EMP solution for 1 h. In Hydrocharis root hair cells, antibodies can be introduced into the cell without enzymatic digestion of the cell wall (Tominaga et al. 1997). Specimens were washed with EMP solution three times and incubated with a monoclonal antibody against α-tubulin (Amersham) in phosphate buffered saline (PBS) for 1 h. After washing with PBS solution three times, specimens were incubated with FITC-conjugated antimouse IgG (American Qualex, San Clemente, CA, U.S.A.) in PBS for 40 min. For double-staining of AFs and MTs, 66 nM rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR, U.S.A.) was added to the secondary antibody solution. Specimens were washed once with PBS, mounted on a glass slide in PBS supplemented with 0.1% (w/v) p-phenylenediamine, and observed with a fluorescence microscope (Zeiss Axioshot). Photographs were taken using Kodak T-MAX 400 film.

Results

Effects of NAA—Kelso and Turner (1955) reported that treatment with 500 mg m–1 (2.8 mM) IAA caused complete cessation of the cytoplasmic streaming within 10 min in staminal hair cells of Tradescantia. They also found similar effects with NAA. In the present study, we examined the effect of NAA in CaSO4 solution on root hair cells of Hydrocharis. The pH of the CaSO4 solution was adjusted at 6.5 with KOH. No effect on cytoplasmic streaming was observed when cells were treated with 1 mM NAA (data not shown). Complete inhibition of the cytoplasmic streaming was observed when the NAA concentration was increased up to 10 mM (Fig. 1). This inhibition with NAA was reversible, and the cytoplasmic streaming recovered upon removal of NAA.

Drastic changes in intracellular structures were induced by the NAA treatment (Fig. 2). In root hair cells of Hydrocharis, a reverse fountain type of cytoplasmic streaming is generally observed in young root hair cells; cytoplasm streams acropetally along the subcortical region...
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Fig. 1 The effect of NAA on velocity of cytoplasmic streaming in root hair cells. NAA at 10 mM (pH 6.5) was added at 0 time and removed after 2 h (arrow). Results show means±SE (n=25).

and basipetally along a cytoplasmic strand longitudinally penetrating the vacuole (Kamiya 1959) (Fig. 2a). When cytoplasmic streaming was stopped by treatment with NAA, the transvacuolar strand disappeared (Fig. 2b). During recovery after removal of NAA, streaming occurred helically along the longitudinal direction of the cell in the subcortical region (helical streaming) (Fig. 2c, e). The transvacuolar strand did not recover at this point (Fig. 2d). The pitch of the helix gradually increased with time, and, after several hours, reverse fountain streaming was restored. The cytoplasmic streaming at the subcortical region occurred in parallel to the longitudinal direction of the cell, and the transvacuolar strand appeared (Fig. 2f).

The concentration of NAA had to be increased up to 10 mM to stop the cytoplasmic streaming. This concentration of NAA is very high compared with that reported by Kelso and Turner (1955). Although they did not adjust the pH of their test solutions, they noted that the pHs of these solutions ranged between 4.5 and 5. Hydrophobic weak acids cause acidification of the cytoplasm due to influx of the protonated form in the acidic medium, resulting in inhibition of the cytoplasmic streaming (Shimmen and Tazawa 1985). Since NAA is also a weak acid, a similar situation is expected. Using the pKa value of NAA, 4.236, the concentration of protonated NAA (NAA-H) in CaSO₄ solution (pH 6.5) containing total NAA at 10 mM is calculated to be 0.054 mM. By lowering the pH, 0.054 mM NAA-H can be obtained by adding NAA at the lower total concentrations (Table 1). At pH 4.0, a solution supplemented with 0.085 mM total NAA contains 0.054 mM NAA-H. When cells were treated with a 0.085 mM NAA solution (pH 4.0), cytoplasmic streaming was stopped (Fig. 3b) as in the case of treatment with 10 mM NAA (pH 6.5), and helical streaming was observed after removal of NAA (Fig. 3c, e). Compared with root hair cells treated with 10 mM NAA, however, only older, longer cells showed helical streaming, and the pitch of the helix was longer. Younger, shorter cells did not show helical streaming. The same
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Table 1 The relationship between the concentrations of total NAA ([NAA]) and those of protonated NAA ([NAA-H]) at various pHs

<table>
<thead>
<tr>
<th>pH</th>
<th>[NAA-H] (mM)</th>
<th>Total [NAA] (mM)</th>
</tr>
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<tbody>
<tr>
<td>6.5</td>
<td>0.054</td>
<td>10</td>
</tr>
<tr>
<td>5.0</td>
<td>0.054</td>
<td>0.37</td>
</tr>
<tr>
<td>4.0</td>
<td>0.054</td>
<td>0.085</td>
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</table>

The value 4.236 was adopted for the pKa of NAA.

effect was irreversible, and cytoplasmic streaming was not recover upon removal of NAA. It is probable that the cell suffered irreversible damage due to the drastic acidification of the cytoplasm. Neither cytoplasmic streaming nor intracellular structures were affected when root hair cells were treated with CaSO₄ solution (pH 4.0) lacking NAA (data not shown), indicating that not the low pH but NAA-H affected cytoplasmic streaming and intracellular structures.

The effect of IAA was also examined. The 1 mM IAA solution (pH 4.0) containing 0.849 mM protonated IAA reversibly inhibited cytoplasmic streaming of root hair cells. However, helical streaming was not observed during the recovery process.

Effects of fatty acids—To examine the possibility that result was obtained when root hair cells were treated with a CaSO₄ solution containing 0.37 mM total NAA (0.054 mM NAA-H) (pH 5.0) (data not shown).

The effect of increasing the NAA-H concentration was examined. When cells were treated with a solution (pH 4.0) containing 2 mM total NAA (1.265 mM NAA-H), cytoplasmic streaming was inhibited within 1 min. However, this
acidification of cytoplasm due to influx of NAA-H is the cause of inhibition of the cytoplasmic streaming, we carried out similar experiments using fatty acids. When root hair cells were treated with 2 mM acetic acid in CaSO$_4$ solution (pH 4.0), the cytoplasmic streaming stopped within 20 min (Fig. 4b). Removal of acetic acid resulted in recovery of the cytoplasmic streaming, but helical streaming was not observed (Fig. 4c). A similar result was obtained with treatment with 2 mM propionic acid in CaSO$_4$ solution (pH 4.0), but the time required for inhibition of cytoplasmic streaming was shorter (within 5 min).

We also examined decanoic acid, which is more hydrophobic than acetic acid and propionic acid. Treatment with a solution containing 0.03 mM decanoic acid in CaSO$_4$ solution (pH 4.0), inhibited cytoplasmic streaming within 60 min. Helical streaming was observed after removal of this acid (data not shown).

**Effects of derivatives of NAA**—To confirm that NAA inhibited cytoplasmic streaming via acidification of the cytoplasm, experiments were carried out using derivatives of NAA, a-naphthaldehyde, b-naphthaldehyde, a-naphthyl propionate, and b-naphthyl propionate. All these experiments used CaSO$_4$ solution (pH 4.0). a-naphthaldehyde at 1 mM did not inhibit cytoplasmic streaming. b-naphthaldehyde at 0.6 mM inhibited the streaming irreversibly. b-naphthaldehyde seemed to have a nonspecific negative effect; the cytoplasm became drastically disorganized by this treatment. Both a and b-naphthyl propionate at 0.8 mM inhibited the cytoplasmic streaming; this inhibition was reversible. Thus, derivatives containing carboxyl groups reversibly inhibited the cytoplasmic streaming. However, no helical streaming was observed during the recovery process after removal of these NAA derivatives.

**Double fluorescence staining of AFs and MTs**—We previously reported that AFs were co-localized with MTs in the longitudinal direction of the cell and that MTs regulated organization of AFs at the subcortical region of root hair cells (Tominaga et al. 1997). In the present study, we

![Fig. 5](https://academic.oup.com/pcp/article-abstract/39/12/1342/1930453/fig5)
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Fig. 6 Double fluorescence staining of AFs and MTs in root hairs showing helical streaming observed after removal of NAA. The cell was treated with 10 mM NAA (pH 6.5). After cytoplasmic streaming was completely stopped, NAA was removed, and the cell was kept in CaSO₄ solution (pH 6.5). When the cell showed helical streaming, it was subjected to double fluorescence staining. (a) AFs stained by rhodamine-phalloidin. (b) MTs stained by immunofluorescence using anti-tubulin. Bar, 20 μm.

Fig. 7 Double fluorescence staining of AFs and MTs after the original reverse fountain streaming returned. The cell was treated with 10 mM NAA (pH 6.5). After cytoplasmic streaming was completely stopped, NAA was removed, and the cell was kept in CaSO₄ solution. When the cell recovered its original pattern of streaming after helical streaming, it was subjected to double fluorescence staining. (a) AFs stained by rhodamine-phalloidin. (b) MTs stained by immunofluorescence using anti-tubulin. Bar, 20 μm.

Examined the organization of AFs and MTs during treatments with NAA, acetic acid, and IAA to examine the effects of these chemicals on both cytoskeletons (Fig. 5). When cells were treated with 10 mM NAA (pH 6.5), AFs showed a random meshwork orientation at the tip region of root hair cells. At the basal region, AFs were disordered, but longitudinal arrays remained (Fig. 5a). MTs were disrupted (Fig. 5b). When cells were treated with 2 mM acetic acid (pH 4.0), the orientation of AFs was disordered, but organization parallel to the longitudinal direction of the cell remained (Fig. 5c). MTs mostly disappeared (Fig. 5d). When treated with 1 mM IAA (pH 4.0), the parallel array of AFs was disorganized, and MTs were destroyed (Fig. 5e, f).

Next, we examined the organization of AFs and MTs in root hair cells showing helical streaming after treatment with 10 mM NAA solution (pH 6.5). Both AFs and MTs aligned obliquely to the long axis of the cell and localized in the same strands (Fig. 6). When root hair cells recovered their reverse fountain streaming after helical streaming, both AFs and MTs aligned in parallel to the long axis of the cells and they frequently localized in the same strands (Fig. 7).

Discussion

Auxin regulates various physiological activities in plants, including cytoplasmic streaming. Auxin activates cytoplasmic streaming at low concentrations but inacti-
vates or inhibits it at high concentrations (Thimann and Sweeney 1937, Sweeney and Thimann 1937, 1942, Sweeney 1941, 1944, Kelso and Turner 1955, Ayling et al. 1994). However, the mechanism of auxin action on cytoplasmic streaming has remained unsolved.

The present study suggested that auxin at high concentration inhibits cytoplasmic streaming by acidifying the cytoplasm. At higher pHo (6.5), the total concentration of NAA had to be increased up to 10 mM. When the pHo was decreased to either 5.0 or 4.0, NAA inhibited cytoplasmic streaming at 0.37 mM or 0.085 mM, respectively. Using the pKa value of NAA, the concentration of NAA-H was calculated to be 0.054 mM in all cases (Table 1). Shimmen and Tazawa (1985) demonstrated that various fatty acids inhibited cytoplasmic streaming in Characeae by acidifying the cytoplasm. The same situation was also expected for root hair cells of Hydrocharis. Acetic acid, propionic acid, and decanoic acid inhibited cytoplasmic streaming at low pHo (4.0). The relationship between the concentration of protonated weak acids and the time needed for complete inhibition is summarized in Table 2. It appears that the extent of cytoplasm acidification is dependent on the rate of influx of the protonated weak acids. Furthermore, the rate of influx is dependent on both the concentration of protonated weak acid and hydrophobicity of the molecule. The results summarized in Table 2 supports these ideas. Inhibition by propionic acid occurred more quickly than that by acetic acid at similar concentrations (Exp. 5, 6). Decanoic acid and NAA, which are supposed to be more hydrophobic, were effective in inhibiting cytoplasmic streaming at much lower concentrations (Exp. 1–3, 7). The time needed for complete inhibition decreased considerably when the concentration of protonated NAA was increased (Exp. 4).

The possibility of cytoplasm acidification as a cause of inhibition of cytoplasmic streaming is also supported by the experiments using derivatives of NAA. Both α-naphthyl propionate and β-naphthyl propionate reversibly inhibited the cytoplasmic streaming. On the other hand, α-naphthaldehyde at 0.8 mM (pH 4.0) did not inhibit the streaming at all. β-naphthaldehyde seemed to have a non-specific negative effect, drastically disorganizing the cytoplasm.

Although inhibition of cytoplasmic streaming by NAA at high concentrations seems to be unphysiological, the acidification of the cytoplasm by the influx of NAA-H seems to reflect one of the steps in polar transport of auxin in the stems of plants. It is generally accepted that auxin does not travel via the plasmodesmata in moving vertically from cell to cell in the stem. A significant proportion of auxin is in the protonated form in the acidic apoplast. Since protonated auxin is relatively hydrophobic, it can easily flow into the cell by crossing the lipid bilayer of the plasma membrane. In a cytoplasm at neutral pH (about 7.0), most auxin is in the dissociated form, which is relatively hydrophilic and can not passively flow out of the cell. An active transporter localized at the basal end of cells facilitates the efflux of auxin from the cell. This localized efflux guarantees the polar transport of auxin. However, passive influx of protonated auxin is also a important step (Taiz and Zeiger 1991). Rubery and Sheldrake (1973) reported that auxin uptake by plant cells increased when the extracellular pH was lowered, supporting the above model. It is expected that auxin flowing into the cell releases H+ in the neutral cytoplasm. When the concentration of auxin is low, the pH of cytoplasm is maintained due to the pH-stat mechanism of the cell. However, when auxin is applied at high concentration, the release of H+ into the cytoplasm exceeds the capacity of the pH-stat mechanism of the cell, resulting in acidification of the cytoplasm.

During the inhibition of cytoplasmic streaming by weak acids, the bundles of AFs remained, although their organization was extensively disturbed (Fig. 5). Lack of cytoplasmic streaming in the presence of bundles of AFs is ap-

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Chemical</th>
<th>pKa</th>
<th>pHo</th>
<th>Total concentration added (mM)</th>
<th>Concentration of protonated form (mM)</th>
<th>Inhibition time (min)</th>
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<td>60</td>
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<td>3</td>
<td></td>
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<tr>
<td>4</td>
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</tr>
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<td>Decanoic acid</td>
<td>3.7–5.0</td>
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</table>

* The range of pKa of decanoic acid was estimated from the pKa values of formic acid (3.7), acetic acid (4.7), propionic acid (4.9), butyric acid (4.6), pivalic acid (5.0), and heptanoic acid (4.8).
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Parently unreasonable. Perhaps the sliding interaction between myosin and AFs is inhibited by cytoplasm acidification. Although the pH dependency of activities of plant myosin has not been yet studied, it is reasonable to expect a strong pH dependency of myosin as a enzyme. Tazawa and Shimmen (1982) reported significant inhibition of cytoplasmic streaming at low pH in tonoplast-free cells of Characeae.

The present study showed that lowering of pHc induced extensive disordering of the longitudinal organization of AFs. The lowering pH of cytoplasm (pHc) also induced destruction of MTs. We have reported that organization of AFs is regulated by MTs in root hair cells of Hydrocharis (Tominaga et al. 1997). Destruction of MTs might cause disordering of the longitudinal array of AFs. As a cause of destruction of MTs by acidification of cytoplasm, the possible involvement of an increase in cytoplasmic Ca$^{2+}$ must be considered. Felle (1988) reported that acidification of cytoplasm led to a simultaneous increase in cytoplasmic free Ca$^{2+}$ in Riccia and Zea.

Significant changes in the streaming track were induced after treatment with NAA at a higher concentration (10 mM) and high pH (6.5). Treatment with NAA of lower concentration at lower pH did not induce significant helical streaming. Acetic acid and propionic acid did not induce helical streaming, either, suggesting that another factor(s) could be involved in the induction of helical streaming in addition to acidification of cytoplasm. In the medium containing 10 mM NAA (pH 6.5), the concentration of dissociated NAA (NAA$^-\text{a}$) is high. This might be one factor inducing helical streaming. Dissociated NAA could affect the plasma membrane as a detergent, disordering the organization of the cytoskeleton. Induction of helical streaming by decanoic acid, which is supposed to have a strong detergent nature in its dissociated form, supports this possibility.

Fluorescence staining of both AFs and MTs revealed that not only AFs but also MTs aligned obliquely to the long axis of the cell and that they localized in the same strands, indicating an intimate interaction between them. Although the mechanism of the dynamic changes of these two cytoskeleton elements after treatment with NAA at high concentration is not fully elucidated yet, this phenomenon can be a clue to revealing the interaction between AFs and MTs in root hair cells of Hydrocharis.

References


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