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Motile Apparatus in *Vallisneria* Leaf Cells. II. Effects of Cytochalasin B and Lead Acetate on the Rate and Direction of Streaming

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ABSTRACT. The effects of cytochalasin B (CB) and lead acetate, inhibitors of "primary streaming" in Characean cells, were examined on "secondary streaming" in *Vallisneria* mesophyll cells with reference to the cessation of streaming and the reversal of its direction.

Cytoplasmic streaming was reversibly inhibited by CB in the concentration range of 10–100 μg/ml. The ratio of cells showing newly established streaming in the reverse direction after removal of CB to the total number of cells observed before treatment increased depending on the concentration and the duration of treatment, reaching 50% after 24 h for CB treatment at 10–100 μg/ml. Lead acetate at 50–100 mM caused the complete cessation of streaming, but the streaming gradually recovered after its removal; however, lead acetate did not induce a change in the streaming direction. Properties of the motile apparatus in secondary streaming are discussed on this basis.

In leaf cells of the higher aquatic plants *e.g.*, *Elodea* and *Vallisneria*, rotational streaming of the cytoplasm is induced by such external stimuli as irradiation with light or the application of various chemicals. This type of streaming is called "secondary streaming" (4, 6), and that seen in Characean cells is called "primary streaming", as it is steady and ceaseless under natural conditions.

Considerable information of the mechanism of primary streaming has been obtained for the internodal cells of Characeae. The rotational cytoplasmic streaming in the Characean cells generally is understood to be caused by the unidirectional sliding of the endoplasmic organelles equipped with myosin-like protein along bundles of F-actin filaments, all of which are anchored on the stationary chloroplast files. The direction of the streaming is thought to be determined by the polarity of the F-actin composing the bundles of microfilaments (7).

Less is known, however, about the structure and function of the motile system for secondary streaming, though the nature of the photoreceptor involved in light-induced cytoplasmic streaming and chloroplast movement has been clarified to some extent (3, 12, 13). Information obtained from investigations of Characean cells should help us to understand the mechanism of rotational streaming in the leaf cells of aquatic plants. Forde and Steer (2) looked for microfilament bundles in leaf cells of *Elodea*, but failed to find large bundles that might be responsible for the generation of cytoplasmic streaming. We recently have found many bundles of microfilaments in

Vallisneria leaf cells which manifested active cytoplasmic streaming (unpublished results). These bundles, which appear very similar to those in Characean cells, are localized in the peripheral region of the cell. Their orientation coincides well with the direction of streaming. Whether these bundles are generated prior to the induction and establishment of streaming attracted our interest. If so, the mechanism of secondary streaming must be closely related to the stability of these microfilament bundles.

The present study was performed to obtain information on the properties of the motile apparatus in secondary streaming. Using two drugs, cytochalasin B (CB) and lead acetate, we examined i) the cessation of streaming induced by their application and ii) the reversal of the streaming direction after their removal.

As stated above, the endoplasm in Characean cells streams unidirectionally, supposedly due to the polarity of the microfilaments. Reversal of the streaming direction, therefore, can occur only through the disorganization and reconstruction of the microfilament bundles. The stability of these bundles in *Vallisneria* in relation to the mechanism of secodary streaming is discussed.

MATERIALS AND METHODS

Cultures. *Vallisneria asiatica* var. *biwaensis*, from the Seta River in Otsu, Japan, was cultured in tap water in a pail under continuous illumination of about 2,000 lux at 30°C. The bottom of the pail was covered with 10 cm of muddy sand collected from the river. Leaves were harvested when needed by cutting them off at the basal region with a razor.

Light Microscopy. The leaf is made up of four layers of cells, two layers of epidermal cells on each exterior side and two other layers of mesophyll cells occupying the inner part. Leaves were first stripped with a razor to make a sheet consisting of epidermal and mesophyll cell layers. Small pieces of the sheet were then cut off and mounted on a slide in tap water. Mesophyll cells were observed with a phase-contrast microscope (Olympus IMT) equipped with a drawing apparatus. Test and control solutions were applied by irrigation between the cover slip and the slide glass on the microscope stage. To measure the streaming rates, five cells, each from a different piece of sheet, were examined under one test condition. The rates were measured by timing the movement of the cytoplasmic particles that passed the calibrated eyepiece divisions and were normalized to the value of the streaming rate immediately before drug application, which was usually within a range of 10–20 μm/sec. The direction of streaming in the cells was checked before application of the drug with five pieces of stripped leaf. The directions in about twenty cells per sample were inspected and recorded with the drawing apparatus. After the removal of the drug, the directions of the newly established streaming were recorded for the same cells. The ratio of the number of cells in which the streaming direction had been changed by the drug to the total number of cells was calculated.

Chemicals. Cytochalasin B (Imperial Chemical Industries) was dissolved at 20 μg/ml in dimethyl sulfoxide (DMSO), then diluted with tap water to give final concentrations of 1, 5, 10, 50, 100 and 200 μg/ml. A 1% DMSO solution was used for the control medium. Lead acetate (Nakarai Chemicals, Ltd.) was dissolved in tap water to give final concentrations of 10, 50, 100, 200 and 500 mM. The solution of lead acetate was prepared immediately before application.

RESULTS

Effects of cytochalasin B. 1) On the streaming rate: The drug was applied at a concentration of 1, 5, 10, 50, 100 or 200 μg/ml for 1 h. Five cells were examined at each concentration. For about 15 min prior to monitoring of the streaming rates, we illuminated the cells with the light on the microscope stage (about 5,500 lux) to level off the rates. Then 30 min after the start of rate monitoring, the drug was applied by irrigation. After treatment for 60 min, the test solution was replaced by the control solution.

Streaming was inhibited reversibly by the drug in the concentration range of 10–100 μg/ml. Fig. 1 shows the time courses of inhibition and recovery. Within 10 min after drug application, streaming decreased to 50% of the rate immediately before application and was completely inhibited within 50 min. About 150 min after irrigation with the control solution, cells that had been completely inhibited by the concentrations of 10 and 50 μg/ml regained their normal velocity. But recovery in cells inhibited by 100 μg/ml was not complete even after 200 min. We noted a considerable time lag before streaming was observed again, the duration of which depended on the concentration of the drug.

Streaming did not recover in more than 40% of the cells treated with CB at 200 μg/ml. CB at the concentration of 1 μg/ml produced no detectable effect on streaming. CB at 5 μg/ml did not halt streaming, but caused irregularity in its pattern. The track of rotational streaming was disordered in one or two areas of the cell, where a stacked

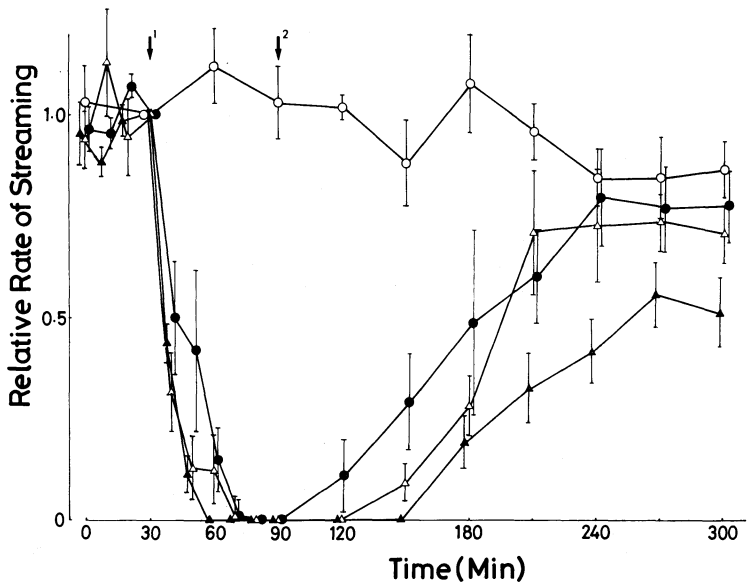

Fig. 1. Effect of CB on the streaming rate. 30 min after the start of velocity measurements (arrow 1), CB was applied at concentrations of 10 (—●—), 50 (—△—) or 100 μg/ml (—▲—). For the control, cells were treated with 1% DMSO (—○—). After treatment for 60 min (arrow 2), the drug was removed by irrigating the cell with the control solution. The rate of streaming immediately before application, which was mostly in the range of 10–20 μm/sec at room temperature, was regarded as 1.00; other data were normalized against this value. Each point represents the mean value for five cells. The length of the vertical bar is the S.E. of the mean.

mass of cytoplasm, like a helmet, containing chloroplasts and other particles was often formed. This helmet-like mass did not shift position and continued to rotate there at least for few hours (Fig. 2).

2) On the streaming direction: Bundles of microfilaments in Characean cells are not affected structurally by cytochalasin B (1, 14). To obtain information of the structural

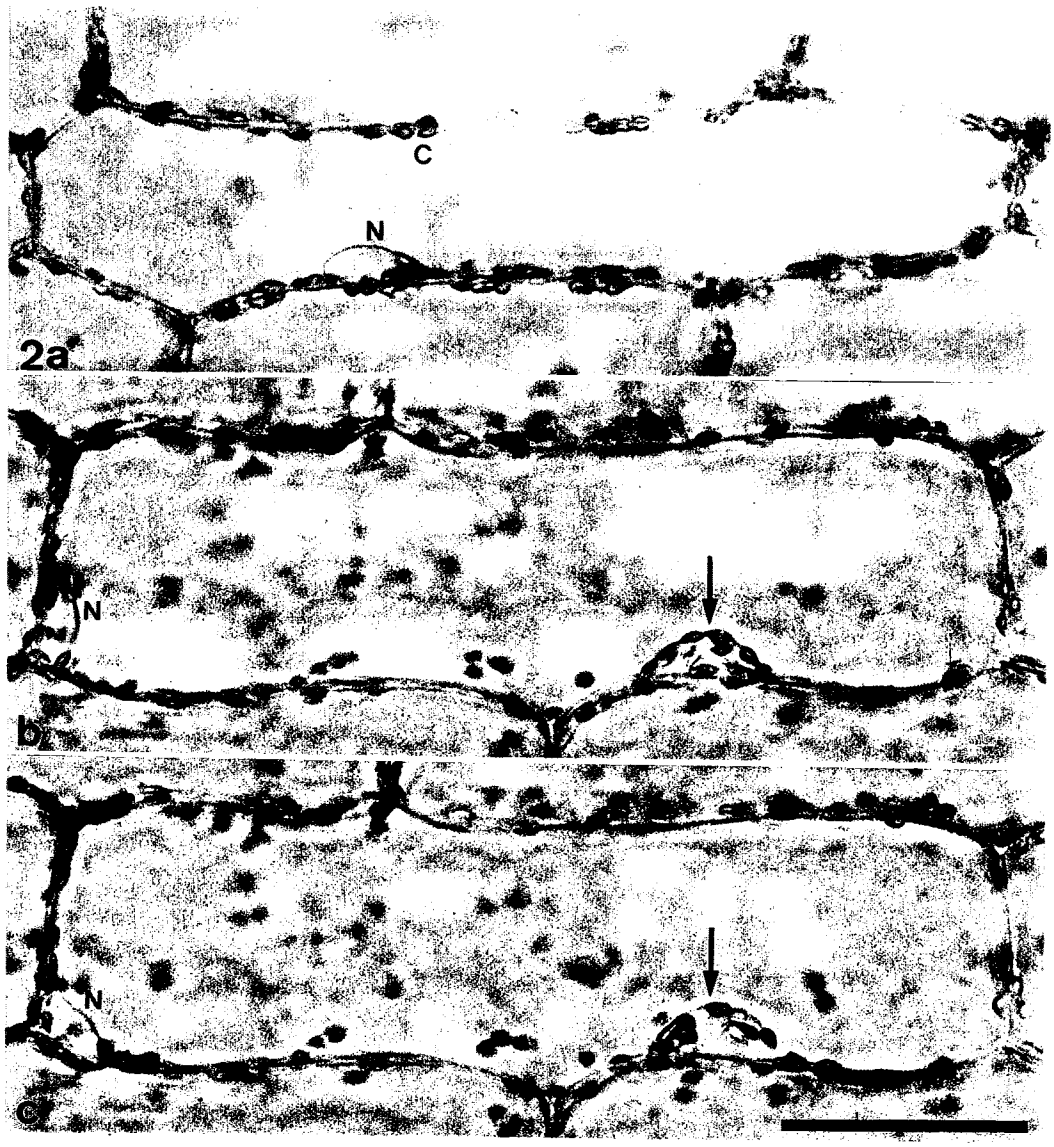

Fig. 2. a) Normal mesophyll cells of *Vallisneria*. The thin layer of cytoplasm containing the nucleus (N), chloroplasts (C) and other particles shows rotational streaming. b) The cell treated with 5 μg/ml CB. The arrow points to a helmet-like mass of cytoplasm containing several chloroplasts. The nucleus is far from this mass. c) The same cell (as in 2b) after 10 sec. The nucleus has moved anticlockwise, but the helmet-like mass has not moved from its original position. Scale, 50 μm, $\times$650

stability of the microfilaments in *Vallisneria* cells, we checked the reversal of the streaming direction caused by drug treatment at concentrations of 10, 50 and 100 μg/ml. As a control, cells were immersed in a solution of 1% DMSO.

The ratio of reversed cells to the total cells examined increased gradually depending on the concentration and the duration of treatment, finally reaching a plateau of 50% on the average (Fig. 3). With treatment at concentrations of 50 and 100 μg/ml, the plateau was reached within 3 h, and at 10 μg/ml within 24 h. In the control, the reversal remained a few percent.

The fact that the reversal of the streaming direction was observed in 50% of the cells suggests that the bundles of microfilaments are disorganized completely by CB in every cell, and that after the removal of the drug the bundles are regenerated independent of the original polarity, because the probability of regenerated microfilaments having reversed polarity is 50%.

Effects of lead acetate. 1) On the streaming rate: Lead acetate also has a reversible inhibitory effect on cytoplasmic streaming in Characean internodal cells, though the mechanism of inhibition is not clear (5). The reversibility of its action is useful for this investigation; therefor, the mode of inhibition was first examined.

The drug was applied at concentrations of 10, 50, 100, 200 or 500 mM for 1 h. When the cells were treated with 10 mM of the drug, cessation of the streaming was observed in 70–80% of the treated cells. In the other cells, streaming was not completely inhibited. Lead acetate at a concentration higher than 100 mM (*i.e.*, 200 and 500 mM) was toxic. In more than 30% of the treated cells, streaming was not resumed after removal of the drug and most of the cells died before long.

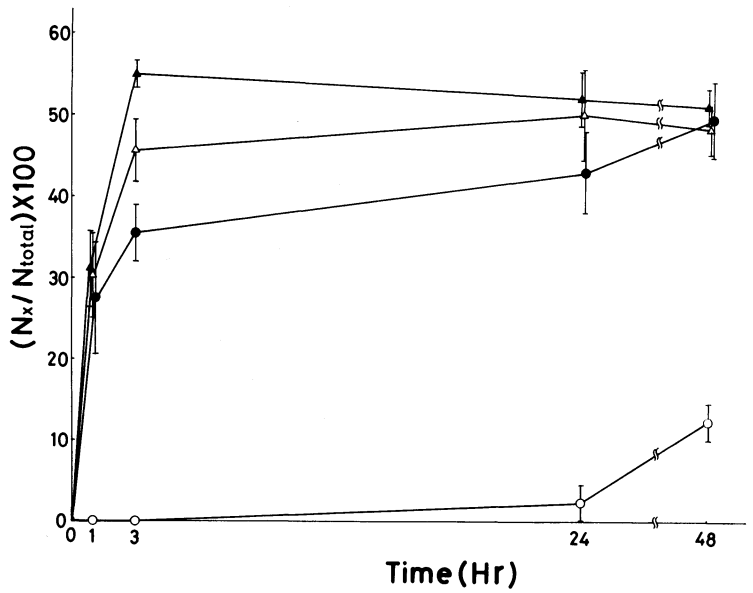

Fig. 3. Effect of CB on the streaming direction. The drug was applied at concentrations of 10 (—●—), 50 (—△—) or 100 μg/ml (—▲—). Control cells were treated with 1% DMSO solution (—○—). Each point represents the mean value for five samples of about twenty cells each. Ordinate: Percentage of cells which reversed their streaming direction after the removal of the drug (N_x) to the total cells examined (N_{total}). Abscissa: Duration of the drug treatment.

Reversible inhibition was observed with treatments at 50 and 100 mM. Fig. 4 shows the time courses of the inhibition of streaming and its recovery. Streaming in every treated cell was inhibited completely within 30 min at 100 mM, and within 45 min at 50 mM. Disturbances in the streaming track and the stack of cytoplasm, observed with CB treatment, did not occur with lead acetate. Cells inhibited completely at 50 mM resumed their normal velocity within 90 min after the drug had been washed out. The recovery in cells that had been treated at 100 mM was slightly delayed.

We noted that streaming started without a time lag after removal of the drug as was observed with CB treatment.

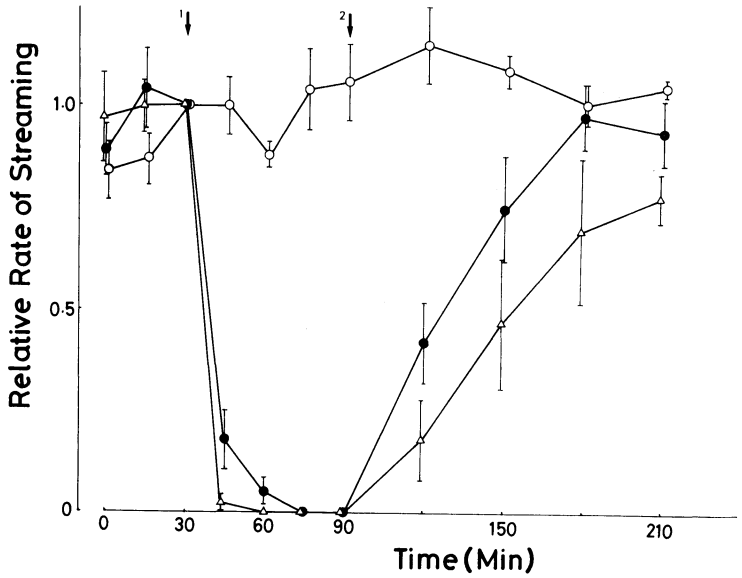

Fig. 4. Effect of lead acetate on the streaming rate. As in the case of CB, lead acetate was applied at the concentrations of 50 (—●—) or 100 mM (—△—). Tap water was used as the control medium (—○—). Each point represents the mean value for five cells. The length of the vertical bar is the S.E. of the mean.

TABLE 1. EFFECT OF LEAD ACETATE ON THE STREAMING DIRECTION

Duration of treatment (h)	Concentration					
	0 mM		50 mM		100 mM	
	N_x*1/N_{total}*2	Mean±S.E.	N_x/N_{total}	Mean±S.E.	N_x/N_{total}	Mean±S.E.
1	0/23 0/21 0/20 0/29 0/28	0.00	0/21 0/24 0/22 0/18 0/30	0.00	0/19 0/23 0/27 0/15 0/22	0.00
3	0/25 0/18 0/31 0/19 0/24	0.00	1/19 0/21 0/22 0/19 1/24	0.02±0.01	0/17 0/26 2/23 0/24 0/18	0.02±0.02

*1 Number of cells which reversed streaming.

*2 Total number of cells examined in one sample.

2) *On the streaming direction*: Effects of the drug at 50 and 100 mM were examined as described above. The treatment periods were set at 1 and 3 h because treating cells for a longer period (such as 1 day) killed even those immersed in rather dilute solutions (*i.e.*, 50 mM). Results are shown in Table 1. Lead acetate produced no reversal of the streaming direction in almost all the cells. This differs sharply from the results obtained for CB treatment, and suggests that the microfilament bundles must be structurally stable during the cessation of cytoplasmic streaming caused by lead acetate.

DISCUSSION

The present study showed: 1) Streaming was completely inhibited by CB at 10–100 μg/ml, but with good reversibility. CB at 5 μg/ml broke the track of rotational streaming locally and induced irregular movement of the cytoplasm. 2) When streaming had ceased due to the presence of CB, reversion of its direction occurred in about 50% of the treated cells after the removal of the drug. 3) Lead acetate at 50–100 mM quickly halted streaming. After its removal, streaming in the same direction was gradually restored.

The cessation of streaming in *Vallisneria* caused by CB and lead acetate is strong evidence that its motile mechanism is homologous to that in Characean cells. This also is supported by the fact that many bundles of microfilaments are localized in the peripheral region of *Vallisneria* cells (unpublished results). However, there seems to be a considerable difference between the structural stability of microfilament bundles in Characeae and *Vallisneria*. The structural integrity of the Characean bundles is preserved when streaming ceases during CB treatment (1, 14). However, the structural integrity of the microfilaments in *Vallisneria* is assumed not to be maintained during CB treatment because of the reversal of the streaming direction. Our next step will be to use electron microscope to check the extent to which these bundles are broken down by the drug.

Lead acetate inhibits cytoplasmic streaming in *Vallisneria* with no reversion of the streaming direction after the drug is removed. The mechanism of this inhibition is not clear and must be further investigated. However, we obtained important evidence about the integrity of the microfilament bundles; simple cessation of streaming for a few hours does not result in their disorganization.

Based on the data for CB treatment, the microfilament bundles in *Vallisneria* cells do not seem to be as rigidly constructed as those in Characean cells. However, their integrity is not so labile that the simple cessation of streaming would lead to disintegration. One result of our preliminary experiment suggested that the microfilaments have a tendency to become disorganized when the cells are kept in darkness. When cells that had been kept in darkness were irradiated with light, the cytoplasmic particles in their pericline walls usually moved around along newly formed, diversely directed cytoplasmic tracks. These streamlets were finally united and established steady rotational streaming along the anticline walls of the cell (cf. 12, 13). These processes seem to be closely related to regeneration of the microfilament bundles which become partly or completely disordered in darkness. We must now determine what factor governs the stability of the microfilament bundles.

Our tentative conclusions are 1). The motile mechanism of secondary streaming, as far as the microfilaments are concerned, is homologous to primary streaming once

rotational streaming is established. 2). The motile system of secondary streaming differs in the structural stability of the microfilament bundles from that of primary streaming.

Cytoplasmic organelles equipped with myosin-like protein need to be investigated further. Myosin-like protein already has been extracted from *Elodea* and partially purified (11).

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