MOTILE APPARATUS IN VALLISNERIA LEAF CELLS. I. ORGANIZATION OF MICROFILAMENTS

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SUMMARY

The organization of the microfilaments in epidermal cells of Vallisneria leaves was investigated with respect to the induction of cytoplasmic streaming (secondary streaming). In many of the epidermal cells, cytoplasm exhibited rotational streaming along the anticlinal wall of the cell after exposure to intense light. In these cells, many bundles of 6–7 nm microfilaments were observed in the cortex around the anticlinal wall. The bundles were arrayed in parallel to the streaming direction. They were recognized usually as 10–40 closely packed dense dots in cross-section. The spacing between bundles was not even. Bundles tended to form groups of 4 to 5 in which the spacing between bundles was usually 0.3 and 0.5 μm.

The microfilaments were identified as F-actin. Together with the fact that rotational streaming in Vallisneria cells was inhibited by cytochalasin B, the motile mechanism of secondary streaming was concluded to be similar in its essential features to the cytoplasmic streaming seen in Characean cells (primary streaming).

INTRODUCTION

In leaf cells of higher aquatic plants, for example, Elodea and Vallisneria, rotational streaming of the cytoplasm is induced by external stimuli, such as irradiation by visible light or application of various chemicals. This type of streaming is called 'secondary streaming', while the streaming seen in Characean cells is called 'primary streaming', because it is continuous under natural conditions (Hauptfleisch, 1892; Kamiya, 1959).

The nature of the photoreceptor involved in light-induced movement of the cytoplasm and the chloroplast has been clarified to some extent (Haupt & Schönbohn, 1970; Seitz, 1972, 1974); however, little is known about the structure and function of the motile system responsible for secondary streaming.

Most of the recent advances in our understanding of cytoplasmic streaming have...
been made through observations and experiments with Characean cells. The rotational cytoplasmic streaming in Characean cells is generally understood to be caused by unidirectional sliding of endoplasmic organelles along bundles of microfilaments anchored on the stationary chloroplast files. The sliding organelles were shown to be equipped with myosin-like protein (Nagai & Hayama, 1979a, b). The microfilaments are mainly composed of F-actin (Palevitz, Ash & Hepler, 1974; Palevitz & Hepler, 1975; Williamson, 1974). The direction of streaming is supposed to be determined by the polarity of F-actin (Kersey, Hepler, Palevitz & Wessells, 1976). This information should help us understand the mechanism of secondary streaming.

Forde & Steer (1976) looked for microfilaments, one of the indispensable components of the motile apparatus, in leaf cells of Elodea. However, they did not recognize the significance of bundles of microfilaments for streaming.

The present paper describes the structural organization of the microfilament bundles in Vallisneria cells.

MATERIALS AND METHODS

Plant

Vallisneria gigantea Graebn was purchased at a tropical fish shop and cultured in a bucket with soil at the bottom and filled with tap water. It was illuminated alternately with 12-h periods of dark and light from fluorescent lamps of about 2000 m-2 cd sr.

A piece of leaf, about 10 cm long, was cut from a healthy plant and then chopped into smaller pieces about 1 mm long. These were floated on distilled water, and the air trapped in the intercellular space was evacuated for 5 min. After that the materials were continuously illuminated at about 300 or 3000 m-2 cd sr for 1 to 2 days.

Induction of cytoplasmic streaming

In 70–80% of the cells that had been illuminated continuously with intense light (about 3000 m-2 cd sr), cytoplasmic rotation was induced within 12 h, while in cells kept under low-intensity light (about 300 m-2 cd sr), the cytoplasm was quiescent. To observe the process of induction of streaming by EGTA, a piece of chopped leaf was mounted in distilled water, then the water was replaced by a solution of 5 mm EGTA and 10 mm Tris-maleate buffer (pH 7.0). Photographs were taken at appropriate stages in the induction process with a Zeiss photomicroscope it with differential interference optics.

Thin-sectioning

Chopped pieces of leaf that had been exposed to intense light were mounted in distilled water and monitored with a light microscope. Epidermal cells in which rotational streaming was observed were photographed for identification during subsequent sectioning. Cells kept under low-intensity light were also monitored under low-intensity illumination. Each group of cells was then immersed in cold fixative consisting of 2% glutaraldehyde, 6 mm MgCl2 and 45 mm potassium phosphate buffer (pH 6.6) for 2 h. After washing twice with the salt solution, they were post-fixed for 2 h with 2% OsO4 prepared with the same salt solution. After dehydration through a graded series of ethanol, the cells were embedded in Spurr’s medium (Spurr, 1969). Each epidermal cell to be examined was trimmed, then sectioned on a LKB ultratome. Thin sections were stained with a solution of uranyl acetate in 25% methanol and lead citrate before examination with a JEM 100-C type electron microscope at 80 kV.
Negative staining

To obtain cytoplasm uncontaminated by cell wall, chopped leaf was treated with Driselase (Kyowa Hakko, Tokyo) prepared as described by Ohiwa (1977) to make protoplasts. Chopped leaf was floated on a salt solution containing the enzyme (10 mg/ml) and 300 mM NaCl, and kept at 25–27 °C under continuous illumination of 3000 mcd sr. Within 5–7 h protoplasts were obtained. They were washed several times to remove the enzyme and cell debris with the solution to which 2 mM CaCl₂ has been added and then were suspended in a small volume of a salt solution containing 100 mM KCl, 2 mM MgSO₄, 15 mM EGTA, 2 mM DTT, 1 mM ATP and 10 mM Tris-maleate buffer (pH 7.0). The suspension was centrifuged to break the protoplasts into fragments. A drop of cytoplasmic suspension was placed on a Formvar/carbon-coated grid, and then negatively stained with a 1% aqueous solution of uranyl acetate. Some grids were treated with heavy meromyosin from skeletal muscle (0.05 mg/ml of 100 mM KCl) before negative staining.

Centrifugation of cells

To examine anchoring of the microfilaments on the cortex of the cell, a piece of leaf was centrifuged at 8000 g for 30 min at 4 °C in a chamber designed for light microscopy. The direction of the rotation in each cell was checked before and after centrifugation, and the percentage of cells showing reversal of the direction was observed.

RESULTS

Induction of cytoplasmic streaming

A Vallisneria leaf is made up of epidermal cells on each surface and a few layers of mesophyll cells occupying the inner part. Observations of the induction of streaming were mainly made on epidermal cells. In epidermal cells that had been kept under low-intensity light, the cytoplasmic particles stayed still and almost all chloroplasts were distributed on the wall facing the exterior, which is called the periclinal wall. On irradiation of intense light, small particles and then chloroplasts began to move in diverse directions. At an early stage of induction of streaming cytoplasmic fibrils along which small particles actively moved were often visible on the periclinal wall, although they were extremely ephemeral. Chloroplasts were transferred in time from the periclinal wall to side walls (which are called anticlinal walls). Streamlets were finally united to establish the steady rotational streaming around the anticlinal walls. The direction of rotation differed from cell to cell: some were clockwise and others were counterclockwise. Once cyclosis had been established in each cell, the direction of streaming no longer changed easily.

A similar pattern of streaming induction was observed when cells were treated with EGTA of appropriate concentration. An example of the steps of the induction process is shown in Fig. 1. Fig. 1A shows a surface view of epidermal cells before application of the drug. Fig. 1B shows the chloroplast distribution at 10 min after the application of EGTA solution. The steady rotational streaming was usually established in 60 to 70% of the epidermal cells by 30 to 40 min after the treatment (Fig. 1C). However, the induced streaming stopped in a few hours, even when the cells were kept in the same solution.

In mesophyll cells which had been kept under low-intensity light, the cytoplasm also stayed still. Chloroplasts were distributed around the walls perpendicular to the
Fig. 1. Surface views of epidermal cells. Cells had been kept under low-intensity light before treatment with 5 mM EGTA containing 10 mM Tris-maleate buffer at pH 7.0. A. Before drug treatment. The cytoplasm is still and the chloroplasts are distributed on the periclinal wall. B. 10 min after drug application. Many chloroplasts have been transferred from the periclinal to the anticlinal wall. Some remain on the periclinal wall. C. 30 min after drug application. Rotational streaming is taking place around the anticlinal wall.
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leaf surface. They started to stream along these walls in much shorter time, usually in 3–5 min, than in epidermal cells. The mesophyll cells continued to stream even after the cytoplasm in epidermal cells had ceased to move.

Microfilament system in epidermal cells

Microfilaments in bundles are one of the indispensable components for cytoplasmic streaming in Characean cells. The same was expected to be the case in Vallisneria cells, because cytochalasin B reversibly inhibits the streaming (Ishigami & Nagai, 1980). To reveal their involvement and localization in Vallisneria cells, ultrastructural investigation was performed. Observations were made only with epidermal cells because fixation of mesophyll cells was unsatisfactory.

Fig. 2A is a low-magnification electron micrograph of an epidermal cell such as that shown in Fig. 1C, in which active cyclosis was manifested before fixation. Sections were made parallel to the streaming loop. The cytoplasm was evenly distributed around the anticlinal walls. Under higher magnification, clear parallel arrays of microfilaments as shown in Fig. 2B, C were often observed. The microfilaments in bundles were localized extremely close to the cytoplasmic surface membrane. The longitudinal axis of the microfilament bundles was parallel to the streaming direction. Much rough-surfaced endoplasmic reticulum existed along the bundles.

In sections perpendicular to the streaming loop, i.e. perpendicular to the periclinal wall, the cytoplasm and the chloroplasts were mostly distributed in the vicinity of the anticlinal walls as shown in Fig. 3A. Higher magnification of the cytoplasm around the anticlinal wall revealed closely packed electron-dense dots (arrows), usually 10 to 40 in number. These represented cross-sections of 6–7 nm microfilaments in bundles (Fig. 3B, C). These bundles were not evenly distributed along an anticlinal wall but were in groups of 4 to 6 (Fig. 4, arrows). In such a group, the distance between adjacent bundles was usually observed to be 0.3–0.5 μm, which is similar to the spacing seen in Characean cells (Nagai & Rebhun, 1966).

In epidermal cells which had been kept under low-intensity light, the cytoplasm and almost all chloroplasts were on the periclinal wall as shown in Fig. 1A. Fig. 5A shows an electron micrograph of such a cell sectioned perpendicularly to the periclinal wall. Most of the cytoplasm and chloroplasts are on the periclinal wall and a thinner layer of the cytoplasm is on the anticlinal walls. Cross-sections of microfilaments (Fig. 5B, C) were found in the thin layer of cytoplasm on the anticlinal walls. We noted that the bundles were more loosely packed than those in the streaming cells and also less frequently observed.

Identification of microfilaments

These microfilaments were suspected of being F-actin. Before testing this possibility, the cells were treated with Driselase to avoid contamination by fibrous components from the cell wall. Isolated protoplasts (Fig. 6) were broken mechanically into fragments in salt solution.

In the cytoplasmic suspension thus obtained, bundles of microfilaments were often observed (Fig. 7A). The twisted and beaded structure of each microfilament, a
Fig. 2. A. A section of an epidermal cell with cytoplasm streaming around the anticlinal wall. Sectioning was parallel to the streaming loop. The cytoplasm and chloroplasts are evenly distributed. \( \text{chl} \), chloroplast; \( n \), nucleus. B, C. Microfilament bundles (\( \text{mf} \)) lying in the vicinity of the cell membrane (\( \text{cm} \)). The longitudinal axis of a bundle is parallel to the streaming direction. Rough-surfaced endoplasmic reticulum (\( \text{er} \)) is close to the bundles.
Fig. 3. A section of an epidermal cell similar to that in Fig. 2. Sectioning was done perpendicular to the streaming loop. The cytoplasm is mainly distributed along the anticlinal wall. A, C. Enlargement of the cytoplasm in the vicinity of the anticlinal wall. Closely packed electron-dense dots representing cross-sections of 6–7 nm microfilaments in bundles are obvious.
Fig. 4. A micrograph showing the cytoplasm at the anticlinal wall. Arrows point to places where cross-sections of the microfilament bundles can be seen.

characteristic feature of F-actin, was clearly seen (arrows). The transverse periodicity of about 40 nm was also obvious. Fig. 7B shows an optical diffraction pattern of the area representing a paracrystalline array. The pattern was similar to that obtained from a paracrystal of muscle F-actin (Moore, Huxley & DeRosier, 1970). Furthermore, the microfilaments were decorated with muscle heavy meromyosin to form arrowhead complexes (Fig. 7C).
Fig. 5. A. A section of an epidermal cell which was kept under low-intensity light before fixation. Sectioning was perpendicular to the periclinal wall. The cytoplasm and the chloroplasts are on the periclinal wall. Only a thin layer of the cytoplasm is observed around the anticlinal wall. B, C. Enlargement of the vicinity of the anticlinal wall side. Electron-dense dots representing microfilaments in bundles are localized in the vicinity of the cell membrane. They seem to be less closely packed than those in Fig. 3B, C.
Fig. 6. Isolated protoplasts from a leaf of *Vallisneria*.
They are clearly free of cell debris.

**Centrifugation of cells**

All the electron micrographs showed that the bundles of microfilaments were localized in the vicinity of the cytoplasmic membrane or in the subcortical layer of the cell. They must be somehow anchored in the cortex. To study the anchoring, cells were centrifuged at a moderate centrifugal force of 8000 g for 15 min. The endoplasm and chloroplasts that had all been shifted to the centrifugal end always started to stream back along the anticlinal wall after a few minutes. The direction of the recovered rotation in each cell was compared with the direction of rotation before centrifugation. In all 150 cells the direction of recovered rotation was the same as that before centrifugation. The result coincides well with the observation made with *Elodea* cells by Beams (1949), that reversal of the direction was observed in only 7% of the cells after centrifugation at a centrifugal force as high as 350000 g for 30 min. From these observations, we can reasonably conclude that the microfilament bundles are kept anchored, and the polarity of the microfilaments is fixed in each cell during centrifugation, as is the case with Characean cells. This is further support for the view that the microfilament bundles must provide the track along which cytoplasmic particles can move.
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DISCUSSION

The involvement of microfilament bundles in Vallisneria epidermal cells was revealed by the present study. The microfilaments are mainly composed of F-actin. The idea that they are indispensable for rotational cytoplasmic streaming in these cells is supported by the following facts: (1) streaming is inhibited by cytochalasin B, a drug that also has inhibitory action on cytoplasmic streaming in Characean cells (Bradley, 1973; Williamson, 1972); (2) microfilaments are anchored in the cortical gel layer of the cell and their longitudinal axis coincides well with the direction of streaming, suggesting that they serve as tracks along which the endoplasm can stream; (3) the bundles of microfilaments are sufficiently abundant compared with Characean cells (Kamitsubo, 1966, 1972; Nagai & Rebhun, 1966). Thus, the motile mechanism of secondary streaming, as far as the microfilaments are concerned, is analogous to primary streaming after rotational streaming is established.

In epidermal cells kept under low-intensity light we observed microfilament bundles, even though the cytoplasm remained stationary in these cells. In such cells...
the microfilaments were packed in less well ordered array and were less frequently encountered than was the case in streaming cells. Rotational streaming in these cells was induced by EGTA in a short time. The function of the motile system must therefore be inhibited in some way so that the cytoplasm is kept immobile. The inhibitory mechanism seems to be closely related to secondary streaming. The effectiveness of EGTA at a proper concentration strongly suggests the role of Ca$^{2+}$ in the inhibitory mechanism. It has been shown in Characean cells that the free Ca$^{2+}$ concentration should be $10^{-7}$ M or less for cytoplasmic streaming to occur at maximum rate (Williamson, 1975; Tazawa, Kikuyama & Shimmen, 1976).

A myosin-like protein has been extracted and partially purified from *Egeria densa* (*Elodea*), a close relative of *Vallisneria* (Ohsuka & Inoue, 1979), but the mode of myosin involvement in streaming remains to be investigated.

We wish to express our sincere thanks to Professor N. Kamiya of the National Institute of Basic Biology for continuous interest and valuable criticism throughout the period of this study. We are indebted to Dr K. Kuroda for her kind guidance in light microscopy, and to Dr R. Kamiya for taking the photographs of the optical diffraction pattern. Rabbit heavy meromyosin was kindly supplied by Dr A. Inoue.

This work was partly supported by grants-in-aid from the Japanese Ministry of Education, Science and Culture.

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(Received 16 June 1980)