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Reconstitution of Cytoplasmic Streaming in *Characeae*

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Summary

The active sites of actin of one *Characeae* species were found to interact with the endoplasmic factor from a different species. Protoplasm was suqueezed out of cells of *Chara australis* with vacuoles that had been perfused beforehand with a medium containing EGTA and Mg·ATP. Centrifugation of this protoplasmic mixture divided it into the supernatant composed of endoplasmic granules and the precipitate composed of chloroplasts and nuclei. When the endoplasmic granular aggregates were introduced into a tonoplast-free *Nitella axilliformis* cell treated with NEM to inactivate the endoplasmic factor, they became attached to the *Nitella* gel and streamed longitudinally with the polarity. Treatment of the endoplasmic granules with the strong Mg^{2+}-chelator CyDTA (1,2-cyclohexane diamine *N*, *N'*-tetraacetic acid) irreversibly inhibited reconstitution of the cytoplasmic streaming.

Keywords: Actin; *Chara;* Cytoplasmic streaming; Myosin; *Nitella.*

Abbreviations

APW artificial pond water, *ATP* adenosine-5'-triphosphoric acid, *CyDTA* cyclohexanediamine-N,N'-tetraacetic acid, *DTT* dithiothreitol, *EGTA* ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid, *HMM* heavy meromyosin, *NEM* N-ethylmaleimide, *PEP* phosphoenolypyruvate, *PIPES* piperazine-N,N'-bis-(2-ethanesulfonic acid), *PK* pyruvate kinase, *PMSF* phenylmethylsulfonylfluoride.

1. Introduction

The motive force of cytoplasmic streaming in *Nitella* is generated at the sol-gel interface (KAMIYA and KURODA 1956). Cytoplasmic fibrils were found at the sol-gel interface or at the inner surface of the stationary layer of chloroplasts with light (KAMITSUBO 1966b) and electron microscopy (NAGAI and REBHUN 1966). KAMITSUBO (1966a, 1972) demonstrated that these fibrils are indispensable for the streaming. They are composed of microfilaments (NAGAI and REBHUN 1966) which can be decorated with arrowhead-like appendages of muscle HMM (PALEVITZ *et al.* 1974, WILLIAMSON 1974) and stained with fluorescent actin antibody (WILLIAMSON and TOH 1979). The relationship between the direction of the arrowheads and that of the streaming has also been clarified (KERSEY *et al.* 1976). The involvement of actin evokes the idea that myosin might be involved in the motive force generation of the streaming. CHEN and KAMIYA (1975) showed the presence of a NEM-sensitive component in the endoplasm. KURODA and KAMIYA (1975) showed that the endoplasmic component can be substituted by muscle HMM with retainment of the ability to rotate chloroplasts in the endoplasm. The endoplasm is also more sensitive to heat treatment than the actin bundle (KAMITSUBO 1982). These results suggest that myosin, which is sensitive to NEM and heat, is located in the endoplasm and that the motive force is generated by interaction between actin fibrils and the putative myosin. Myosin has been isolated from *Nitella* (KATO and TONOMURA 1977), but its localization in the cell remains unknown. Mg·ATP was demonstrated to be the direct energy source for the streaming (WILLIAMSON 1975, TAZAWA *et al.* 1976, SHIMMEN 1978).

The factor in the endoplasm which cooperates with actin fibrils to cause cytoplasmic streaming needs to be identified. To approach this problem, we tried to reconstitute the streaming from isolated components using tonoplast-free cells prepared by vacuolar

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perfusion with a medium containing EGTA (TAZAWA *et al.* 1976). In such cells, chemicals or cell organelles introduced by intracellular perfusion have easy access to the actin bundles located in the cytoplasm.

2. Materials and Methods

Male strains of dioecious *Chara australis*[1] and *Nitella axilliformis* were cultured in plastic buckets containing tap water with soil and rotten leaves at the bottom. The culture was kept in an air-conditioned room (27 °C) with 14 hours of light (1.5 W/m^2 at the surface) and 8 hours of dark. Intrernodal cells were used throughout the experiments. The experimental media compositions are shown in Table 1 and the flow diagram of the experimental procedures in Fig. 1.

Table 1. *Compositions of perfusion media (in mM)*

	Media	
	Mg·ATP	CyDTA
EGTA	5	0
CyDTA	0	5
ATP	1	1
$MgCl_2$	6	0
PIPES	30	30
K^+	71	68
DTT	1	1
Sorbitol	200	200
PMSF	10 μM	10 μM
pH	7.0	7.0

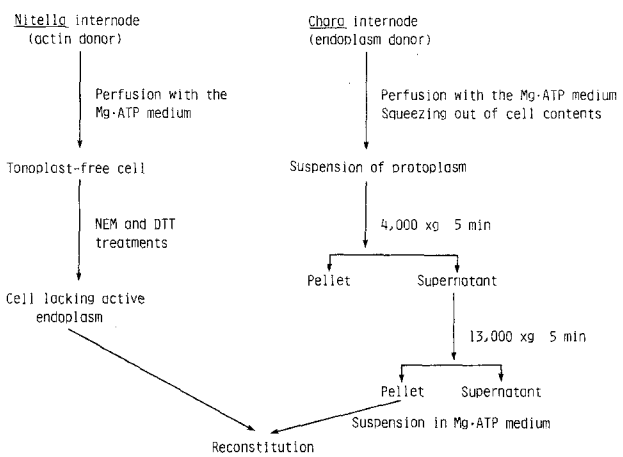

Fig. 1. Flow diagram of procedures for reconstitution of the streaming. For explanation, see the text

[1] The material was supplied by Dr. W. J. LUCAS (University of California, Davis) who named it *C. corallina* (LUCAS and SHIMMEN 1981). Since it is dioecious, we refer to it as *C. australis* after IMAHORI (1977).

2.1. Preparation of Nitella Internodes Lacking the Active Endoplasmic Factor

The internodal cell of *Nitella* was cut at both cell ends and then perfused with the Mg·ATP medium. Next, both opened cell ends were closed by ligation with strips of polyester thread (TAZAWA *et al.* 1976). The cell was kept in artificial pond water (APW) containing 0.1 mM each of KCl, NaCl and $CaCl_2$. After microscopic confirmation of tonoplast disintegration (TAZAWA *et al.* 1976), the cell was transferred to APW containing 5 or 10 mM NEM. After 20 minutes, the cell was transferred to APW containing 1 mM DTT to remove free NEM. The streaming was not recovered after washing with DTT. It has been reported that NEM irreversibly inactivates the endoplasmic factor responsible for streaming but not the actin bundle (CHEN and KAMIYA 1975).

2.2. Isolation of Chara Endoplasm

Isolated endoplasm was obtained from internodal cells of *Chara australis* selected for their large size (800 μm in diameter and more than 10 cm long). Endoplasm from 20 to 40 cells was required for one reconstitution experiment. The natural cell sap of *Chara* internodes was replaced with Mg·ATP medium by the perfusion technique. The cell contents including protoplasm and perfusion medium were squeezed out on a piece of Parafilm (American Can Company) and gently mixed with a Pasteur pipette. Next, this mixture was centrifuged at 4,000 × g for 5–10 minutes at room temperature to discard the chloroplasts and nuclei which precipitated. The supernatant containing endoplasmic granules was suspended in the Mg·ATP medium and then centrifuged at 14,000 × g for 5–10 minutes at 4 °C. Most of the granules were collected at the centrifugal end. The precipitate was suspended in the Mg·ATP medium in which the ATP concentration had been fixed at 1 mM by adding 10 mM PEP and 1 mg/ml PK.

Reconstitution was carried out by perfusing the inactivated *Nitella* cell with the suspension of *Chara* endoplasm.

3. Results

The micrograph of an endoplasm suspension of miscellaneous granules isolated from *Chara* is shown in Fig. 2. Brownian movement but not active movement was observed under the × 40 objective.

When the tonoplast-free *Nitella* cell treated with NEM and DTT was perfused with the Mg·ATP medium, the streaming was not recovered, indicating that it was irreversibly inhibited by NEM. When the *Nitella* cell lacking active endoplasm was perfused with the endoplasm suspension from *Chara* cells, active movement was not observed at first. However, after about 4 minutes, granules attached to the inner surface of the lower chloroplast layer began to move in the longitudinal direction along the files of chloroplasts (Fig. 3 *a*). When the cell was placed upside down, the movement continued on the new upper side. After a while, granules were seen moving on both upper and lower sides of the cell (Fig. 3 *b*). The granule movement was observed on both sides of the indifferent line; the

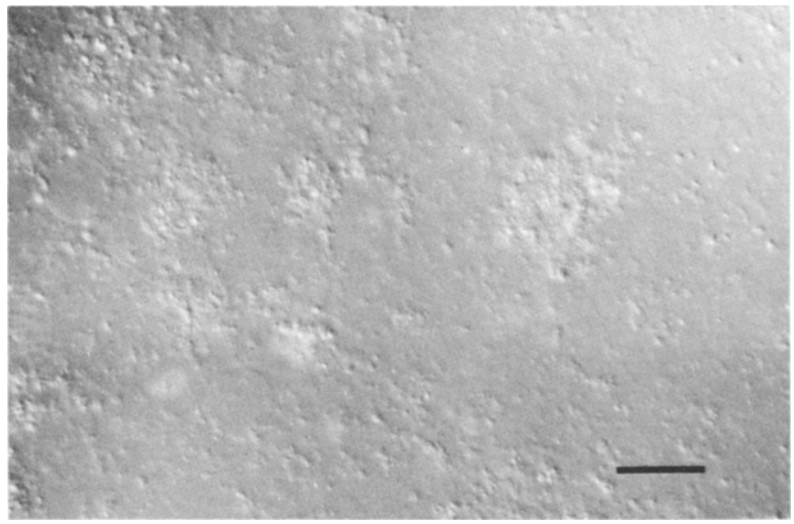

Fig. 2. Nomarski micrograph of endoplasm isolated from *Chara* internodes. Endoplasm was suspended in the Mg·ATP medium. ×40 objective lens. Marker bar represents 20 μm

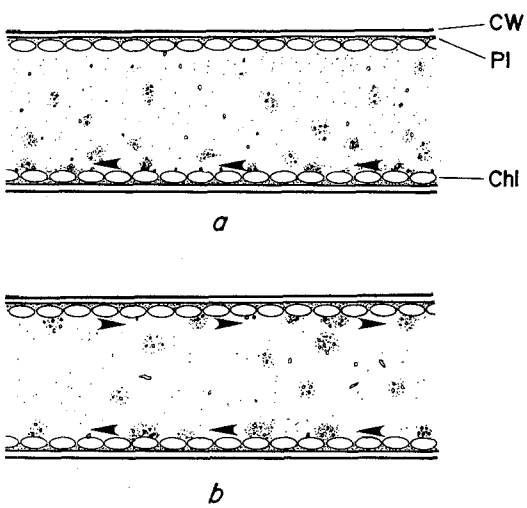

Fig. 3. Schematic representation of reconstituted streaming. (*a*) Streaming occurred along the lower chloroplast layer. (*b*) When the cell was placed upside down, streaming occurred along both lower and upper chloroplast layers. Arrows indicate the streaming direction. *CW* cell wall, *Pl* plasmalemma, *Chl* chloroplasts

movement on one side was in the direction opposite that on the other side, as in the case of normal cytoplasmic streaming. Occasionally, granules on one side moved in the wrong direction for a short distance (ca. 100 μm) but then soon corrected their direction. Table 2 shows the highest rate of granule movement in each cell. Since the speed of normal streaming was 110 μm/sec, the speed of the reconstituted streaming was half or less than half of the normal rate.

A previous paper (SHIMMEN 1978) reported that in tonoplast-free cells of *Nitella*, the streaming was

Table 2. *Rate of reconstituted cytoplasmic streaming*

Cell No.	Streaming rate (μm/sec)
1	50
2	50
3	33
4	30
5	25
6	22
7	20
8	20
9	16

reversibly inhibited by depletion of ATP but irreversibly by Mg^{2+} depletion. To check whether the actin bundle or the endoplasmic factor is irreversibly inactivated by Mg^{2+} depletion, either the *Nitella* cell (actin donor) or the endoplasm from *Chara* was pretreated with the CyDTA medium. To see the effect of Mg^{2+} depletion on actin bundles, *Nitella* internodes were perfused with the CyDTA medium. Since CyDTA binds strongly with both Ca^{2+} and Mg^{2+}, the streaming which is dependent on Mg^{2+} stopped after disintegration of the tonoplast. The Mg^{2+}-depleted *Nitella* cell was treated with NEM and DTT, then was perfused with the Mg·ATP medium containing a suspension of *Chara* endoplasm. The streaming was reconstituted in this case. To see the effect of Mg^{2+} depletion on the endoplasm, *Chara* internodes were perfused with the CyDTA medium and the endoplasm was isolated by centrifugation. It was washed once with

Mg·ATP medium and resuspended in Mg·ATP medium containing 10 mM PEP and 1 mg/ml PK to keep the $[ATP]_i$ constant. This endoplasm suspension was introduced into the *Nitella* internode which had been perfused with the Mg·ATP medium and treated with NEM and DTT. Active movement of granules was scarcely observed. Occasionally, some granules moved irregularly for a short distance (500 μm) but soon stopped.

4. Discussion

This is the first report of successful reconstitution of cytoplasmic streaming in *Characeae* cells with intact actin bundles and the endoplasmic component. Heavy meromyosin or subfragment 1 from rabbit muscle forms arrowhead structures around the microfilaments (F-actin) of plant cells, *Characeae* (PALEVITZ *et al.* 1974, WILLIAMSON 1974), *Physarum* (ALLÈRA *et al.* 1971, NAGAI and KATO 1975), *Mougeotia* (KLEIN *et al.* 1980), *Vallisneria* (YAMAGUCHI and NAGAI 1981), and *Vaucheria* (BLATT *et al.* 1980), indicating that actin and myosin from different kingdoms can interact with each other. The present study showed that actin and the myosin-like component from a different species of *Characeae* can cooperate to drive the cytoplasmic streaming.

Only granules attached to the surface of chloroplasts where the actin bundles are located could move along the actin bundles, those detached from the chloroplasts could not. This indicates that the factor which interacts with the actin bundle is located on the surface of the moving granules. This might be also the case in normal streaming. NAGAI and HAYAMA (1979) found endoplasmic granules with filamentous projections which bind to actin bundles in the absence of ATP. WILLIAMSON (1979) reported the existence of filamentous structures in the endoplasm which were irreversibly destroyed by treatment with high Ca^{2+} concentrations.

A previous paper (SHIMMEN 1978) reported that in the tonoplast-free cell of *Nitella*, depletion of Mg^{2+} from the cytoplasm caused irreversible inhibition of the streaming. The present study showed that not the actin bundles but the endoplasm is inactivated by the Mg^{2+} depletion, which may directly affect the functionally important filamentous structures in the endoplasm.

In one series of experiments, we selectively inactivated the endoplasm by heating (50 °C) the cell, since the endoplasmic factor is more sensitive to heat treatment than the actin bundle (KAMITSUBO 1982). The *Nitella* streaming which had been stopped by heat treatment was recovered upon introduction of *Chara* endoplasm (data not shown). This shows clearly that the heat-labile component is not the actin bundles but the component in the endoplasm. However, as the reconstitution was occasionally unsuccessful, NEM treatment is better for selective inactivation of the endoplasm.

In the present study, mixture of aggregates of unidentified granules of endoplasm was used for the reconstitution. In future work, the endoplasm should be separated into its different components to identify the one responsible for the streaming.

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