Accelerated Sliding of Pollen Tube Organelles along Characeae Actin Bundles Regulated by Ca²⁺

Tadashi Kohno and Teruo Shimmen
Department of Botany, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

Abstract. Pollen tubes show active cytoplasmic streaming. We isolated organelles from pollen tubes and tested their ability to slide along actin bundles in characean cell models. Here, we show that sliding of organelles was ATP-dependent and that motility was lost after N-ethylmaleimide or heat treatment of organelles. On the other hand, cytoplasmic streaming in pollen tube was inhibited by either N-ethylmaleimide or heat treatment. These results strongly indicate that cytoplasmic streaming in pollen tubes is supported by the “actomyosin”–ATP system. The velocity of organelle movement along characean actin bundles was much higher than that of the native streaming in pollen tubes. We suggested that pollen tube “myosin” has a capacity to move at a velocity of the same order of magnitude as that of characean myosin. Moreover, the motility was high at Ca²⁺ concentrations lower than 0.18 μM (pCa 6.8) but was inhibited at concentration higher than 4.5 μM (pCa 5.4). In conclusion, cytoplasmic streaming in pollen tubes is suggested to be regulated by Ca²⁺ through “myosin” inactivation.

Pollen tubes show active cytoplasmic streaming. This cytoplasmic streaming may be responsible for the transport of substances and organelles indispensable for tip growth. When the cytoplasmic streaming is inhibited by cytochalasin B, tip growth stops (Mascarenhas and Lafountain, 1972; Franke et al., 1972), even though secretory vesicles are produced (Picton and Steer, 1981). Cytoplasmic streaming in pollen tubes has been suggested to be supported by the actomyosin system based on the following findings. The actin filaments emerge from the grain and traverse into the pollen tube to the tip. When pollen tubes are treated with cytochalasin B, actin filaments disappear and cytoplasmic streaming stops, whereas colchicine affects neither the actin filaments nor the cytoplasmic streaming (Mascarenhas and Lafountain, 1972; Franke et al., 1972; Condeelis, 1974; Perdue and Parthasarathy, 1985). Thus, the involvement of actin filaments in the cytoplasmic streaming of pollen tubes has been well documented. However, the involvement of myosin in the cytoplasmic streaming of pollen tubes has not yet been established.

The free Ca²⁺ concentration was reported to be highest at the tip region and decreases towards the base (Reiss and Nobiling, 1986; Nobiling and Reiss, 1987). When the Ca²⁺ gradient was disrupted, an accumulation of secretory vesicles at the tip region was disrupted and tip growth was inhibited (Herth, 1978; Reiss and Herth, 1979). Localized Ca²⁺ was thought to limit the vesicle fusion at the tip area (Picton and Steer, 1981). On the other hand, we will suggest that the Ca²⁺ regulation of cytoplasmic streaming may be one of the key processes in controlling tip growth.

Cell motility is generally studied by using isolatedcontractile proteins or demembranated cell models. Myosin isolations were done from characean cells (Kato and Tonomura, 1977) and from two species of higher plant cells (Ohsuka and Inoue, 1979; Vahey et al., 1982). Demembranated cell models were made only in some giant algal cells using the methods of tonoplast removal (Williamson, 1975; Tazawa et al., 1976; Fukui and Nagai, 1985) or plasma membrane permeabilization (Shimmen and Tazawa, 1983; La Claire, 1984). Recently, a novel system to study actomyosin-based motility has been developed. In characean internodal cells, bundles of actin filaments are fixed at the inner surface of the stationary chloroplast layer. It has become possible to induce sliding of foreign myosin along these actin bundles (Sheetz and Spudich, 1983; Shimmen and Yano, 1984). Foreign organelles also showed active movement (Shimmen and Tazawa, 1982; Adams and Pollard, 1986). Since it was demonstrated that actin filaments of characean cells lack Ca²⁺ sensitivity (Shimmen and Yano, 1986), myosin-linked Ca²⁺ regulation can also be studied using this trans-situ method (Vale et al., 1984; Kohama and Shimmen, 1985). Since the Ca²⁺ regulation of the cytoplasmic streaming may be a key process of tip growth, we examined the possibility of myosin-linked Ca²⁺ regulation of cell organelle movement in pollen tubes using Ca²⁺-insensitive characean actin bundles.

Here we demonstrated that pollen tube organelles can move along characean actin bundles and the characteristics of this translocator were similar to those of skeletal muscle myosin. However, unlike skeletal muscle myosin, the movement of this translocator along actin bundles was much faster and was inhibited by Ca²⁺ at physiological concentrations.
**Materials and Methods**

**Pollen Tube Culture**

Pollen of *Lilium longiflorum*, collected 2 d after flowering, was stored at 4°C and used within 3 d. The pollen was sown in glass vials and allowed to germinate for 2.5 h at 25°C in a medium containing 7% (wt/vol) sucrose, 1.27 mM Ca(NO₃)₂, 162 μM boric acid, 0.99 mM KNO₃, and 30 mM KH₂PO₄. pH 5.2 (Dickinson, 1968). Germinated pollen was collected on a glass microfiber filter (GF/F; Whatman Inc., Maidstone, England).

**Isolation of Pollen Tube Organelles**

Pollen tubes were resuspended in homogenization buffer (one anther's pollen per 1 ml) containing 5 mM EGTA, 6 mM MgCl₂, 30 mM Pipes, 71 mM KOH, 200 mM sorbitol, 1% (wt/vol) casein or BSA, 1 mM dithiothreitol (DTT), 100 μg/ml leupeptin, pH 7.0 and homogenized by 150-250 strokes with a mortar and pestle at 0°C. Casein and BSA had been previously dialyzed against distilled water. The homogenate was cleared of pollen grains and cell wall debris by hand-operated centrifugation. This cleared homogenate was introduced into characean cells. To see the effect of Ca²⁺, pollen tubes were homogenized in homogenization buffer containing 1% BSA and various concentrations of CaCl₂ (pH 7.0). The free Ca²⁺ concentration was calculated by using a computer program by Drs. S. Oiki and Y. Okada (Kyoto University, Japan) (personal communication). The H⁺ activity coefficient was adopted from Harned and Owen (1958). The absolute stability constants for the chelators (EDTA and ATP) and the divalent cations (Ca²⁺ and Mg²⁺) were recalculated to compensate for the effect of ionic strength (Shimmen et al., 1982). EDTA purity was 98.32% (lot. No. BS1953; Dojindo Laboratory, Kumamoto, Japan).

**Preparation of the Tonoplast-free Characean Cell**

Preparation of tonoplast-free cells was carried out as reported previously (Shimmen and Yano, 1984). Three species of Characeae—*Niella axilliformis, Nitellogia obtusa*, and *Chara corallina*—were used. Both ends of the internodal cell were cut open, and the cell sap was replaced with the homogenate for 2.5 h at 25°C in a medium containing 7% (wt/vol) sucrose, 1.27 mM Ca(NO₃)₂, 162 μM boric acid, 0.99 mM KNO₃, and 3.0 mM KH₂PO₄, pH 5.2 (Dickinson, 1968). Germinated pollen was collected on a glass microfiber filter (GF/A; Whatman Inc., Maidstone, England).

**Observation of Movement**

The movement of the pollen tube organelles was observed using a Nomarski microscope (Nikon Optiphot-NT) with a 40× objective and recorded with a video camera (CTC 5600JS; Ikegami Tsushiuki Co., Ltd., Tokyo, Japan) and recorder (HG601A; Matsushica Electric Industrial Company Ltd., Osaka, Japan) at 23-25°C. The velocity of the movement was measured for organelles that moved smoothly for more than 20-120 μm. To obtain the ratio of motile organelles, the microscopic field was focused on the inner surface of the chloroplasts where actin filaments are anchored. Motile and stagnant organelles were counted for 10 s. 100 organelles were analyzed in each characean cell and each experiment was repeated four to five times at a given Ca²⁺ concentration. Photomicrographs of moving organelles in characean cells (Fig. 1) were taken with video-enhanced contrast differential interference contrast (DIC) microscopy (Olympus BH, objective 60×).

**Results**

Only Brownian movement was observed in the isolated cytoplasmic suspension when viewed under a 40× objective. When the cytoplasmic suspension from the pollen tubes was introduced into a tonoplast-free characean cell (Tazawa et al., 1976) whose native endoplasm had been inactivated by EDTA and removed (Shimmen and Tazawa, 1982; Shimmen and Yano, 1984), active movement along the characean cell's long axis was observed (Fig. 1). The direction of movement of pollen tube organelles was opposite on the two sides of the indifferent zone where the polarity of actin filaments is reversed (Kersey et al., 1976), offering evidence that movement was induced along the polarity of the actin bundles. This movement was dependent on ATP (Table I, Exps. 1 and 3). When the characean cell was treated with NEM, which also irreversibly inactivates characean endoplasm with respect to the cytoplasmic streaming (Chen and Kamiya, 1975), pollen tube organelle movement was as active as in the EDTA-treated cells (Table I, Exp. 2), indicating that characean myosin did not contribute to the sliding of pollen tube organelles.

Since skeletal muscle myosin is extremely heat labile (Yasui et al., 1958, 1960) and NEM sensitive (Yamaguchi et al., 1973; Shibata-Sekiya and Nomura, 1975), we studied the effect of these treatments on pollen tube organelles. Pollen tubes were treated with 2 mM NEM for 5 min and organelles were isolated. Organelles isolated from NEM-treated pollen tubes could not move along characean actin cables (Table I, Exp. 4). In addition, pollen tube organelles treated at 50°C for 2 min after isolation also lost their motility.

![Figure 1. Sequence of video-enhanced DIC micrographs of pollen tube organelle movement in a characean cell. Pollen tube organelles are moving from right to left along the long axis of the characean cell. Elapsed times in seconds are indicated at right. Bar, 10 μm.](image-url)
Table I. Requirement for Pollen Tube Organelle Movement

| Exp. | Pollen tube organelles | Characean cell | [ATP] mM | Motility |
|------|------------------------|----------------|----------|----------|
| 1    | None                   | EDTA           | 1        | +        |
| 2    | None                   | NEM then DTT   | 1        | +        |
| 3    | None                   | EDTA           | 0*       | -        |
| 4    | 50°C, 2 min            | EDTA           | 1        | -        |
| 5    | NEM then DTT           | EDTA           | 1        | -        |

* The ATP associated with the pollen tube homogenate was removed by centrifugation of the organelles.

Discussion

This study demonstrates that pollen tubes contain translocator, which can generate a sliding force along characean actin bundles in an ATP-dependent manner. The sensitivity of the translocator associated with pollen tube organelles to NEM and heat was quite similar to that of skeletal muscle myosin. Since the cytoplasmic streaming of pollen tube was also inhibited by NEM or heat treatment, it is suggested that the movement of pollen tube organelles along characean actin bundles was remarkably sensitive to Ca$^{2+}$. (Fig. 3). Since the velocity distributions varied among different batches of Characeae, motility was evaluated by counting organelles. When pollen tubes were homogenized in buffers containing <0.18 μM Ca$^{2+}$ (pCa 6.8), the translocator was active and >75% of organelles moved along the actin bundles. However, when the pollen tubes were homogenized in a buffer containing 2.1 μM Ca$^{2+}$ (pCa 5.7), the translocator was almost inactive and only 10% of organelles moved. Only 1% of the organelles moved when the homogenization buffer contained 4.5 μM Ca$^{2+}$ (pCa 5.4), and movement completely stopped when the homogenization buffer contained 37 μM Ca$^{2+}$ (pCa 4.5). To see whether the inhibition by Ca$^{2+}$ was reversible, we added EGTA to pollen tubes that had been homogenized in a buffer containing 37 μM Ca$^{2+}$ (pCa 4.5) to lower the Ca$^{2+}$ concentration to 0.12 μM (pCa 6.9) (final EGTA concentration was 25 mM). In this case only 12.0 ± 4.4% of the organelles moves. So EGTA could only partly reverse the inhibition due to Ca$^{2+}$.
translocator, putative pollen tube myosin, is also working in situ to generate the motive force of the cytoplasmic streaming. The possibility that the actomyosin system generates the motive force for native pollen tube streaming is also supported by the observation that the actin filaments emerge from the grain and traverse into the pollen tube to the tip (Perdue and Parthasarathy, 1985). In addition, when pollen tubes are treated with cytochalasin B, actin filaments disappear and cytoplasmic streaming stops, whereas colchicine affects neither the actin filaments nor the cytoplasmic streaming (Mascarenhas and Lafountain, 1972; Franke et al., 1972; Perdue and Parthasarathy, 1985). These data strongly indicate that the actomyosin system is responsible for cytoplasmic streaming in pollen tubes.

The putative myosin in pollen tubes has a capacity to move on the actin filaments at a velocity of the same order of magnitude as that of the Characeae. There are several factors that may be responsible for the acceleration of the movement of pollen tube organelles in characean cells. (a) Actin bundles in characean cells are so thick that pollen myosins can make good contact with actin filaments. (b) In pollen tubes, the cytoplasmic streaming in one direction is close to that in the opposite direction so that the flows are affected by the drag from the counter streaming (there is no indifferent zone in pollen tubes). (c) The viscosity of the cytosol in pollen tubes may be higher than that of the homogenization buffer in tonoplast-free characean cells.

The maximal sliding velocity between myosin and actin filaments in skeletal muscle contraction is \( \approx 6 \mu m s^{-1} \) (Crowder and Cooke, 1984). A similar velocity has been reported for the sliding of isolated skeletal muscle myosin filaments along isolated muscle actin filaments (Higashi-Fujime, 1985) or for the sliding of beads coated with skeletal muscle myosin along characean actin bundles (Sheetz and Spudich, 1983). By contrast, organelles of amebas move along characean actin bundles at 0.24 \( \mu m s^{-1} \) (Adams and Pollard, 1986). However, pollen tube organelles moved on characean actin bundles at a velocity of the same order of magnitude as that of cytoplasmic streaming of Characeae. Myosins of plant cells with cell walls may be equipped with an ability to induce rapid movement along actin filaments due to the high activity of the individual myosin molecules or due to their organization (e.g., thick filaments).

Ca\(^{2+}\) inhibited the motility of pollen tube organelles in characean cells. Since characean actin filaments lack Ca\(^{2+}\) sensitivity (Shimmen and Yano, 1986), it is concluded that the Ca\(^{2+}\)-sensitizing component is associated with pollen tube organelles. Organelles inactivated by Ca\(^{2+}\) only partially recovered their motility when the Ca\(^{2+}\) concentration was subsequently decreased. The partial recovery may result from the dilution of some component(s) responsible for the reactivation of myosin which had been inactivated by Ca\(^{2+}\). It is also possible that myosin was partially inactivated by a Ca\(^{2+}\)-activated protease, even though a protease inhibitor (leupeptin) and BSA were included in the homogenization buffer. The effect of Ca\(^{2+}\) on motility of pollen tube organelles was opposite to the effect of Ca\(^{2+}\) on the skeletal muscle and the scallop myofibril where Ca\(^{2+}\) activates motility (Lehman and Szent-Györgyi, 1975). But the characteristic that the motility changed at \( \approx pCa 6 \) within a narrow range of pCa (about a unit of pCa) is similar. In the plant kingdom, other examples of Ca\(^{2+}\) inhibition have been reported including cytoplasmic streaming in Characeae (Tomina et al., 1983) and the actin-activated myosin ATPase in Physarum (Kohama and Kendrick-Jones, 1986).

Both membrane and cell wall materials must be continuously supplied at the tip region for the tip growth of pollen tubes, and a mechanism to capture these materials at the tip region is necessary. Cytochalasin B inhibits both tip growth and cytoplasmic streaming. However, it does not inhibit vesicle production (Pficon and Steer, 1981). It seems that the tip growth requires cytoplasmic streaming as a transport system. On the other hand, the Ca\(^{2+}\) gradient is also thought to be necessary for tip growth. When the Ca\(^{2+}\) gradient is destroyed by incubating pollen tubes with the Ca\(^{2+}\) ionophore A23187 in the absence of external Ca\(^{2+}\), tip growth stops even if cytoplasmic streaming is active (Herth, 1978). At the same time, the vesicular zone at the tip is reduced and numerous vesicular contents are irregularly integrated in the pollen tube wall not only in the tip but also along the distance of the pollen tube wall (Reiss and Herth, 1979). The Ca\(^{2+}\) gradient seems to be necessary to localize the growth at the tip. It has been suggested that the localized Ca\(^{2+}\) limit exocytosis at the tip region (Reiss and Herth, 1979). In addition, our present results suggest the possibility that vesicles in the flowing cytoplasm may be captured at the very tip due to an inhibition of the "actomyosin"-based movement by localized free Ca\(^{2+}\).

This is the first report of a successful reconstitution of active movement of organelles from higher plant cells along characean actin bundles. The findings also suggest a physiological role for the higher concentration of free Ca\(^{2+}\) at the tip region in tip-growing pollen tubes. In plant materials the purification of myosin and/or the preparation of a demembranated cell model are difficult. Therefore, studies on the regulation of plant cell motility have been carried out only in limited materials. Using the present method, we may be able to analyze the actomyosin system in many plant cells.

We thank Professor M. Tuzawa and Dr. R. Wayne for their valuable discussion and critical reading of this manuscript. We also thank Professor E. Kamitsubo for use of his video-enhanced DIC microscopy. We also thank Dr. S. Oki and Dr. Y. Okada for the use of a computer program for Ca\(^{2+}\)-EGTA buffer.

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Received for publication 24 June 1987, and in revised form 4 January 1988.

References

Adams, R. J., and T. D. Pollard. 1986. Propulsion of organelles isolated from Acanthamoeba along actin filaments by myosin-1. Nature (Lond.). 322: 754-756.

Blinks, J. R., W. G. Wier, P. Hess, and F. G. Prendergast. 1982. Measurement of Ca\(^{2+}\) concentrations in living cells. Prog. Biophys. Mol. Biol. 40:1-114.

Chen, C. C. W., and N. Kamiya. 1975. Localization of myosin in the internal cell of Nitella suggested by differential treatment with N-ethylmaleimide. Cell Struct. Funct. 1:1-9.

Condeelis, J. S. 1974. The identification of F actin in the pollen tube and prooplast of Amaryllis belladonna. Exp. Cell Res. 88:435-439.

Crowder, M. S., and R. Cooke. 1984. The effect of myosin sulphhydril modification on the mechanics of fibre contraction. J. Muscle Res. Cell Motil. 5:131-146.

Dickinson, D. B. 1968. Rapid starch synthesis associated with increased respiration in germinating lily pollen. Plant Physiol. 43:1-8.

Franke, W. W., W. Herth, W. J. Van Der Woude, and D. J. Morré. 1972. Tubular and filamentous structures in pollen tubes: possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. Planta. 105:317-341.
Kohno and Shimmen

Accelerated Sliding of Pollen Tube Organelles

1543