Evidence for Active Interactions between Microfilaments and Microtubules in Myxomycete Flagellates

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Abstract. We have previously observed the apparent displacement of microfilaments over microtubules in the backbone structure of permeabilized flagellates of *Physarum polycephalum* upon addition of ATP (Uyeda, T. Q. P., and M. Furuya. 1987. *Protoplasma.* 140:190–192).

We now report that disrupting the microtubular cytoskeleton by treatment with 0.2 mM Ca^{2+} for 3–30 s inhibits the movement of the microfilaments induced by subsequent treatment with 1 mM Mg-ATP and 10 mM EGTA. Stabilization of microtubules by pretreatment with 50 μM taxol retarded both the disintegrative effect of Ca^{2+} on the microtubules and the inhibitory effect of Ca^{2+} on the subsequent, ATP-induced movement of the microfilaments. These results suggest that the movement of the microfilaments depends on the integrity of the microtubular cytoskeleton. EM observation showed that the backbone structure in control permeabilized flagellates consists of two arrays of microtubules closely aligned with bundles of microfilaments of uniform polarity. The microtubular arrays after ATP treatment were no longer associated with microfilaments, yet their alignment was not affected by the ATP treatment. These results imply that the ATP treatment induces reciprocal sliding between the microfilaments and the microtubules, rather than between the microfilaments themselves or between the microtubules themselves. While sliding was best stimulated by ATP, the movement was partially induced by GTP or ATPγS, but not by ADP or adenylyl-imidodiphosphate (AMP-PNP). AMP-PNP added in excess to ATP, 50 μM vanadate, or 2 mM *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA) inhibited the sliding. Thus, the pharmacological characteristics of this motility were partly similar to, although not the same as, those of the known microtubule-dependent motilities.

It is well recognized that interactions between cytoskeletal elements, as well as interactions between the cytoskeleton and other cell components, are each functionally important in cell metabolism and motility (reviewed in reference 23). Colocalization of microfilaments and microtubules, two major and ubiquitous components of the cytoskeleton, has been observed in a variety of cells (e.g., references 6, 13, 14, 21, 27, and 28). There has been, however, no report of direct and active interactions between the two, either in vivo or in vitro.

Swarm cells of the myxomycete *Physarum polycephalum* have a pair of flagella which emanate from the anterior end of the elongated cell body. Previous EM observations revealed that cytoplasmic microtubules form a conical structure, called the flagellar cone, which extends posteriorly from the basal body (1, 10, 16, 26, 38). Our previous observation by fluorescence microscopy indicated that the flagellates additionally contain a thick bundle of microfilaments, which we have called the backbone structure, which is coaligned with a bundle of microtubules in the flagellar cone (see Fig. 3, *a–c*). The backbone structure runs along the dorsal axis of these elongated cells, starting from a region close to the basal body, and appears to be crucial for the maintenance of the elongated shape of these cells (28, 29). We examined possible interactions of microfilaments and microtubules in the backbone structure and found that the microfilaments in the permeabilized cells appeared to slide along the microtubules upon treatment with ATP (30).

Here we report an improved fixation protocol for EM and demonstrate that the backbone structure actually consists of closely coaligned bundles of microfilaments and arrays of microtubules. Furthermore, we provide pharmacological data to suggest that the apparent translocation of microfilaments is active and depends on an intact microtubular cytoskeleton.

Materials and Methods

Cells

Flagellates of *Physarum polycephalum* were prepared in 10 mM potassium phosphate buffer, pH 6.6 (buffer P), as described previously (28).

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Reactivation of Triton-permeabilized Flagellates

An equal volume of double-strength permeabilization buffer (0.2% Triton X-100, 4% polyethyleneglycol, 4 mM $MgCl_2$, 2 mM EGTA, 0.2 mM dithiothreitol, 50 μg/ml leupeptin, and 20 mM Pipes, pH 7.0) was added to the suspension of flagellates in buffer P. Then, a solution of 10 mM ATP (Oriental Yeast Co., Tokyo, Japan) in the permeabilization buffer was added, and incubation was continued until fixation of the cells with 1% glutaraldehyde. Solutions of 10 mM GTP (Yamasa Shoyu Co., Chosi, Japan), adenosine-5'-*O*-(3-thiophosphate) (ATPγS[1]; Boehringer-Mannheim GmbH, Mannheim, FRG), and adenylyl-imidodiphosphate (AMP-PNP; Boehringer-Mannheim GmbH) in the permeabilization buffer were pretreated with 0.1 mg/ml hexokinase and 2 mM glucose for 30 min and then added to the permeabilized flagellates in place of ATP. Effects of vanadate were examined by addition of 1 or 10 mM solutions of $NaVO_3$ in 0.1 M Tris-Cl, pH 8.8. The effects of *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA; Burroughs-Wellcome & Co., Raleigh, NC) were examined by addition of aliquots of an 8 mM stock solution in water.

Treatment of Permeabilized Flagellates with Ca^{2+}

Flagellates were pretreated for 1 min in permeabilization buffer containing 50 μM taxol and 0.5% DMSO or 0.5% DMSO alone. They were then treated with 0.2 mM Ca^{2+} by addition of 1/10 vol of a 12.2 mM $CaCl_2$ solution and fixed with 5 mM ethyleneglycol bis-(succinic acid *N*-hydroxysuccinimide ester) (EGS) (9). All experiments described above were performed at 25°C.

Fluorescence Microscopy

Flagellates fixed with glutaraldehyde were double stained for microfilaments with rhodamine-phalloidin (Rh-Ph; Molecular Probes, Inc., Junction City, OR), and for microtubules with a monoclonal antibody against yeast α-tubulin and FITC-labeled secondary antibody as described previously (29). Flagellates fixed with EGS were attached to polylysine-coated coverslips using a centrifugal cell collector (Tomy Seiko Co., Tokyo, Japan), quenched with 0.1 M glycine for 10 min (9), and stained as above.

Quantitation of the Relative Movement between Microfilaments and Microtubules

Fixed, permeabilized flagellates stained with Rh-Ph were observed under a fluorescence microscope equipped with a night-vision TV camera (model CTC-9000; Ikegami Tsushinki Co., Tokyo, Japan). The position of the anterior end of the backbone microfilaments was determined from the fluorescence image, and the position of the basal body, which corresponds to the anterior end of the microtubular cytoskeleton (1, 26, 38), was determined from the phase-contrast image. The distance between the anterior end of the backbone microfilaments and the basal body was measured using a video micrometer.

Electron Microscopy

Flagellates in buffer P were permeabilized and extracted by addition of an equal volume of a double-strength modified permeabilization buffer (1% Triton X-100, 2 mM $MgCl_2$, 6 mM EGTA, and 20 mM Pipes, pH 7.0) for 3 min. The resultant cytoskeletons were simultaneously washed and prefixed by a 5-min centrifugation at 1,000 *g* through a step gradient of 6% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in modified permeabilization buffer, and 2% glutaraldehyde and 12% Percoll in modified permeabilization buffer. The resultant pellet was treated sequentially with the following solutions: 2% glutaraldehyde, 50 mM lysine, 0.1 M phosphate buffer, pH 7.3, for 15 min (4); 2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.3, for 2 h; and 0.5% tannic acid (Nakarai Pure Chemicals Co., Kyoto, Japan), 0.1 M phosphate buffer, pH 7.3, for 5 min. After rinsing with water, the fixed cytoskeletons were treated with 2% uranyl acetate overnight on ice, dehydrated in acetone, and embedded in epoxy resin. Cytoskeletal microfilaments were labeled with 1 mg/ml α-chymotryptic rabbit skeletal muscle myosin subfragment 1 (S1) (36) in the primary permeabilization buffer (11). Intact, unextracted cells were fixed by the method of Boyles et al. (4) with some minor modifications and embedded in epoxy resin as described above. Thin sections were cut, poststained with Reynold's lead citrate, and observed under a JEOL JEM-100C electron microscope.

1. *Abbreviations used in this paper*: AMP-PNP, adenylyl-imidodiphosphate; ATPγS, adenosine-5'-*O*-(thiotriphosphate); EGS, ethyleneglycol bis-(succinic acid *N*-hydroxysuccinimide ester); EHNA, *erythro*-9-[3-(2-hydroxynonyl)]adenine; MTA, microtubular array, Rh-Ph, rhodamine-phalloidin; S1, subfragment 1.

Results

EM of the Backbone Structure in Permeabilized Flagellates

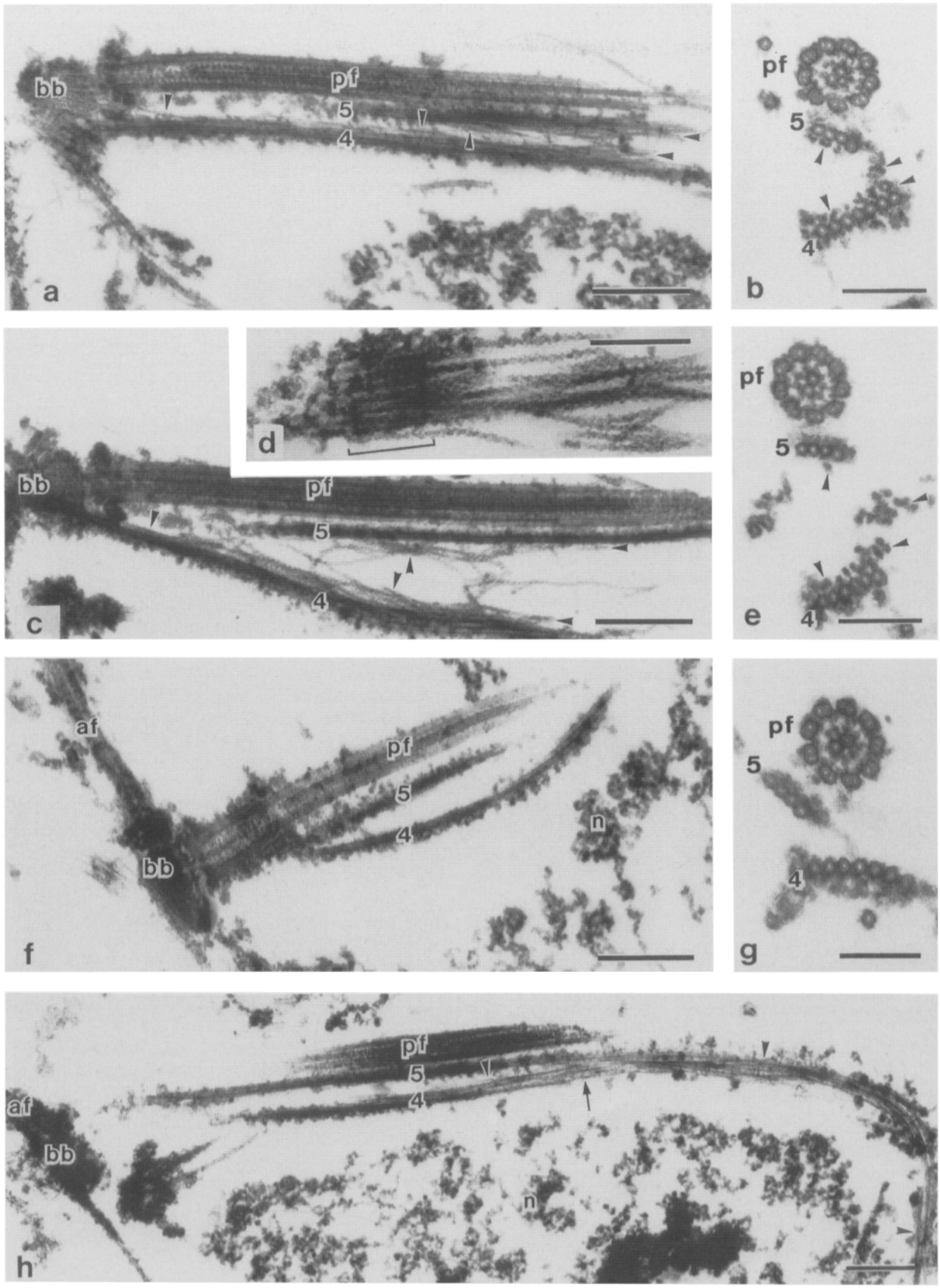
We have visualized the ultrastructural organization of microfilaments, as well as microtubules, in the permeabilized flagellates by using an improved fixation protocol. A prominent bundle of microfilaments in the dorsal curvature of the cell originates from a region close to the basal body and extends posteriorly along the long axis of the cell (Fig. 1 *a*). In the anterior region of the cell, this bundle of microfilaments closely coaligns with microtubular array (MTA) 4 (Fig. 1 *a*), a linear array of five to eight microtubules which constitutes the flagellar cone and extends posteriorly from the basal body (16, 38). In transverse section, some microfilaments in the bundle were found in close association with the microtubules in MTA 4 (Fig. 1 *b*). MTA 5, another linear array of four microtubules which extends posteriorly from the basal body between the posterior flagellum and MTA 4 (16, 38), was also associated with a bundle of microfilaments. The number of microfilaments that associated with MTA 5 was fewer than that associated with MTA 4 in the anterior region.

The two bundles of microfilaments were relatively tightly packed and closely associated with MTAs 4 and 5 in the anterior 3–4-μm region. However, they fused into one loose, thick bundle at a level between the middle and the posterior end of the nucleus (Fig. 1 *h*), where MTAs 4 and 5 were no longer detectable (16; see also Fig. 1 *h*). This loose bundle of microfilaments extended towards the posterior end of the cell loosely associating with one or two microtubules (data not shown), which are most likely an extension of microtubules in MTA 5 (16). These characteristics of the bundle of microfilaments and the arrays of microtubules are compatible with our previous observation by fluorescence microscopy of the backbone structure (28). Thus, we have concluded that this complex structure is the backbone structure.

Decoration of Microfilaments with S1

Treatment of the permeabilized flagellates with S1 resulted

Figure 1. Electron micrographs of permeabilized flagellates. (*a* and *b*) Thick (gold) longitudinal (*a*) and thin (silver) transverse (*b*) sections of the anterior region of a control permeabilized flagellate. Two parallel arrays of microtubules, MTAs 4 and 5, underlay the posterior flagellum. Two bundles of microfilaments closely associate with MTAs 4 and 5. (*c–e*) Anterior portion of a permeabilized flagellate labeled with S1. In thin transverse section (*e*), the microfilaments are thicker than those in control permeabilized flagellates. In thick longitudinal section (*c*), arrowhead structures are formed along the microfilaments. Higher magnification (*d*) of the oblique longitudinal section of an MTA 4 (*bracket*) and associated microfilaments shows that all arrowhead structures on the microfilaments, when recognizable, point to the right, that is, to the posterior of this cell. (*f* and *g*) Anterior portion of a permeabilized flagellate which was treated with ATP before



fixation. Thin, transverse (*g*) and thick, slightly oblique, longitudinal (*f*) sections show that neither MTA 4 nor 5 is associated with any microfilaments. (*h*) Lower magnification of the anterior portion of a control permeabilized flagellate. Two tight bundles of microfilaments fuse into one loose, thick bundle as indicated by the arrow. (*4*) MTA 4; (*5*) MTA 5; (*af*) anterior flagellum; (*bb*) basal body; (*n*) nucleus; (*pf*) posterior flagellum. Arrowheads indicate bundles of microfilaments. Bars: (*a*, *c*, *f*, and *h*) 0.5 μm; (*b*, *d*, *e*, and *g*) 0.2 μm.

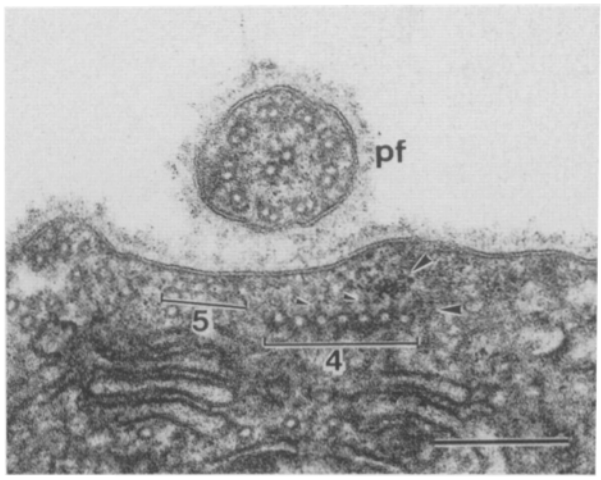


Figure 2. Dorsal portion of a thin, transverse section of the anterior region of an unextracted flagellate. Arrowheads indicate some of the dense dots that are clustered very close to, or associated with, MTA 4. (*4*) MTA 4; (*5*) MTA 5; (*pf*) posterior flagellum. Bar, 0.2 μm.

in thickening of the backbone microfilaments in the transverse section (Fig. 1 *e*), indicating that the bundle consists of actin filaments (11). In longitudinal sections, all the observed arrowhead structures along the microfilaments (11) pointed posteriorly or away from the basal body (Fig. 1 *d*).

EM of Unextracted Cells

In the transverse section of the anterior region of unextracted flagellates, fixed by the method of Boyles et al. (4), a number of dense dots, of 5–7 nm diameter, were observed very close to or associated with MTA 4 (Fig. 2). The structural similarities between the images of permeabilized cells (Fig. 1, *b* and *e*) and an unextracted cell (Fig. 2) indicates that these dots represent the transverse sections of microfilaments.

EM of Backbone Structure After ATP Treatment

EM of permeabilized flagellates treated with ATP before fixation showed that MTAs 4 and 5 still originated from the proximity of the basal body, but both were no longer associated with microfilaments (Fig. 1, *f* and *g*).

The number of microtubules contained in MTA 4 was 6.44 ± 0.86 in 200 random transverse sections of ATP-treated permeabilized flagellates while it was 6.37 ± 0.81 in control permeabilized flagellates. Similarly, MTA 5s in both ATP-treated and control permeabilized flagellates contained four microtubules. Thus, the numbers of microtubules contained in MTAs 4 and 5 were not decreased by the ATP treatment.

Effects of Ca^{2+} on the Cytoskeleton of Permeabilized Flagellates

1.2 mM $CaCl_2$ was added to a suspension of flagellates that had been permeabilized for 1 min in the presence of 1 mM EGTA and 0.16 μM Rh–Ph. Immunofluorescence staining, using tubulin-specific antibody (Figs. 3 *f* and 4 *b*), and EM (data not shown) showed that the flagella and many of the microtubules in the flagellar cone were disintegrated within 30 s of the addition of Ca^{2+}. Longer treatment with Ca^{2+} almost completely disrupted the microtubules. By contrast, the basal body was apparently unaffected by such Ca^{2+} treatment, as revealed by phase-contrast (Fig. 3 *e*) and EM observation (data not shown). The organization of the microfilamentous cytoskeleton was also not noticeably affected by

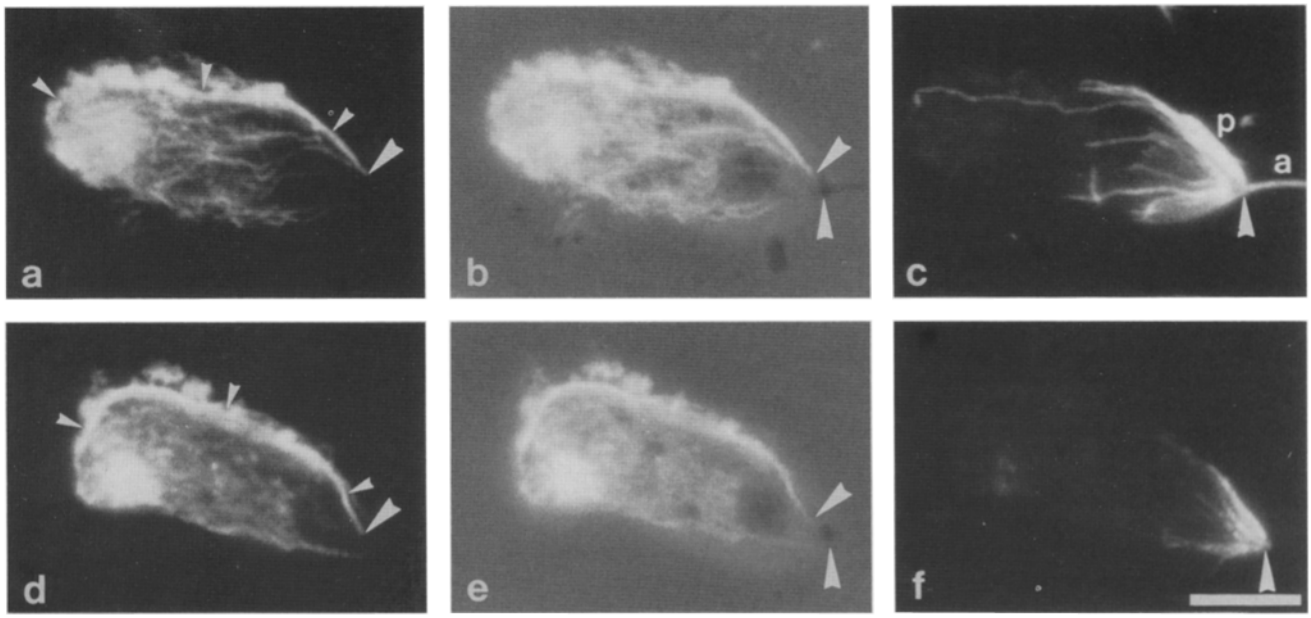


Figure 3. Effects of Ca^{2+} treatment on the cytoskeleton of permeabilized flagellates. Flagellates were permeabilized for 1 min with the permeabilization buffer containing 0.16 μM Rh–Ph, treated with Ca^{2+} for 30 s, and fixed with EGS (*d–f*). Control cells were fixed without the Ca^{2+} treatment (*a–c*). Cells were shown by Rh–Ph fluorescence (*a* and *d*); double exposure for Rh–Ph fluorescence and phase-contrast (*b* and *e*); and FITC–antitubulin immunofluorescence (*c* and *f*). Large arrowheads indicate the anterior end of the backbone microfilaments and the basal body. Small arrowheads indicate the backbone structure. (*c*) Anterior (*a*) and posterior (*p*) flagellum. Bar, 5 μm.

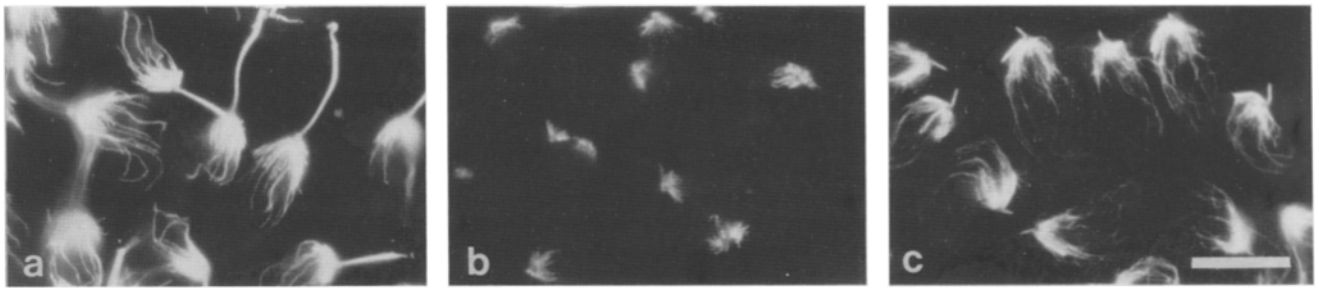


Figure 4. Effects of taxol on Ca^{2+}-induced disintegration of the microtubular cytoskeleton. Flagellates, permeabilized for 1 min in the presence (*c*) or absence (*b*) of 50 μM taxol, were treated with Ca^{2+} for 30 s, and fixed with EGS. Control cells (*a*) were permeabilized in the absence of taxol and fixed without the Ca^{2+} treatment. Cells were attached to the coverslips, flattened by overlaying agarose sheets (39), and stained with tubulin-specific antibody. Bar, 10 μm.

such Ca^{2+} treatment, as the backbone microfilaments reached almost to the basal body, as judged by staining with Rh-Ph (Fig. 3 *d*) and EM observation (data not shown).

The destructive effect of Ca^{2+} on the microtubular cytoskeleton was partially retarded by pretreatment of permeabilized flagellates with 50 μM taxol for 1 min. In such cells, the basal portion of the flagella and many of the microtubules in the flagellar cone were retained after incubation with Ca^{2+} for 30 s (Fig. 4 *c*).

Effects of Pretreatment with Ca^{2+} on the ATP-induced Relative Movement between Microfilaments and Microtubules

Pretreatment of permeabilized flagellates with 1 mM EGTA and 0.16 μM Rh-Ph for 1 min before reactivation with 1 mM Mg-ATP and 10 mM EGTA did not affect the apparent relative movement between the backbone microfilaments and the microtubular cytoskeleton or basal body (data not shown). However, pretreatment of permeabilized flagellates with Ca^{2+} instead of EGTA for 3 s inhibited the change in position of the microfilaments relative to the basal body (Fig. 5, *a–c*).

The inhibitory effect of pretreatment with Ca^{2+} on the subsequent ATP-induced relative movement was examined quantitatively by measurements of the distance between the anterior end of the backbone microfilaments and the basal body (Fig. 6). This mean distance in permeabilized flagellates reactivated with 1 mM Mg-ATP and 10 mM EGTA for 15 s was 3.6 μm, while it was 0.6 μm in unreactivated cells, an average increase of 3.0 μm. The inhibitory effect of Ca^{2+} was nearly complete even after 3 s of incubation with Ca^{2+}, when the increase in the mean distance after the subsequent reactivation with Mg-ATP and EGTA was 0.3 μm.

Preincubation of permeabilized flagellates with 50 μM taxol for 1 min before the Ca^{2+} treatment significantly retarded the inhibitory effect of Ca^{2+} on ATP-induced movement (Fig. 5, *d–i*). When taxol-treated, permeabilized flagellates were incubated with Ca^{2+} for 10 s and then reactivated with Mg-ATP and EGTA, the mean distance between the anterior end of the backbone microfilaments and the basal body increased by 1.7 μm (Fig. 6).

Requirements for Nucleotides and Sensitivity to Inhibitors

GTP induced the relative movement between microfilaments and microtubules at 1 mM. However, 0.1 mM GTP only weakly stimulated movement, while 0.1 mM ATP induced considerable movement (Table I). A thiophosphate analogue of ATP (ATPγS) also induced the relative movement. Although the movement induced by ATPγS was slower than that induced by ATP, the final displacement induced by ATPγS was comparable to that induced by ATP (Table I). Neither ADP nor AMP-PNP, a nonhydrolyzable analogue of ATP, induced any movement. Excess AMP-PNP retarded the movement induced by ATP (Table I).

Two ATPase inhibitors each inhibited the ATP-induced relative movement (Table I). The relative movement was inhibited by 50 μM vanadate, but 10 μM vanadate had little effect on the movement. EHNA inhibited the relative movement at 2 mM. EHNA has been shown to perturb the organization of microfilaments in various types of cells (25), but fluorescence microscopy of permeabilized flagellates stained with Rh-Ph suggested that the microfilamentous cytoskeleton was not affected by EHNA under our present experimental conditions (data not shown).

Discussion

Ultrastructure of the Backbone Structure

Previous EM observations of thin-sectioned myxomycete flagellates, which used conventional double-fixation protocols using glutaraldehyde and OsO_4, successfully revealed the ultrastructure of the microtubular cytoskeleton (1, 10, 16, 26, 38). However, none of these earlier studies visualized any microfilamentous cytoskeleton.

Hence, we devised a fixation protocol that allows preservation and visualization of microfilaments in *Physarum* flagellates. The combination and modification of published fixation methods designed for this purpose (3, 4, 20), namely, (*a*) permeabilization and extraction of cells before fixation; (*b*) inclusion of lysine in the glutaraldehyde fixative; (*c*) treatment with tannic acid; (*d*) omission of the OsO_4 treatment; (*e*) en bloc staining with uranyl acetate; and (*f*) dehydration with acetone resulted in acceptable preservation and visualization of microfilaments, as well as microtubules, in thin sections. These sections clearly demonstrated that the anterior portion of the backbone structure in control permeabilized flagellates consists of closely associated and coaligned bundles of microfilaments and arrays of microtubules (Fig.

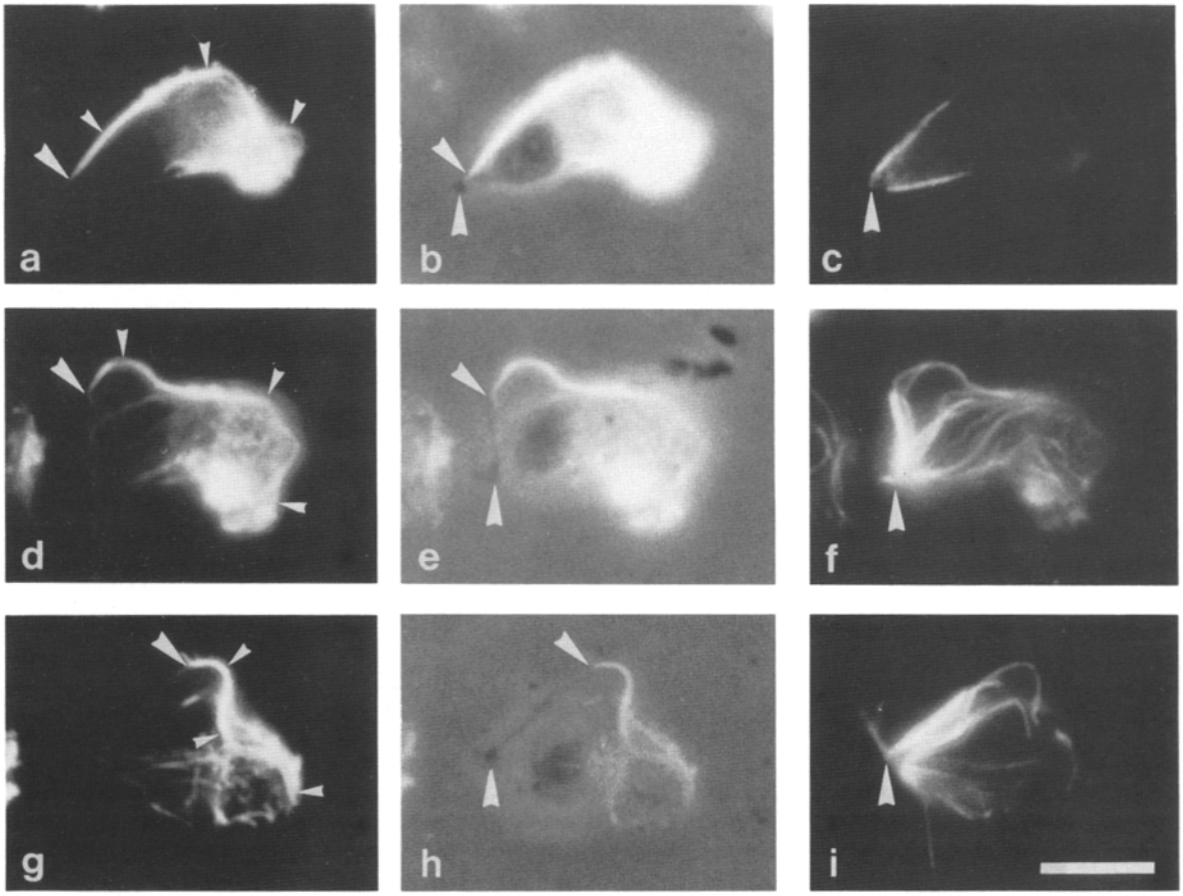


Figure 5. Effects of pretreatment with Ca^{2+} on the ATP-induced relative movement between microfilaments and microtubules. Flagellates were permeabilized for 1 min with the permeabilization buffer that contained 0.16 μM Rh-Ph in the absence (*a–c*) or presence (*d–i*) of 50 μM taxol, pretreated with Ca^{2+} for 5 s, reactivated with 1 mM Mg-ATP and 10 mM EGTA for 15 s, and fixed with EGS. Cells are shown by Rh-Ph fluorescence (*a*, *d*, and *g*); double exposure for phase-contrast and Rh-Ph fluorescence (*b*, *e*, and *h*); and FITC-antitubulin immunofluorescence (*c*, *f*, and *i*). Large arrowheads indicate the anterior end of the backbone microfilaments and the basal body. Thus, the distance between the two large arrowheads is the displacement between the two cytoskeletal systems. Small arrowheads indicate the backbone structure. Bar, 5 μm.

1, *a* and *b*). Since similar images were observed in unextracted flagellates (Fig. 2), the close association of the bundle of microfilaments and microtubules is not an artifact which resulted from extraction of cells before fixation.

The Apparent, Relative Sliding between Microfilaments and Microtubules Is an Active Movement

We reported previously that the microfilaments and microtubules in the backbone structure of permeabilized *Physarum* flagellates apparently slide over each other upon addition of ATP (30). However, we did not demonstrate unequivocally that the apparent movement was active and not a result of passive drifting or polarized depolymerization of the microfilaments.

As an active process, a requirement for an energy source in the form of nucleotides might be expected. We have found that ATP, GTP, and ATPγS all support movement while ADP and the nonhydrolyzable analog of ATP, AMP-PNP, did not. These results suggest the activity of a mechanochemical enzyme. However, phosphorylation of proteins is also energy dependent. Phosphorylation of MAP 2, isolated from mammalian brain, modifies its ability to form cross-links between microfilaments and microtubules (15, 21). Perhaps the treatment with ATP, GTP, or ATPγS caused dissociation of microfilaments from microtubules by modifying the cross-links, so that the microfilaments may drift passively away from the microtubules. However, this explanation is unsatisfactory in light of the result that disruption of microtubules by Ca^{2+}, which should release microfilaments from microtubules, had no effect on the position of the microfilaments within the cells (Fig. 3, *d–f*).

It is also unlikely that depolymerization of actin from the anterior end of the backbone microfilaments caused the apparent relative movement, since pretreatment with 0.16 μM Rh-Ph (Fig. 5, *d–i*) or with 100 μM phalloidin for 3 min (result not shown), thereby inhibiting depolymerization of microfilaments (37), did not affect the relative movement.

Dependence of the Relative Movement on the Intact Microtubular Cytoskeleton

We examined the effects of disruption of the microtubular cytoskeleton with Ca^{2+} (24). The Ca^{2+} treatment of flagellates permeabilized in the absence of Ca^{2+} caused both disintegration of the microtubular cytoskeleton and inhibition of

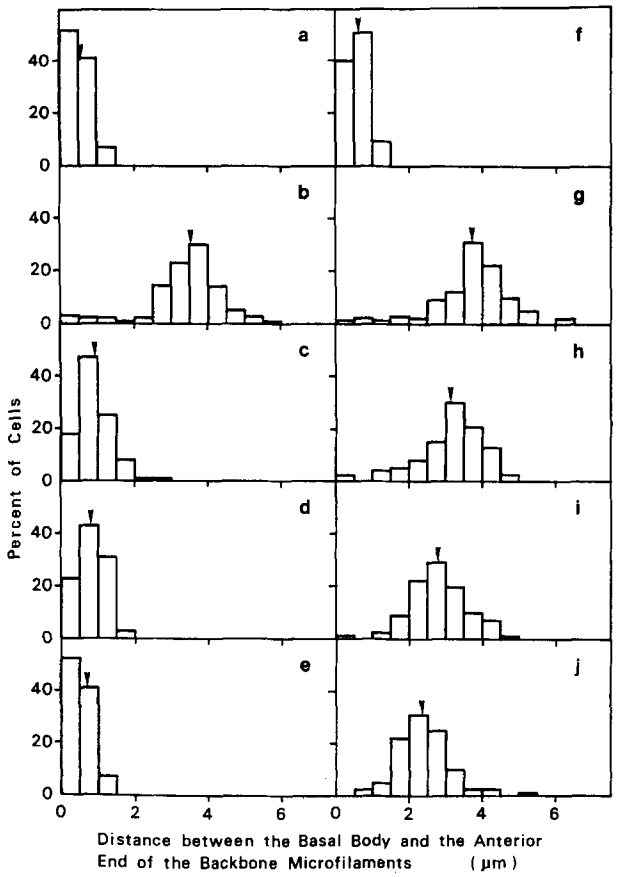


Figure 6. Effects of pretreatment with Ca^{2+} on the ATP-induced relative movement between microfilaments and microtubules. Flagellates were permeabilized for 1 min with the permeabilization buffer that contained 0.16 μM Rh–Ph in the absence (*a–e*) or presence (*f–j*) of 50 μM taxol, pretreated with Ca^{2+}, and reactivated with 1 mM Mg-ATP and 10 mM EGTA. After incubation for 15 s, cells were fixed with EGS, and the distance between the anterior end of the backbone microfilaments and the basal body was measured. Cells were reactivated with Mg-ATP and EGTA after 3 (*c* and *h*), 6 (*d* and *i*), or 10 s (*e* and *j*) of pretreatment with Ca^{2+}. Control cells, which were not treated with Ca^{2+}, were fixed before (*a* and *f*) or after (*b* and *g*) the reactivation with Mg-ATP and EGTA. Each histogram was constructed from the measurements of 100 cells. Arrowheads indicate the mean distances.

Table 1. Effects of Nucleotides and Inhibitors on the Relative Movement in Permeabilized Flagellates

Nucleotides and inhibitors	Distance between the basal body and the anterior end of the backbone microfilaments: After 30 s	After 10 min
	μm ± SD	*μm ± SD*
None	0.4 ± 0.2	0.6 ± 0.2
1 mM ATP	4.4 ± 1.2	4.7 ± 1.6
1 mM GTP	2.3 ± 0.8	3.9 ± 1.1
1 mM ADP	0.5 ± 0.3	0.7 ± 0.4
1 mM AMP-PNP	0.3 ± 0.2	0.6 ± 0.2
1 mM ATPγS*	0.6 ± 0.5	3.7 ± 1.5
0.1 mM ATP	3.9 ± 1.3	
0.01 mM ATP	1.2 ± 0.4	
0.1 mM GTP	0.7 ± 0.3	
1 mM ATP +		
2.5 mM AMP-PNP	0.7 ± 0.5	
10 μM vanadate	4.6 ± 1.7	
50 μM vanadate	1.4 ± 1.5	
0.1 mM ATP‡ +		
0.5 mM EHNA	3.3 ± 1.1	
2 mM EHNA	1.4 ± 0.7	

Flagellates were treated for 15 s with the permeabilization buffer, incubated for 30 s or 10 min with nucleotides, and fixed. Inhibitors were contained in the initial permeabilization buffer. Each result represents the mean of 50 measurements ± SD.
* The concentration of EGTA was 10 mM.
‡ Effect of EHNA was examined at 0.1 mM ATP (5).

the relative movement induced by the subsequent addition of Mg-ATP and EGTA. Pretreatment of permeabilized flagellates with taxol, an agent specific for the stabilization of microtubules (22), retarded both the disintegrative effect of Ca^{2+} on microtubules and the inhibitory effect of Ca^{2+} on the subsequent relative movement (Figs. 4–6).

Permeabilization of flagellates in the presence of Ca^{2+} disrupts not only microtubules but also the microfilamentous cytoskeleton (29). However, the Ca^{2+} treatment after 1 min of permeabilization in the absence of Ca^{2+} showed no noticeable effects on the microfilaments under the present experimental conditions, indicating that the effect of Ca^{2+} was specific for the microtubules. This was probably due to the loss of myxamoebal fragmin (31), which is responsible for the lability of microfilaments in Ca^{2+}, by rapidly diffusing out of cells after permeabilization in the absence of Ca^{2+} (32).

These results indicate that the relative movement between the microfilaments and the microtubules depends on the intact microtubular cytoskeleton and cannot be produced by the activity of the microfilamentous structures alone.

Mechanism of the Relative Movement between Microfilaments and Microtubules

In summary, the relative movement between microfilaments and microtubules in the backbone structure of *Physarum* flagellates is an active movement dependent on hydrolysis of nucleotides and on the intact microtubular cytoskeleton. It is also evident that this relative movement is driven by the activity of the backbone structure itself, because the movement can be observed in isolated preparations of the backbone structure (30). Therefore, we speculate that some active sliding between cytoskeletal filaments in the backbone structure is driving this movement. So far, two groups of mechanochemical proteins that can cause relative sliding between two cytoskeletal filaments depending on hydrolysis of nucleotides have been identified; one is the myosins, which include proteins that cause relative sliding between microfilaments, and the other is the dyneins, which include the proteins that cause relative sliding between microtubules (23). Here, we discuss the mechanism of the relative movement based on the comparison of its pharmacological properties with those of myosins and dyneins and on the ultrastructure of the backbone structure.

First, it could be argued that the relative movement is caused by a myosin-dependent contraction of the microfilaments in the backbone structure, and that the microtubules merely serve as a track for the contraction of the microfila-

ments. It is, however, unlikely that such contraction is crucial to this relative movement, because the movement was inhibited by 50 μM vanadate and reactivated by ATPγS (Table I). By contrast, greater than millimolar concentrations of vanadate are required to inhibit ATPase activity of skeletal muscle myosin (8, 12), and ATPγS is a poor substrate of skeletal muscle myosin (2). These results seemed to hold true for the myosin in *Physarum* also, because pharmacological properties of ATP-induced contraction of permeabilized *Physarum* amoebas was similar to those of skeletal muscle myosin ATPase activity (unpublished results). Thus, the differential reactivity by ATPγS and differential sensitivity to vanadate suggest that myosin-dependent contractability is neither necessary nor sufficient for the relative movement. In addition, S1 labeling showed that the microfilaments in the backbone structure have a uniform polarity, which is an unfavorable alignment for myosin-dependent contraction. Furthermore, the backbone structures of some portion of the ATP-treated permeabilized flagellates were extruded out of the cell bodies (30; see also Fig. 5, *d–f*), a result which is indicative of extension, rather than contraction, of the backbone structure.

We, therefore, speculate that the relative movement was caused by an active process which resulted in extension by reciprocal sliding between cytoskeletal filaments in the backbone structure. Logically, the sliding could take place along three interfaces. Firstly, sliding might take place between two populations of microfilaments, one of which is tightly connected to microtubules, depending on a myosin-like protein. Secondly, sliding might take place between two population of microtubules, one of which is tightly connected to microfilaments, depending on a dynein-like protein. Thirdly, microfilaments and microtubules might slide over each other.

The pharmacological data are inconsistent with the involvement of myosin in this relative movement, as was discussed above, and is therefore inconsistent with the first possibility. In addition, if the first possibility was the case, some of the microfilaments should remain associated with the microtubules after the ATP treatment. However, MTAs 4 and 5 in the ATP-treated, permeabilized flagellates were not associated with any microfilaments (Fig. 1, *f* and *g*).

As to the second possibility, this relative movement was inhibited by 50 μM vanadate and 2 mM EHNA. Therefore, it shares a relatively high sensitivity to vanadate and EHNA with sperm axonemal dynein (5, 8, 12) and MAP 1C or cytoplasmic dynein (17, 35). However, the nucleotide requirements of the relative movement was distinct from those of dyneins, as movements induced by axonemal and cytoplasmic dyneins are not reactivated with GTP (7, 17), and axonemal dynein cannot hydrolyze ATPγS (18). Thus, the pharmacological properties of this relative movement are distinct from, although partially similar to, those of dyneins, suggesting that dyneins may not be involved in this relative movement. Some pharmacological properties of this relative movement are similar to those of kinesins, another class of microtubule-dependent, mechanochemical proteins (33). However, kinesin-dependent relative sliding between two cytoskeletal filaments has not yet been observed (34).

The results of our EM observation more strongly argue against the second possibility. If sliding takes place between microtubules, the number of microtubules contained in MTAs 4 and/or 5 in random sections should decrease. However, such a decrease was not observed.

The third possibility outlined above, that is, active sliding between microfilaments and microtubules, is not inconsistent with any of our experimental results and therefore we consider it most likely. A yet undescribed mechanochemical factor may be responsible for this relative movement. Alternatively, this movement of microfilaments over microtubules may well represent a novel role for previously known microtubule-based mechanochemical proteins in cell movement. In fact, some similarities between pharmacological properties of this relative movement and those of dyneins and kinesins were observed. In addition, one of the axonemal dynein subunits in *Chlamydomonas* was recently shown to be associated with actin (19).

Biological Significance of this Sliding Motility

What could the biological significance of this sliding motility between microfilaments and microtubules, which we observed in the permeabilized flagellates treated with ATP, be for the live flagellates? It should be noted that living flagellates have a high intracellular ATP concentration, but do not show this displacement between microfilaments and microtubules when fixed. Displacement was never observed unless cells were treated with both Triton X-100 and ATP. One possible explanation for this apparent discrepancy between the effects of ATP in vivo and in vitro is that, in live flagellates, the microfilaments are connected to the microtubules at one end or along their length by some linkage, which is lost after the Triton treatment. Production of a sliding force between the two elastic bundles of filaments, linked to each other, might stretch the backbone structure into an isometric tension, maintaining the elongated shape of these cells.

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