1994

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Recommended Citation

Kuznetsov, S. A., D. T. Rivera, F. F. Severin, D. G. Weiss, and G. M. Langford. 1994. "Movement of Axoplasmic Organelles on Actin Filaments from Skeletal Muscle." Cell Motility and the Cytoskeleton 28 (3): 231–42.

Journal of Cell Science 107, 2291-2298 (1994)
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Movement of axoplasmic organelles on actin filaments assembled on acrosomal processes: evidence for a barbed-end-directed organelle motor

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SUMMARY

The directionality of the actin-dependent motors on squid axoplasmic organelles was determined using actin filaments assembled on the barbed ends of acrosomal processes. Acrosomal processes were isolated from *Limulus polyphemus* sperm and incubated in monomeric actin under conditions that promoted barbed end assembly only. Newly assembled actin was stabilized and stained with rhodamine-phalloidin and the presence of filaments at the barbed ends of the acrosomal processes was verified by fluorescence microscopy and negative contrast electron microscopy. Axoplasmic organelles that dissociated from extruded axoplasm were observed by video microscopy to move along the newly assembled actin filaments at an average velocity of 1.1±0.3 μm/second. All organelles moved in the direction away from the acrosomal fragment and towards the tip of the actin filaments. Therefore, the actin-dependent organelle motor on axoplasmic organelles is a barbed-end-directed motor like other myosins analyzed. These findings support the conclusions that axoplasmic organelles are driven by a myosin-like motor along actin filaments and that these filaments as well as microtubules function in fast axonal transport.

Key words: squid axoplasm, organelle movement, acrosomal process, actin filament, axonal transport, directionality of motor

INTRODUCTION

Unconventional myosins represent a diverse group of proteins (for review see Endow and Titus, 1992; Titus, 1993; Fath and Burgess, 1994), many of which are thought to function as organelle motors. Evidence for the role of these myosins as organelle motors has accumulated rapidly over the past few years (Fukui et al., 1989; Baines et al., 1992; Yonemura and Pollard, 1992; Kellerman and Miller, 1992; Wagner et al., 1992; Espreafico et al., 1992; Lillie and Brown, 1992; Coluccio and Conaty, 1993; Conrad et al., 1993). Brush border myosin I has been shown on the surfaces of cytoplasmic vesicles in intestinal epithelial cells (Drenckhahn and Dermietzel, 1988) and myosin IA has been shown on small vesicles in *Acanthamoeba* (Baines et al., 1992). In addition, differential centrifugation has been used to show that myosins cosediment with Golgi membranes in intestinal epithelia (Fath and Burgess, 1993), with small vesicles in *Acanthamoeba* (Adams and Pollard, 1986) and with contractile vacuoles in *Dictyostelium* (Zhu and Clarke, 1992).

Direct evidence for a role for myosin in the transport of organelles along actin filaments has been more difficult to obtain. Some of the earliest data showing directed movement of organelles along actin filaments came from studies of motility in plant cells and protozoa. Organelles isolated from *Chara* were shown to move on *Nitella* actin cables at speeds similar to those observed in intact cells (Shimmen and Tazawa, 1982; Kachar, 1985). The actin-dependent movement of organelles in *Chara* strongly implicated myosin as the putative motor. Using the *Nitella* actin cables assay, Adams and Pollard (1986) showed that vesicles from *Acanthamoeba* moved along actin filaments and this movement could be inhibited by antibodies to *Acanthamoeba* myosin I. More recently, genetic and molecular genetic experiments have provided further evidence that myosins function as organelle motors. The mouse Dilute and yeast MYO2 proteins, members of the myosin V class of unconventional myosins, have been implicated as organelle motors in studies of organisms that express the mutant forms of these proteins (Mercer et al., 1991; Johnston et al., 1991).

Axoplasm from the squid giant axon, the biological preparation originally used to provide direct evidence for microtubule-based organelle motility (Allen et al., 1985; Vale et al., 1985a,b; Weiss et al., 1988) has proven to be one of the best systems in which to study actin-dependent organelle movement. In recent papers (Kuznetsov et al., 1992, 1994), we demonstrated that axoplasmic organelles have the ability to move on actin filaments. Organelle movement on actin filaments was unidirectional, ATP-dependent, and AMP-PNP-

and nocodazole-insensitive. The velocity of organelle translocation on actin filaments was similar to that on microtubules and hence we concluded that actin-dependent motors are likely to be involved in fast axonal transport. These studies provided the first evidence for the presence of both actin-based and microtubule-based motors on axoplasmic vesicles and for the ability of vesicles to switch from one filament type to another. As proposed by Atkinson et al. (1992) microtubules appear to provide tracks for the movement of vesicles over distances of many micrometers and, in the case of the axon, principally parallel to the longitudinal axis, while actin filaments provide tracks for the local distribution of vesicles to sites along the axon, e.g. the delivery of Golgi-derived vesicles to the plasma membrane and the movement of synaptic vesicles within the axon terminal.

In this paper we provide further evidence for the presence of myosin motors on the surfaces of axoplasmic organelles. Here we show that axoplasmic organelles move towards the barbed ends of actin filaments nucleated by acrosomal processes. Therefore, the myosin motors on these organelles have properties in common with other myosins, i.e. the myosin I and II classes of proteins, that have been shown to generate force towards the barbed ends of actin filaments (for review see Pollard et al., 1991; Warrick and Spudich, 1987). The ability of organelles to move on acrosomal process-nucleated skeletal muscle actin filaments supports our earlier results, which showed that organelles are able to move on coverslips coated with skeletal muscle actin filaments (Kuznetsov et al., 1994), and provides another in vitro method to assay motor activity of myosin-like proteins purified from axoplasm and other sources.

MATERIALS AND METHODS

Materials

Squid (*Loligo pealei*) and horseshoe crabs (*Limulus polyphemus*) were obtained from the Department of Marine Resources of the Marine Biological Laboratory (Woods Hole, MA). Monomeric actin from rabbit skeletal muscle (a gift from Michael Bermann) was prepared according to the method of Spudich and Watt (1971) and further purified by gel filtration chromatography on Sephacryl S-300. Monomeric actin (20 μM) was stored for up to 2 weeks at 0°C in 2 mM Tris-HCl buffer (pH 8.0) with 1 mM ATP, 0.05 mM $MgCl_2$ and 0.2 mM dithiothreitol. Calmodulin (CaM), rhodamine-labelled phalloidin (TRITC-phalloidin), cytochalasin B and nocodazole (Sigma Chemical Company) were made as stock solutions and used at the concentrations indicated in the text.

Preparation of acrosomal processes in TAMDC buffer

Sperm were collected from horseshoe crabs as described previously (Tilney, 1975), then resuspended in 30 mM Tris buffer (pH 8.0) with 3 mM $MgSO_4$ and 1% Triton X-100 to induce the false discharge (acrosomal process) and to remove membranes (Tilney, 1975). Acrosomal processes were gently sheared into short fragments, 2-10 μm long, by several passages through the tip of a Pasteur pipette. The sheared acrosomal processes were spun for 5 minutes at 3,000 ***g*** to remove nuclei and attached axonemes. After centrifugation, the supernatant contained primarily short fragments of acrosomal processes and an occasional flagellar axoneme as determined by video and electron microscopy. The acrosomal processes were pelleted by spinning for 10 minutes at 10,000 ***g*** and then resuspended in 25 mM Tris buffer (pH 7.5) plus 2 mM $MgCl_2$ to remove the detergent. The centrifugation procedure was repeated 5 times, after which the acrosomal processes were resuspended in TAMDC (motility assay) buffer (25 mM Tris, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 25 μg/ml CaM).

Barbed-end assembly of actin filaments on acrosomal processes

To a preparation of acrosomal processes resuspended in TAMDC buffer, monomeric actin from skeletal muscle was added to a final concentration of 0.5 μM. This mixture was incubated for 1 hour at room temperature followed by an overnight incubation on ice. After assembly of the actin filaments, a sample was diluted 10-fold into 0.5 μM TRITC-phalloidin to stabilize and stain the actin filaments. After 1 hour of staining (a longer staining time was used to produce more highly fluorescent acrosomal processes), a 4 μl droplet of the solution containing acrosomal processes with polymerized actin filaments (or without polymerized actin filaments for control experiments) was placed on a plastic surface (20 mm × 13 mm rectangle of Scotch tape 3M) and gently covered with a no. 0 coverslip (22 mm × 26 mm). The coverslip was incubated in the solution for 5 minutes in the dark to allow adsorption of the acrosomal processes to the glass surface, then rinsed with TAMDC buffer before use.

Preparation of axoplasmic organelles from extruded axoplasm

Axoplasmic organelles were obtained from the giant axon of the squid by diffusion from bulk axoplasm. Squid giant axons were dissected and extruded on microscope slides as previously described (Allen et al., 1985; Weiss et al., 1990). Immediately after extrusion, 25 μl of TAMDC buffer was added to each slide. After addition of the buffer, a glass coverslip with or without adsorbed acrosomal processes was applied to the extruded axoplasm and the preparation was sealed with valap (vaseline:lanolin:paraffin; 1:1:1, by weight). Axoplasmic organelles were allowed to accumulate at the margins of the bulk axoplasm for 10-15 minutes at room temperature, after which a high density of organelles could be seen. Movement of organelles on actin filaments nucleated by acrosomal processes was assayed at the margins of the bulk axoplasm (between 20 and 60 μm from the bulk) by AVEC-DIC (Allen video-enhanced contrast differential interference contrast) microscopy. In these experiments, the TAMDC buffer contained 50 μM cytochalasin B and 100 μM nocodazole to prevent polymerization of endogenous actin and to depolymerize microtubules that dissociated from the bulk axoplasm. For control experiment involving movement of organelles on endogenous actin filaments, 0.5 μM TRITC-phalloidin was added to the buffer and cytochalasin B was omitted.

AVEC-DIC and fluorescence microscopy

The movement of axoplasmic organelles on nucleated skeletal muscle actin filaments or endogenous neuronal actin filaments was observed by AVEC-DIC microscopy (Allen et al., 1981, 1985; Weiss et al., 1989) and video intensified fluorescence microscopy (Weiss et al., 1989). A Zeiss Axiophot microscope (C. Zeiss, Inc., Thornwood, NY) equipped with oil immersion condenser (NA 1.4) and ×100 DIC Plan Neofluar oil objective (NA 1.32) were used. The mercury arc lamp (HBO 100) for DIC was modified to generate full condenser aperture illumination by the addition of an Ellis fiberoptic light scrambler (Technical Video, Ltd, Woods Hole, MA). A Hamamatsu C2400-07 Newvicon camera was used to acquire DIC images and a Hamamatsu C2400-97 intensified CCD camera system (Hamamatsu Photonics, Inc., Bridgewater, NJ) for fluorescence images. The video signals were first subjected to analog contrast enhancement (Allen et al., 1981, 1985; Weiss et al., 1989), followed by real-time digital image processing in the following steps: subtraction of an out-of-focus background (mottle pattern), accumulation or averaging of images to increase signal to noise ratio, and finally selection of the desired range of gray levels (Allen and Allen, 1983; Inoué, 1986; Weiss and Maile,

1993). The analog and digital processing of fluorescence and DIC signals was performed in parallel using two ARGUS 10 real time image processors (Hamamatsu Photonics Inc.). A Mitsubishi BV-1000 SVHS video recorder was used to record processed images.

In order to reduce potential photo-damage to the organelle motors during observation by fluorescence microscopy, we observed a given field in the preparation by AVEC-DIC microscopy before fluorescence microscopy. In addition, bleaching of the fluorescent label on actin filaments and photo-damage to the motors was minimized by depleting molecular oxygen in the preparations by the addition of 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase and 0.02 mg/ml catalase (Sigma Chemical Company) (Kishino and Yanagida, 1988; Collins et al., 1990).

Motile activity and velocity measurements

The level of motile activity (number of organelles moving/minute per field) on endogenous actin filaments in a given preparation was determined by counting the total number of organelles exhibiting directed motion on the tracks invisible by AVEC-DIC microscopy. Three areas of the preparation, 22 μm × 25 μm (the size of the video field), at a distance of 20-60 μm from the bulk axoplasm were selected for analysis and the number of organelles moving in each area during a period of 20 seconds was counted. A given organelle was counted only once during the 20 second interval. The average for the 3 areas was used as the measure of motile activity. In analyzed areas, the presence of an actin filament network was confirmed by fluorescence microscopy.

Motion analysis was performed by first extracting the *x*- and *y*-coordinates of moving organelles from a series of recorded video images either automatically (XY-Tracker C1055, Hamamatsu Photonics Inc.) or manually using a mouse to measure the distance directly in projected video images (Weiss et al., 1989). The displacement vs time plots were quantitatively evaluated using the motion analysis and statistical software packages PARTI-MOVI and SPSS as previously described (Weiss et al., 1988, 1991).

Electron microscopy

A 5 μl droplet of the solution containing isolated acrosomal processes or the acrosomal processes with polymerized actin filaments was applied to Formvar/carbon-coated copper grids. The samples were stained using 1% aqueous uranyl acetate and examined in a Zeiss 10C electron microscope (Zeiss, Inc., NY).

SDS-gel electrophoresis

Isolated monomeric actin and acrosomal processes were analyzed by discontinuous SDS-PAGE with a 4% stacking gel and a 10% resolving gel (Laemmli, 1970).

RESULTS

Organelle motility on endogenous actin filaments in TAMDC (motility assay) buffer

In a previous study (Kuznetsov, et al., 1992), we showed that a network of actin filaments formed at the margins of axoplasm after it had been extruded from the squid giant axon and incubated in buffer for 15-30 minutes. Axoplasmic organelles that dissociated from the bulk axoplasm were observed to move along these actin filaments at an average velocity of about 1 μm/second, which is approximately the rate of fast axonal transport. We originally characterized the actin-dependent movement of organelles (Kuznetsov et al., 1992) in axoplasmic dissociation buffer (100 mM K^+-aspartate, 18 mM taurine, 10 mM betaine, 7.5 mM glycine, 4 mM HEPES, pH 7.2, 2.0 mM $MgCl_2.6H_2O$, 1.4 mM $CaCl_2$, 1.4 mM EGTA, 2.0 mM ATP). This buffer was derived from the artificial axoplasmic buffer developed by Brady et al. (1982) and contained the major ions and low molecular mass components found in axoplasm. In a subsequent study (Kuznetsov et al., 1994), we showed that the addition of CaM to axoplasmic dissociation buffer reduced the variation among axoplasmic preparations and maintained a high level of motile activity (number of organelles moving/minute per field); therefore, CaM was added to the buffer for motility assays. For the studies to be reported here, we chose a buffer (TAMDC buffer: 25 mM Tris-HCl, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 25 μg/ml CaM) that was originally developed for the isolation and characterization of cytoplasmic myosins (Cheney et al., 1993b) because of the increasing body of evidence that myosin-like motors are responsible for organelle movement along actin filaments in axoplasm. In order to establish the suitability of the TAMDC buffer for motility studies, we determined the motile activity and the velocity of axoplasmic organelles on endogenous actin filaments in this buffer.

Axoplasm from a freshly dissected axon was extruded and allowed to incubate in TAMDC buffer containing 0.5 μM TRITC-phalloidin for 15-30 minutes at room temperature. During the incubation period, endogenous actin filaments assembled on the coverslip surface at the margins of the bulk axoplasm and many organelles were observed to move. The level of motile activity on endogenous actin filaments (Table 1A), was found to be similar to that observed in preparations made in axoplasmic dissociation buffer containing CaM (Kuznetsov et al., 1994). The average velocity of movement of organelles (diameter <400 μm) was found to be 0.9±0.2 μm/second, an average velocity identical to that in axoplasmic buffer (Table 1A). Since the frequency and velocity of organelle motility on endogenous actin filaments in the TAMDC buffer were equivalent to the values obtained in axoplasmic buffer, we conducted all of the motility experiments described here in this buffer.

Barbed-end assembly of actin filaments on acrosomal processes: polarity marker for actin filaments

To determine the direction of force production by the actin-dependent motors on axoplasmic organelles we needed to

Table 1. Comparisons of motile activity and velocity of axoplasmic organelles (diameter <400 nm) in TAMDC and axoplasmic dissociation buffers containing 3 μM CaM

Buffer	Motile activity (organelles/min per field)	Average velocity (μm/s)
(A) Movement of organelles on endogenous actin filaments		
TAMDC buffer*	82±21 (*N*=9)	0.9±0.2 (*N*=51)
Axoplasmic buffer†	99±20 (*N*=12)	0.9±0.3 (*N*=63)
(B) Movement of organelles on exogenous actin filaments nucleated by acrosomal processes		
TAMDC buffer	–	1.1±0.3 (*N*=20)‡
Axoplasmic buffer	–	1.0±0.4 (*N*=42)§

*TAMDC buffer: 25 mM Tris-HCl (pH 7.5), 5 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 25 μg/ml CaM.

†Axoplasmic buffer: 100 mM K^+-aspartate, 18 mM taurine, 10 mM betaine, 7.5 mM glycine, 4 mM HEPES, pH 7.2, 2.0 mM $MgCl_2.6H_2O$, 1.4 mM $CaCl_2$, 1.4 mM EGTA, 2.0 mM ATP.

‡Skeletal muscle actin filaments were nucleated by acrosomal processes.

§Skeletal muscle actin filaments were adsorbed to the coverslip surface (Kuznetsov et al., 1994).

observe organelle movement on actin filaments whose polarity could be established unambiguously. Actin filaments of known polarity were obtained by polymerizing skeletal muscle actin in the presence of acrosomal processes. The actin filaments that polymerized on acrosomal processes at an actin concentration that promoted only barbed-end assembly, provided a means to distinguish the barbed and pointed ends of the filaments and therefore, to determine the direction of movement of organelles relative to the polarity of the actin filaments.

The preparation of rabbit skeletal muscle actin used for these studies contained pure G-actin as determined by SDS-PAGE. On overloaded gels, no actin-binding proteins or other contaminating proteins were present (data not shown). The polypeptide composition of the preparations of acrosomal processes was also determined by SDS-PAGE and the banding pattern was found to be identical to that described by Bullitt et al. (1988).

Barbed-end assembly of actin filaments was achieved by incubating the acrosomal fragments in 0.5 μM skeletal muscle actin overnight in TAMDC buffer. At this actin concentration, bundles of actin filaments assembled preferentially at one end of each acrosomal process, as determined by negative contrast electron microscopy (Fig. 1).

At the other end of the acrosomal process, a few short actin filaments were sometimes seen. The end with a newly grown bundle of actin filaments is defined as the barbed or fast-growing end of the acrosomal process (Tilney at al., 1983). The actin bundles ranged between 1 and 5 μm in length at the barbed ends of the acrosomal fragments.

The presence of actin filament bundles on one end of the acrosomal fragments was verified by fluorescence microscopy after staining with 0.5 μM TRITC-phalloidin. Fluorescence due to the specific uptake of rhodamine-phalloidin was used to routinely verify that the structures visible by AVEC-DIC microscopy contained polymerized actin. The staining by TRITC-phalloidin of acrosomal processes with newly assembled actin filament bundles at the barbed end did not promote additional assembly of actin at the pointed end because the concentration of monomeric actin in solution was well below that required for pointed-end assembly (the addition of the staining solution diluted the sample 10-fold).

By AVEC-DIC microscopy, the acrosomal fragments with newly assembled actin filaments on the barbed end were visible as high contrast fibers each with a low contrast structure at one end (Fig. 2). Infrequently, an axonemal fragment was seen in the acrosomal preparations but it could be distinguished by differences in contrast and morphology in AVEC-DIC microscopy and by the absence of staining with TRITC-phalloidin when viewed in the fluorescence mode. The absence of a low contrast bundle of filaments at one end of a high contrast fiber and the movement of organelles along the fiber itself were other parameter used to identify easily the axonemal contaminants. On two occasions, organelles were observed to move along an axonemal fragment that was identified by the absence of staining with TRITC-phalloidin and the absence of a bundle of filaments at one end of the high contrast fiber. Organelle movement on axonemal microtubules, previously described by Gilbert et al. (1985), was different from that along the actin filaments in the following ways. First, organelles moved along the fiber itself; second, organelles were observed to move in both directions; and third, the average velocity of movement was twofold higher than that on actin filaments (about 2 μm/second; similar to the average velocity of organelle movement along free microtubules dissociated from the bulk axoplasm).

Barbed-end-directed movement of axoplasmic organelles on actin filaments

Actin filaments assembled at the barbed ends of acrosomal processes were used to determine the direction of movement of the motors on axoplasmic organelles. Acrosomal processes with actin filament bundles were adsorbed to a coverslip, after which the coverslip was rinsed and placed on a freshly extruded sample of axoplasm in TAMDC buffer containing 100 μM nocodazole and 50 μM cytochalasin B. Nocodazole was added to depolymerize microtubules that dissociated from the bulk axoplasm and cytochalasin B was present to inhibit the assembly of endogenous actin filaments near the bulk axoplasm. The acrosomal process-nucleated actin filaments were stabilized by TRITC-phalloidin and were insensitive to the presence of cytochalasin B. Under these buffer conditions, the only actin filaments at the margins of the bulk axoplasm were those attached to the acrosomal processes. The occasional axonemal fragment introduced as a contaminant of the acrosomal preparations did not depolymerize in the presence of nocodazole but was easily discernible morphologically (see above).

In these preparations, axoplasmic organelles rapidly accumulated at the margins of the bulk axoplasm by free diffusion. The ability of the organelles to move on the actin filaments was determined by AVEC-DIC microscopy. In the region between 20 and 60 μm from the bulk axoplasm, many organelles were observed in rapid Brownian motion and some of these were observed to attach and move along the nucleated actin filaments (Fig. 2). In a video field (22 μm × 25 μm), several bundles of actin filaments with attached acrosomal fragments were visible and most of these showed organelle movement. Both small (<400 nm) and large (>400 nm) organelles were observed to move along actin filaments but none were observed to move on the acrosomal process itself, presumably because the myosin binding sites on these actin filaments were blocked by the actin binding protein scruin (Tilney et al., 1981; Owen and DeRosier, 1993). Each area of the preparation viewed by AVEC-DIC microscopy was viewed also by fluorescence microscopy to confirm that the movement seen was taking place on actin filaments.

The direction of movement of organelles was analyzed from videotape recordings of several different preparations and all organelles were observed to move from the base towards the tip of the actin filament bundle, i.e. barbed-end-directed (Fig. 2). The average velocity was found to be 1.1±0.3 μm/second (n=20), a value similar to that obtained for organelle motility both on networks of endogenous axonal actin filaments and on exogenous skeletal muscle actin filaments (Table 1B). This observation provided strong evidence that organelles obtained by free dissociation from the axoplasm retained the ability to move at a rapid transport rate on exogenous actin filaments. The observation that the average velocity of movement on the nucleated actin filaments was the same as that on endogenous filaments provided confirmation that this assay accurately reflected the activity on endogenous actin filaments and therefore the direction of movement was reliably determined.

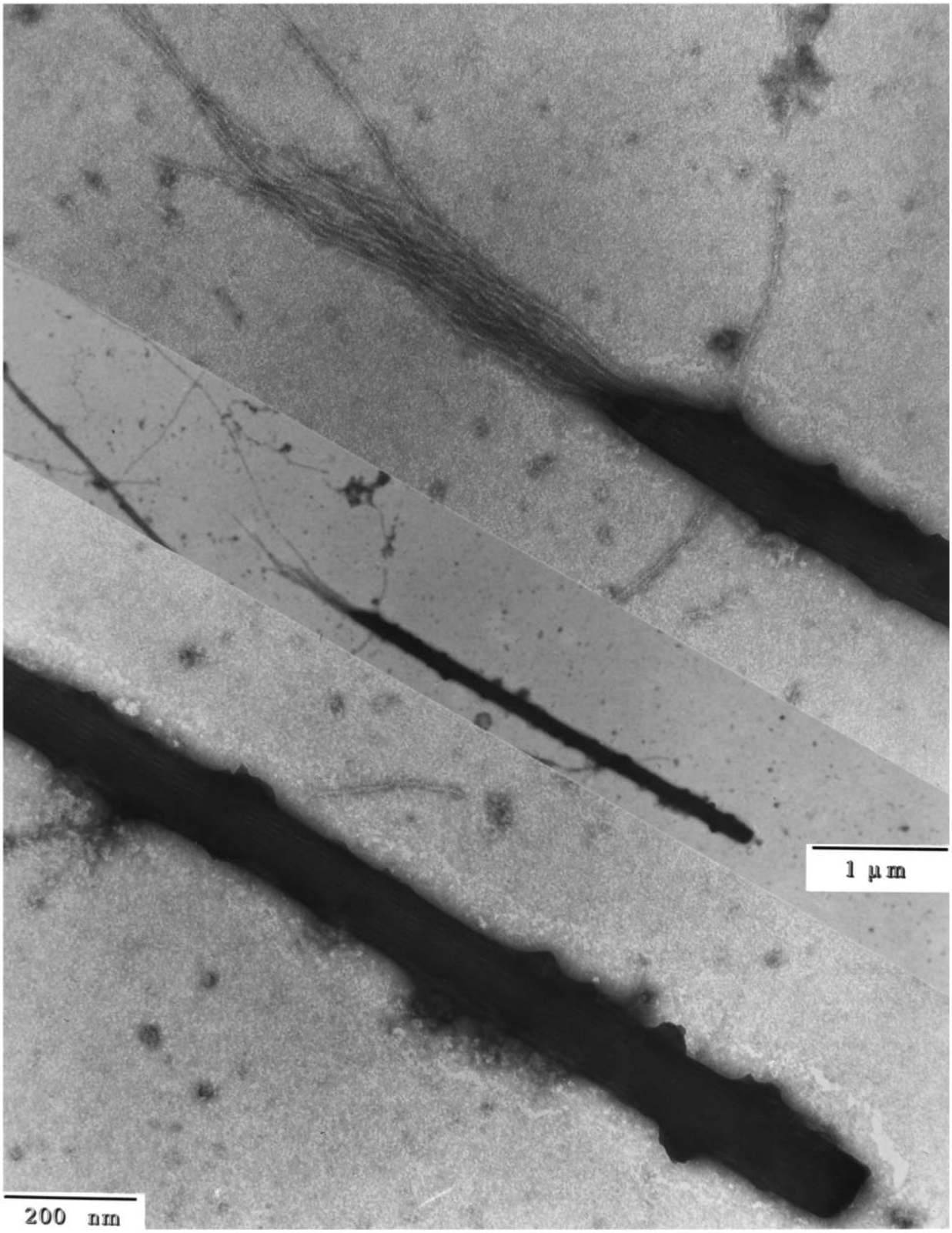

Fig. 1. Electron micrographs of an acrosomal process after incubation overnight in 0.5 µM monomeric rabbit skeletal muscle actin. The middle panel is a low magnification image of the acrosomal process (dark rod) showing a bundle of actin filaments at one end (at left). Polymerization of actin filaments occurred preferentially at this end of the acrosomal process, which is defined as the barbed or fast-growing end. The upper panel is a high magnification image of the barbed end with the bundle of actin filaments. The lower panel is a high magnification image of the pointed end without a bundle of actin filaments. Low mag.: bar, 1 µm. High mag.: bar, 200 nm.

DISCUSSION

In this study we have determined the direction in which axoplasmic organelles move on actin filaments. We have shown that the actin-dependent motor produces force towards the barbed end of the actin filament. These new data provide important information about the type of motor responsible for organelle movement on actin filaments. Since all myosins characterized to date are barbed-end-directed motors, the observations that the actin-dependent motors on axoplasmic organelles are unidirectional and barbed-end-directed lend further support to the idea that these motors are myosin-like proteins, probably one of the unconventional myosins. As predicted, organelles were unable to move along the actin filaments of the acrosomal processes, presumably due to the presence of scruin that sterically blocks the myosin binding sites. This is additional evidence showing that myosin is the motor responsible for force generation. Therefore, we can safely conclude that we have identified an organelle motor in squid axoplasm that is different from the microtubule-based motors known to be present on organelles.

Acrosomal process-nucleated actin filaments functioned as a reliable assay to determine the relative direction of movement on actin filaments. This is the first demonstration of organelle movement at the fast transport rate on actin filaments whose polarity was established by this method. A key feature of the assay that made it work effectively for these experiments was the use of nonhomogenized axoplasmic extracts from which

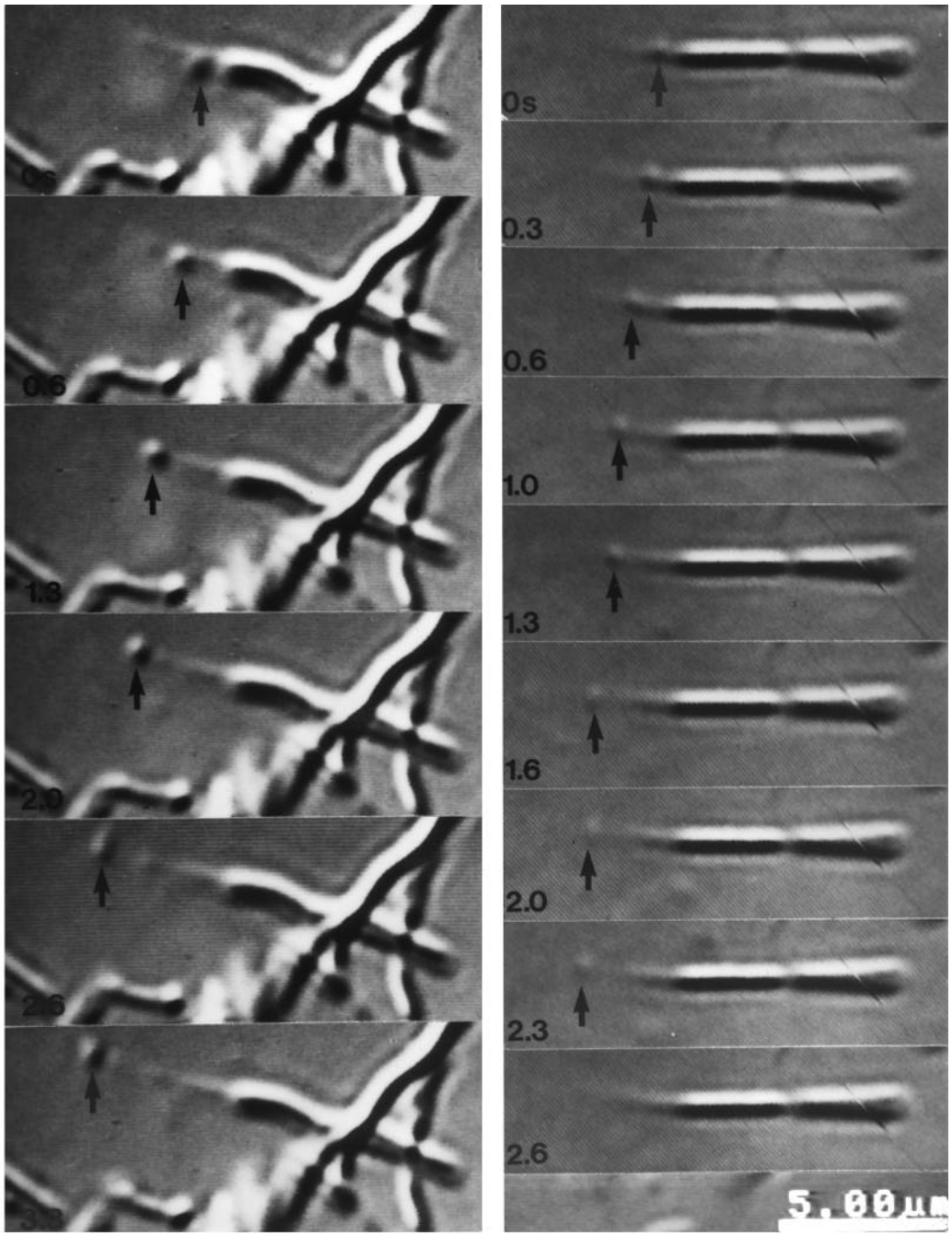

Fig. 2. Barbed-end-directed movement of organelles on actin filaments. Two sets of AVEC-DIC micrographs of axoplasmic organelles (arrows) moving along actin filaments assembled at the barbed end of acrosomal fragments are shown. The acrosomal fragments exhibit high contrast while the actin bundles on which the organelles are moving are faintly visible. The identity of these structures as actin filaments was confirmed by fluorescence microscopy. Time in seconds is given at lower left corner. Bar, 5 µm.

we could obtain organelles. We found that axoplasmic organelles, obtained by free dissociation from the bulk axoplasm, exhibited robust motility. Therefore, care was taken to extrude the axoplasm without mechanical disruption of the cylinder of cytoplasm and to allow the organelles to accumulate at the margins by free diffusion. Under these conditions, organelles moved on actin filaments at a fast transport rate. Adsorption of the acrosomal fragments to the coverslip was another key feature of the assay. Immobilization of the acrosomal processes by adsorption to the coverslip prior to extrusion of the axoplasm made possible the visualization of the movement using video microscopy.

In a recent study by Bearer et al. (1993), using a similar assay to study the movement of organelles obtained by homogenization of squid axoplasm, an occasional organelle was observed to move and the velocity was found to be 0.1 μm/second, i.e. tenfold lower than the value we reported for organelle movement on endogenous actin filaments (Kuznetsov et al., 1992). The loss of motility at the fast transport rate after fractionation of axoplasmic organelles following homogenization suggests that the activity is very sensitive to mechanical shear forces or to substances that may have been either released or modified during homogenization. Alternatively, these results may suggest that the motor involved in movement at the slow rate is different from the one involved in movement at the fast transport rate.

The identity of the actin-dependent motor(s) on axoplasmic organelles has yet to be established but the most likely candidate is one of the unconventional myosins. The list of new myosins has grown rapidly due to the development of molecular techniques that provide means to identify, clone and sequence genes. Cheney et al. (1993a) as well as Goodson and Spudich (1993) have shown by phylogenetic analysis of the myosin superfamily that there are at least eight evolutionarily distinct classes. Most of these myosins have not been functionally characterized. The myosin V class of unconventional myosins is of particular interest to us because of its identification and purification from neuronal cells (Espreafico et al., 1992). Using an antibody to chicken brain myosin V (gift from Cheney and Mooseker), we have been able to show, by western blotting, a polypeptide of M_r 190×10^3 in squid axoplasm that cross-reacts with this antibody and we have been able to purify this protein from squid brain (unpublished data). Therefore, myosin V is a candidate for the organelle motor. Bearer at al. (1993) reported that several high molecular mass bands (all > M_r 200×10^3) on western blots of axoplasm were recognized by an antibody to scallop muscle myosin. The identity of these bands remains unclear and the role of these polypeptides in organelle motility has not been established.

In axoplasm, several different types of organelles move on actin filaments. Based on the sizes and shapes of the organelles observed to move on actin filaments, the types of organelles appear to include mitochondria, ER, and large and small vesicles (Kuznetsov et al., 1992, 1994). A question that arises is whether one motor is responsible for the movement of all types of organelles or whether each type of organelle has a specific type of myosin motor. There are a number of studies showing that myosins of several different classes are present within a given cell (Cheney et al., 1993b; Jung and Hammer, 1990; Titus et al., 1989, 1993). These observations could be interpreted to mean that cells exhibit functional redundancy so that loss of one myosin is compensated for by the others. The results with myosin I-defective mutants in *Dictyostelium discoidium* (Titus et al., 1989) are consistent with this idea. Recent results with the contractile vacuole in *Acanthamoeba* (Doberstein et al., 1993), however, provide evidence for the 'one myosin, one organelle' hypothesis (see Bement and Mooseker, 1993). In the report by Doberstein et al. (1993), myosin IC was shown to be associated specifically with the contractile vacuole and antibodies that inhibit its function were shown to perturb vacuole contraction. Antibodies to myosin IB, a related myosin, were shown to have no effect on vacuole function. Therefore, myosin IC has been shown to be associated with one organelle in these cells and with one specific function. Consequently, it is possible that each of the different types of axoplasmic organelles observed moving on actin filaments may have a unique type of myosin and the motors, once identified, may comprise a diverse group. However, the similarity of the parameters of movement for the different size classes of organelles leads us to argue against this idea and to favor a common motor for all types of organelles.

The acrosomal process-nucleated skeletal muscle actin filaments as well as skeletal muscle actin filaments adsorbed directly to a glass coverslip (Kuznetsov et al., 1994) are both suitable substrata on which to study the motor functions of newly identified myosins. In the case of axoplasmic vesicles, the movement on endogenous actin filaments serves as a useful baseline for comparison of movements in an in vitro assay. Purification of motors and reconstitution of movement should be possible with these in vitro systems.

In summary, we have shown that axoplasmic organelles have motors that move them toward the barbed ends of actin filaments. This finding further supports the conclusion that these organelles have a myosin-like motor on their surface and that actin as well as microtubules function in fast axonal transport. The present work addresses the question of whether different classes of organelles have different types of myosin-like motors.

We gratefully thank Lew and Molly Tilney for their help with the preparation of acrosomal processes from sperm of horseshoe crabs. We thank Michael Bermann for G-actin, Gregory Lewis for assistance with SDS-PAGE, and Marion Shonn for help with library research and preparation of references. We especially thank Bruce Jackson whose program at Massachusetts Bay Community College provided the summer stipend for Dale Johnson. This work was supported by NIH GM48438, NSF MCB-9216634, DFG We790/13-1 and an MBL Fellowship.

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(Received 24 March 1994 - Accepted 26 April 1994)