Movement of Organelles Along Filaments Dissociated from the Axoplasm of the Squid Giant Axon

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Summary
Cytoplasmic filaments, separated from the axoplasm of the squid giant axon and visualized by video-enhanced differential interference contrast microscopy, support the directed movement of organelles in the presence of ATP. All organelles, regardless of size, move continuously along isolated transport filaments at 2.2 ± 0.2 μm/sec. In the intact axoplasm, however, movements of the larger organelles are slow and saltatory. These movements may reflect a resistance to movement imposed by the intact axoplasm. The uniform rate of all organelles along isolated transport filaments suggests that a single type of molecular motor powers fast axonal transport. Organelles can attach to and move along more than one filament at a time, suggesting that organelles have multiple binding sites for this motor.

Introduction
Directed movement of vesicular organelles and mitochondria occurs in almost all types of cells (Schliwa, 1984), yet the cellular machinery for generating this movement has not been identified. Since the function of organelle movement is to transport macromolecules from one compartment of a cell to another, it is not surprising that this process is particularly well developed in neurons where transport occurs bidirectionally in axons as long as a meter or more (Grafstein and Forman, 1980).

Rapid transport of material in axons has been examined using radioactive labels to follow the fate of proteins synthesized in the cell body (Lasek, 1968); these experiments revealed that certain proteins are transported in the anterograde direction of rates up to 400 mm/day. Other proteins are transported in the retrograde direction at rates of up to 200 mm/day (Kristensson and Olsson, 1971; Hendry et al., 1974). Materials moving in both the anterograde and retrograde directions at the fast rate are associated with membrane-limited organelles (Lorenz and Willard, 1976; Smith, 1980; Tsukita and Ishikawa, 1980). Collectively, these transport processes have been termed "fast axonal transport" to distinguish them from "slow axonal transport" (Lasek, 1982), which refers to a net anterograde movement of cytoskeletal and soluble proteins at rates 100 times slower. Only materials transported by fast axonal transport utilize the mechanism of directed organelle movement (Grafstein and Forman, 1980).

The recent application of video processing to polarization-based microscopy (Allen et al., 1981; Inoue, 1981) amplifies image contrast to the extent that structures below the resolution limit of the light microscope can be made visible. Using video microscopy to visualize the squid giant axon, it was shown that organelles less than 200 nm in diameter move parallel to linear elements that could be single cytoplasmic filaments (Allen et al., 1982). In contrast to the saltatory movements of larger organelles, these small organelles moved continuously at rates consistent with those reported for fast anterograde axonal transport. Thcoo organello movemonto contimiento in axo

plasm that is extruded from the squid giant axon (Brady et al., 1982).

Single filamentous elements associated with moving organelles occasionally separate from the edge of the isolated squid axoplasm (Allen et al., 1983; Brady and Lasek, unpublished observations). Here we report the results of dissociating axoplasm to produce extensive fields of isolated filaments where virtually all filaments support the transport of organelles for many hours. We have used video microscopy to examine the characteristics of organelle movements along these single filaments. A major advantage of this system is that organelle movement can be examined in the absence of any impeding interactions exerted on organelles by an organized cytoplasmic gel, so characteristics of organelle movement should reflect directly the underlying molecular processes.

Results
Organelle Movement in Intact Axoplasm
We examined intact, extruded axoplasm from the squid giant axon in order to compare organelle movements in intact axoplasm (Allen et al., 1982; Brady et al., 1982) with organelle movements along isolated filaments in dissociated axoplasm. Organelles in the axoplasm moved in both directions at rates that varied, depending on the size of the organelle, from approximately 2 μm/sec for the smallest vesicles to less than 0.5 μm/sec for the largest mitochondria (see Figure 2). Small organelles moved continuously in one direction; larger organelles frequently stopped (saltatory motion) and sometimes moved backwards for a short distance. These movements in a reversed direction have been interpreted as recoils (Allen et al., 1982); otherwise each organelle moved in only one direction.

Directed Organelle Movement in Dissociated Axoplasm
In dissociated preparations, 1–30 discrete and well-separated filaments per viewing field of 400 μm² adhered to the coverslip around the edge of the bulk axoplasm
Figure 1. Typical Field of Transport Filaments and Associated Organelles

A few small and medium-sized vesicular organelles are indicated by black triangles. A small mitochondrion (black and white arrowhead) contacts two filaments; subsequent observation showed that it was attached to both. Some stationary vesicular organelles are attached to the glass surface (open triangles); vesicles suspended in the medium are outside the plane of focus. Magnification 11,000x. Inset shows transport filaments (black triangles) settled on the glass surface at the edge of the bulk axoplasm (A).

(Figure 1). Filaments were oriented in all directions so the original orientation in the intact axoplasm was no longer apparent.

Most of the filaments were distinguished by their sharp contrast with the substrate, their smooth, uninked contours, and their uniform apparent diameter of approximately 0.2 μm (Figure 1); these filaments were readily distinguished, especially by their lower contrast, from other, less common filaments. The true diameter of these filaments could not be determined from the light microscopic image alone, because structures smaller than 0.2 μm generate an inflated image as a result of their diffraction by light. Nevertheless, these filaments belonged to a recognizable class that we refer to as transport filaments because of their obvious involvement in the organelle movements described below.

Virtually all transport filaments supported directed movements of small particles, and all particle movements were along these filaments. These particles are regarded as organelles rather than macromolecular complexes because they resemble organelles in the intact cytoplasm (Allen et al., 1982), and because particles on isolated transport filaments are membrane-limited as seen by electron microscopy (Schmapp et al., 1985). Also, a variety of evidence suggests that it is organelles that move at rapid rates through axons (Smith, 1980; Tsukita and Ishikawa, 1980; Breuer et al., 1975; Lorenz and Willard, 1978).

A 1:1 dilution of buffer X with water in the presence of 2 mM ATP (motility buffer) provided the best dissociations of transport filaments from the bulk axoplasm and supported long-lasting and reproducible organelle movements. Preparations made in full-strength buffer X yielded fewer isolated filaments and more unattached organelles. In a buffer of lower ionic strength (20 mM KCl, 4 mM MgCl₂, 4 mM EGTA, 10 mM imidazole, pH 7.1), organelles were bound to transport filaments but rarely moved. No movements occurred in the absence of ATP with 1 mM sodium azide and 10 mM 2-deoxyglucose present to block endogenous production of ATP. Under these conditions organelles remained bound to filaments. The transport filaments in motility buffer were not static but many were bending and changing their shape. It was not clear whether the movements of transport filaments could be entirely explained by the movements of the associated organelles.
Organelle Transport along Filaments

Figure 2. Velocities of Small Organelles, Medium-Sized Organelles, and Mitochondria in Intact, Extruded Axoplasm (Anterograde, A, and Retrograde, R, Directions) and along Isolated Transport Filaments from Dissociated Preparations (D).

Organelle velocity decreases with increasing size in intact axoplasm, but all classes of organelles move at the same velocity (2.2 ± 0.2 μm/sec) along isolated filaments.

Movements along transport filaments were smooth and continuous, even for large organelles, and recolts or saltatory movements were infrequent. Organelles continued to move along isolated filaments for many hours, over 12 hr in one instance. Since rates of organelle movement remained constant for at least 90 min and then often showed a gradual decline, rates of directed organelle movement were measured during the early phase (see below). The frequency of organelles moving past reference points on transport filaments also declined with time.

Rates of Organelle Movement

The sizes and shapes of organelles moving through intact axoplasm were similar to the sizes and shapes of organelles moving along isolated filaments in dissociated axoplasm (Figures 1 and 2). Vesicular organelles were classified by size, and mitochondria by their elongate shape. Small organelles were defined as those with an apparent diameter of 0.2 μm, but many were presumably much smaller. Medium-sized organelles varied between 0.2–0.53 μm in diameter, and large organelles were those larger than 0.53 μm. Elongate cylinders with diameters of approximately 0.25 μm and lengths between 0.7 and 3.6 μm were presumed to be mitochondria, though some may have been tubular-vesicular organelles (Smith, 1980; Tsukita and Ishikawa, 1980).

Small organelles in intact squid axons move continuously with a mean rate of 2.5 μm/sec, but movements of larger organelles are considerably slower and intermittent (Allen et al., 1982). In intact, extruded axoplasm (Figure 2), small organelles moved in the anterograde direction at 2.15 μm/sec, and in the retrograde direction at 1.75 μm/sec; medium sized organelles moved at 1.1 μm/sec; and large spherical organelles and mitochondria moved at 0.4 μm/sec during periods of continuous movement over several micrometers (Figure 2). For all but the small organelles, rates of movement were the same in the anterograde and retrograde directions (Figure 2).

Along isolated transport filaments, small- and medium-sized organelles, as well as mitochondria, moved at a mean rate of 2.15 μm/sec (Figure 2), which was remarkably consistent from preparation to preparation (± 0.15 μm/sec; 23 preparations). Large spherical organelles (greater than 0.53 μm in diameter) were very infrequent along isolated transport filaments, but a few measurements indicated that they move somewhat slower than the other organelles.

Interactions of Vesicles with Filaments

Organelles suddenly attached to filaments and, either immediately or after a brief pause, began to move along
them (Figure 3). Some organelles moving along filaments suddenly dissociated from them; attached and stationary organelles also dissociated from filaments (Figure 3). Once an organelle left a filament, it rapidly disappeared into the surrounding medium.

Several observations indicated that organelles have multiple attachment sites for filaments. Mitochondria moved along filaments with any or all of their length attached; unattached segments of mitochondria exhibited Brownian motion (Figure 1). Vesicular organelles and mitochondria sometimes attached to two transport filaments simultaneously (see Figures 1 and 4). Organelles occasionally switched from one filament to another at points where filaments crossed (Figure 5). At some filament crossings every organelle switched filaments, while at others only some of them switched. Organelles typically moved in only one direction along the filaments to which they switched. When mitochondria switched filaments, one end attached to a crossing filament while the other end remained attached to the original filament; the trailing end subsequently detached from the original filament and reattached to the crossing filament. We infer that filament switching by vesicular organelles also requires simultaneous binding to two filaments because organelles rapidly diffused away into the medium once they detached from a transport filament (Figure 3).

Discussion

Comparison of Organelle Movement in the Intact Axoplasm and along Isolated Transport Filaments

Differences between the characteristics of organelle movement in intact axoplasm and along isolated transport filaments provide insight into the mechanism of organelle transport in axons. In intact axoplasm, the larger the organelle, the slower and more discontinuous or saltatory are its movements. It could be supposed that smaller organelles have a higher density of force-generating sites or a different force-generating mechanism; in fact, different myosin ATPases support different velocities of bead movement in vitro (Sheetz et al., 1984). However, along isolated transport filaments, all organelles move at a single rate. This single rate of organelle movement in dissociated axoplasm favors the idea that movements of all organelles are powered by the same species of molecular motor, which might depend on a single type of ATPase.

The velocity of organelle movement along isolated transport filaments, 2.2 μm/sec, is the same as that of the smallest organelles in intact axoplasm. The inverse relationship between organelle size and velocity in the intact axoplasm probably depends on impeding interactions with the axoplasm that could become increasingly important for larger organelles, accounting for their saltatory movement. Differences in size within the class of small organelles could account for the different rates of anterograde and retrograde movement of these organelles in the intact axoplasm (Smith, 1980; Tsukita and Ishikawa, 1980). These differences between organelle movement in intact axoplasm and along isolated transport filaments underscore the ambiguity inherent in pharmacological experiments aimed at blocking transport in intact cytoplasm or cells. Drugs or treatments could affect organelle transport either directly through the transport machinery or indirectly by disrupting the cytokolotolon.

It is clear that the force production for organelle movement does not require the complex tertiary structure of the axonal cytoplasm. This casts doubt upon hypotheses that...
would explain organelle transport by contraction of a cytoplasmic lattice (Ellisman and Porter, 1980) or cytoplasmic streaming through low-viscosity channels (Weiss and Gross, 1982).

Interactions between Organelles and Transport Filaments

Organelles bind to and dissociate from isolated transport filaments. The number of organelles bound to and moving along transport filaments at any moment must, therefore, depend upon the rate constants governing association and dissociation as well as the concentration of organelles and filaments. Dilution of organelles by the medium would favor dissociation over association and, therefore, the number of organelles moving along filaments would decrease as a preparation ages. A function of the microtubule domains in the intact axoplasm (Schnapp and Reese, 1984) might be to confine organelles near transport filaments to shift the equilibrium in favor of binding.

The finding that separate ends of the same mitochondrion can move along separate transport filaments shows that each mitochondrion has several sites that are not only capable of attaching to transport filaments but of interacting with them to generate movement. Recurrent observations of mitochondria moving in intact axoplasm and attaching to more than one filament also led to the conclusion that these organelles contain multiple sites that interact with the force-generating system (Martz et al., 1984). Vesicular organelles also appear to have multiple attachment sites.

Nature of the Molecular Motor

Organelle movement along transport filaments requires ATP, suggesting that an ATPase is involved in this process. In the absence of ATP, organelles bind to filaments without moving along them so organelle binding to filaments may be independent of their directed movement. The static, bound state could be analogous to the condition of rigor that develops between myosin and actin in muscle (Adelstein and Eisenberg, 1980), or dynein and microtubules in cilia (Gibbons and Gibbons, 1974) when ATP is absent. In these two familiar examples, attachment and force generation are functions of a single molecule.

What cytoplasmic filaments are responsible for organelle transport and what is the molecular nature of the ATPase? These questions have been difficult to answer using whole axons or intact axoplasm because of the complexity of these systems. The dissociated filament preparation provides an opportunity to answer these questions, in particular to determine the structure of the transport filaments (Schnapp et al., 1985).

Experimental Procedures

Preparation of Intact and Dissociated Axoplasm

Squid (Loligo pealei) were collected at the Marine Biological Laboratory in Woods Hole, MA. The giant axon was dissected under running sea water and ligatured just distal to the stellate ganglion. The ligatured axon was then removed and placed in Ca+2-free artificial sea water at 2°C, where it could be kept for several hours without any loss of directed organelle movement. Just prior to use, the axon was cleaned of surrounding connective tissue and small nerve fibers, blotted on tissue paper, and washed with 5 μl of a 1:1 dilution of buffer X containing 2 mM ATP (motility buffer). Buffer X (aspartate, 250 mM; taurine, 130 mM; betaine, 170 mM; glycine, 20 mM; MgCl2, 12.9 mM; EGTA, 100 mM; CaCl2, 3.0 mM; glucose, 1 mM; pH 7.2; see Brady et al., 1984) is similar to the internal medium of the squid giant axon. The axon was blotted again, placed on a glass coverslip (#17, Clay Adams, Parsippany, NJ), and transected. Axoplasm was extruded by pulling the axon under a Pasteur pipette covered with polyethylene tubing, or by pushing the axon between the silicone rubber covered tips of 85 forceps.

Intact axoplasm was examined directly by sealing it between two coverslips. To retard air drying, the volume of this simple chamber was reduced by applying a length of silicone grease on either side of the axoplasm. The upper coverslip was then pressed down onto wax spacers until the axoplasm just made contact with its surface.

To make dissociated preparations, the axoplasm was placed on 40 μl of motility buffer on the surface of a coverslip (22 x 60 mm; #0). A second coverslip (22 x 22 mm or 22 x 40 mm) with wax spacers was then placed on top of the axoplasm, and vertical pressure was applied and released to spread out the disrupted axoplasm. The coverslips were then sealed with a mixture (1:1:1) of vaseline–lanolin–paraffin.

Video Microscopy

Axoplasm was viewed in a Zeiss ICM inverted microscope set up for differential interference contrast. Bias retardation was introduced by deGennarmont compensation. The contrast of single transport filaments and of the small organelles on the video monitor was optimal when the bias compensation for the combined optical-video system was between +36 and +120 of a wavelength (how to set up the optical conditions to match optimally the performance of video tubes is controversial; e.g., Inoue, 1981; Allen et al., 1981). The rationale for the parameters selected in this paper, as well as a more detailed account of the video microscopy set-up and operation will be presented elsewhere (Schnapp et al., submitted). The specimen was illuminated by a 100 watt mercury arc, which was heat-filtered and made monochromatic for the 546 nm mercury peak with an interference filter.

The image was projected out the side port of the ICM with a 12.5 x corrected eyepiece and focused onto the Newvicon target of a Dage–MITI video camera (Michigan City, Indiana; series 65, 67, or 68) with a Fujicon 105 mm zoom lens and 2 x telextender. An improvement in image quality was achieved by replacing the zoom lens and telextender with a Zeiss 63 mm luminar lens. A 25 x corrected ocular replaced the 12.5 x ocular to provide comparable magnification.

Visualization of transport filaments required a large amplification of image contrast. The video gain was amplified by increasing the intensity of illumination (by adjusting the optical system for critical balance of Kohler illumination) and by adjusting the gain of the camera amplifier. At such high gain the black level of the Dage cameras had insufficient range to keep the signal from saturating. Range was extended by a simple modification of the camera control or by using the contrast expansion capability of the digital video processor in series with the camera. A mottled background pattern under these conditions of enhanced contrast was subtracted from the real-time video image with a digital video processor (Quantex DS-30; Quantex, Sunnyvale, CA), as previously described with the Hamamatsu system (Allen and Allen, 1983). The analog output of the Quantex went through a video processor (F302-2 Colorado Video sync stripper and #604 video processor, Colorado Video Inc., Boulder, CO) to amplify slices of the gray scale. The output of this analog processor was connected to a video recorder and a video monitor. Photographs of video images were then taken from the video tapes by Image Transform (N. Hollywood, CA), or from digitally averaged video images, using a 50-line Ronchi diffraction grating placed in front of the camera lens to minimize the contrast of the TV scan lines (Figures 1, inset, and 6). Rates of organelle movement along filaments having straight segments that were at least 4 μm long were calculated from distances measured on the video monitor.

Acknowledgments

We thank Raymond Laser, Scott Brady, Susan Gilbert, and Robert Allen for many helpful discussions and for sharing unpublished observations with us. We would also like to thank Robert Allen for use of his
video microscopy system during the initial phase of this project, Dr. Hideshi Fujiwaka for assistance in using it, and John Murphy for preparation of the figures. R. D. V. is a trainee in the National Institutes of Health Medical Scientist Training Program (GM 07365). This work was supported in part by Hoechst-Rousselle Pharmaceuticals and NIH (GM 33351; M. P. S.). M. P. S. is an established investigator of the American Heart Association.

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Received September 10, 1984; revised December 10, 1984

References


