Axonal Transport of Mitochondria along Microtubules and F-Actin in Living Vertebrate Neurons

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Abstract. A large body of evidence indicates that microtubules (MTs) conduct organelle transport in axons, but recent studies on extruded squid axoplasm have suggested that actin microfilaments (MFs) may also play a role in this process. To investigate the separate contributions to transport of each class of cytoskeletal element in intact vertebrate axons, we have monitored mitochondrial movements in chick sympathetic neurons experimentally manipulated to eliminate MTs, MFs, or both. First, we grew neurons in the continuous presence of: (*a*) cytochalasin E to create neurites which had never contained MFs; or (*b*) nocodazole or vinblastine to produce neurites which had never contained MTs. Mitochondria moved bidirectionally at normal velocities along the length of neurites which contained MTs and lacked MFs, but did not even enter neurites grown without MTs but containing MFs. In a second approach, we treated established neuronal cultures with cytoskeletal drugs to disrupt either MTs or MFs in axons already containing mitochondria. In cytochalasin-treated cells, which retained MTs but lacked MFs, average mitochondrial velocity increased in both directions, but net directional transport decreased. In vinblastine-treated cells, which lacked MTs but retained essentially normal levels of MFs, mitochondria continued to move bidirectionally but the average mitochondrial velocity and excursion length were reduced for both directions of movement, and the mitochondria spent threefold as much time moving in the retrograde as in the anterograde direction, resulting in net retrograde transport. Treatment of established cultures with both drugs produced neurites lacking MTs and MFs but still rich in neurofilaments; these showed a striking absence of any mitochondrial motility. These data indicate that axonal organelle transport can occur along both MTs and MFs in vivo, but with different velocities and net transport properties.

ORGANELLE transport in animal cells is widely considered to be an exclusively microtubule-dependent process. Evidence from various cell types indicates that microtubule (MT)[1]-dependent movement is essential to transport and position in several classes of organelles, including the Golgi apparatus (Corthésy-Theulaz et al., 1992; Ho et al., 1989), endoplasmic reticulum (Terasaki et al., 1984, 1986), and lysosomes (Hollenbeck and Swanson, 1990). Axonal organelle transport in particular has been thought to be entirely MT-dependent. This view developed from a large body of morphological and pharmacological studies (reviewed by Grafstein and Forman, 1980), and was reinforced by the images of axoplasm obtained by freeze-etch electron microscopy (Hirokawa, 1982; Hirokawa and Yorifuji, 1986), by the observations of reactivated axoplasmic organelle transport using video-enhanced microscopy (Allen et al., 1985; Brady et al., 1985; Schnapp et al., 1985), and by the subsequent discovery and characterization of the MT-based motors kinesin (Brady, 1985; Vale et al., 1985; Scholey et al., 1985) and cytoplasmic dynein (Vallee et al., 1988; Shpetner et al., 1988; Schnapp and Reese, 1989; Schroer et al., 1989).

However, there has also existed in parallel a more diffuse body of studies indicating a role for actin microfilament (MF)-based motility in organelle transport. First, other eukaryotic cells, such as the giant alga *Nitella*, have well-developed systems of actomyosin-based organelle transport; indeed, this was among the first powerful systems established for the in vitro study of organelle motility (Sheetz and Spudich, 1983). Second, axonal organelle transport has been shown to continue after MT disruption

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1. *Abbreviations used in this paper*: MF, actin filament; MT, microtubule; NF, neurofilament; R-phal, rhodamine-conjugated phalloidin; R123, rhodamine 123.

in at least one study (Brady et al., 1980), and to be partially inhibited by the introduction of agents that specifically disrupt MFs (Goldberg, 1982; Goldberg et al., 1980; Brady et al., 1984). Finally, since the general demonstrations that neuronal tissue was rich in actin (Fine and Bray, 1971) and myosin (Burridge and Bray, 1975), several myosin isotypes have also been detected there, including myosins I (Li and Chantler, 1992), II (Sun and Chantler, 1991, 1992; Mochida et al., 1994; Li et al., 1994), V (Espreafico et al., 1992; Cheney et al., 1993*a*), and VI (Hasson and Mooseker, 1994), and it has been suggested that these may be involved in neuronal organelle transport (Mooseker, 1993).

Thus, it should not seem entirely heretical that recent re-examinations of the role of MF-based transport in axoplasm have suggested that MFs and their associated motor proteins might also support axonal organelle movement. Since Kuznetsov and co-workers (1992) first observed MT-independent organelle movements in extruded axoplasm that were strongly indicative of actomyosin-based motility, it has been demonstrated that isolated axoplasmic organelles have myosin on their surfaces, and can move toward the barbed end of MFs in vitro (Bearer et al., 1993; Kuznetsov et al., 1994; Langford et al., 1994). However, the role of MF-based transport and its significance relative to MT-based transport in intact axons remain unclear.

In this study, we sought to address these questions directly by quantitative analysis of organelle movement in cultured neurons. We specifically followed the movement of mitochondria, since they can be unambiguously identified in live neurons by staining with lipophilic cationic dyes (Johnson et al., 1981; Magrassi et al., 1987), they exhibit a broad range of motile behaviors and velocities within axons (Martz et al., 1984; Forman et al., 1987; Morris and Hollenbeck, 1993), and their motility is profoundly regulated by the physiological state of the neuron (Morris and Hollenbeck, 1993). In addition, some evidence has already suggested that mitochondria can enter regions of the cell which lack MTs but contain MFs (Bradley and Satir, 1979). To investigate the contributions of the different cytoskeletal filament systems to the transport of mitochondria within living vertebrate axons, we adapted techniques originated by Heidemann and colleagues (1990) to grow neurites in the absence of MTs, and by Marsh and Letourneau (1984) to grow neurites which lack MFs. We report that mitochondria are transported along both MTs and MFs in living cells, but that the motile behaviors of mitochondria are dramatically different on the two cytoskeletal tracks.

Materials and Methods

Materials

Unless otherwise specified, all materials and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Rhodamine 123 (R123) and rhodamine-conjugated phalloidin (R-phal) were obtained from Molecular Probes, Inc. (Eugene, OR). Extracell coverslips were obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). DM1A anti-tubulin was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Texas red, horse radish peroxidase–, and alkaline phosphatase–conjugated secondary antibodies were obtained from Vector Labs (Burlingame, CA).

Neuronal Cell Culture and Drug Treatments

Sympathetic chain ganglia were dissected from 9–11 d chicken embryos, dissociated, and grown on coverslips as previously described (Hollenbeck and Bray, 1987). Cells were grown in Liebovitz L-15 medium supplemented with 10% fetal bovine serum, 0.6% glucose, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml nerve growth factor, and 0.5% methylcellulose. Coverslips were treated with 1 mg/ml polylysine overnight at 4°C, then with 10 μg/ml laminin in Hanks balanced salt solution for 0.5–2 h before use. To grow neurites lacking MFs from sympathetic neurons, we modified the technique of Marsh and Letourneau (1984) and grew cells for 3 d in the continuous presence of 20 μg/ml cytochalasin E diluted into growth medium from a 4 mg/ml stock in DMSO. To grow neurites lacking MTs, we modified the technique of Lamoreaux et al. (1990) and cultured cells on polylysine/laminin-treated coverslips for 1 d in the continuous presence of 100–200 nM vinblastine diluted into culture medium from a 4 mM stock in methanol. Alternatively, cells could be grown on Extracell coverslips for 1 d in the continuous presence of 0.4 μg/ml nocodazole diluted from a stock of 0.2 mg/ml in DMSO. To depolymerize the MFs in established cultures (grown in normal medium for 1 d before experimental treatment), we treated them with 20 μg/ml cytochalasin for 30 min, and then either transferred them to the microscope for low light level video microscopy or fixed them for immunofluorescent staining. To depolymerize the MTs in established cultures, we treated them with 1 μM vinblastine in culture medium without methylcellulose for 60 min before fixing or transferring live to the microscope. To depolymerize both MFs and MTs in established cultures, we incubated the cultures in vinblastine-containing medium for 30 min, and then in vinblastine plus cytochalasin-containing medium for an additional 30 min prior to either fixation or transferring live to the microscope. Control cells were treated simultaneously with the same doses of methanol as vinblastine-treated cells (0.025%) and/or with the same dose of DMSO as cytochalasin-treated cultures (0.5%).

Preparation for Immunofluorescence Microscopy and Electron Microscopy

For light microscope fluorescent staining of MTs, NFs, or MFs, cells were simultaneously fixed and permeabilized in 2% formaldehyde, 0.1% glutaraldehyde, 0.12 M sucrose, 0.5% Triton X-100, 2 mM EGTA, in HBSS, pH 7, at 37°C for 15 min, and then changed to the same solution without Triton for 8–10 min. Cells were blocked in 3% BSA in PBS for 15 min at 25°C or overnight at 4°C, and then incubated for 1 h in primary antibody: either monoclonal antibody DM1A to label MTs (Breitling and Little, 1986) and/or polyclonal antibody NF70 to label neurofilaments (NFs) (Hollenbeck and Bray, 1987). For double-labeling of MTs and NFs, primary antibodies were applied simultaneously, and secondary antibodies in sequence. Secondary antibodies were FITC-conjugated horse anti–mouse IgG, FITC-conjugated goat anti–rabbit IgG, or Texas red–conjugated donkey anti–rabbit IgG, all at 1 μg/ml. MFs were labeled after secondary antibody treatment by incubation for 1 h in 0.1mg/ml R-phal. Coverslips were mounted in 1 mg/ml p-phenylenediamine in 90% glycerol, 10% 10× PBS (Johnson and Araujo, 1981) and observed using a Zeiss IM35 microscope with a 63× Planapochromatic objective. Control and drug-treated cells were fixed, processed, embedded, and sectioned for transmission electron microscopy as previously described (Hollenbeck, 1993).

Measurements of Mitochondrial Motility

To view mitochondria in living, drug-treated cells, we added 0.5–1 μg/ml R123 to the drug-containing medium 30 min before the end of drug treatment, and then rinsed the cells with drug-containing medium without dye. Cultures were placed on a Zeiss IM35 microscope with a 37°C air curtain stage warmer and R123-labeled mitochondria were viewed by epifluorescence illumination with a tungsten light source. Images were captured with a Hamamatsu intensified CCD camera and recorded with a Panasonic AG-7300 sVHS video recorder after frame averaging with an Image-1 Image Analysis System (Universal Imaging Corp., West Chester, PA). Due to the high density of mitochondria in control cells, these axons were viewed by video-enhanced phase contrast microscopy, using a Hamamatsu Newvicon camera and an Image-1 image analysis system to provide background subtraction and contrast enhancement. (Phase-dark organelles which were 0.5-μm across and at least 1-μm long were known to be mitochondria based on extensive direct comparisons between phase contrast and R123 epifluorescence images.) Movements of individual mitochondria were quantified from video tape recordings with a Mark-V video

measurement system (M. Walsh Electronics, San Dimas, CA) driven by Measure software (gift of Steven M. Block, Princeton University, Princeton, NJ) as previously described (Morris and Hollenbeck, 1993). In vinblastine-treated cultures, to ensure that we were observing true mitochondrial motility rather than the results of possible axonal retraction or bulk axoplasmic flow, we applied three selection criteria: (*a*) Only axons that did not move relative to the substratum during the observation period were considered. (*b*) Only axons showing bidirectional and/or saltatory mitochondrial movement were considered; those showing slow, simultaneous, and unidirectional movement of all mitochondria were rejected. (*c*) Only mitochondrial movements within sections of the axon which were thin, of uniform caliber, and lacking varicosities were considered. To prevent bias in selection of moving vs. stationary mitochondria for measurement, all visible mitochondria which met the above criteria were tracked for the entire time that they remained in focus. All mitochondrial displacement data for one drug condition were then pooled. In cultures treated simultaneously with vinblastine and cytochalasin, we ensured detection of any mitochondrial movements that might take place by monitoring all mitochondria visible in a viewing field which met only criterion (*a*) above for the entire time they remained in focus.

Results

Mitochondrial Distribution in Neurites Grown in the Absence of F-Actin or Absence of MTs

To determine if MFs or MTs were necessary or sufficient for the transport of mitochondria in neuronal processes, we began by adapting previously published techniques for growing neurites which lack MFs but contain MTs (Marsh and Letourneau, 1984), or which lack MTs but contain MFs (Lamoureux et al., 1990). Although the effects of these procedures on the MTs and MFs have been extensively characterized, we examined our cultures using anti-tubulin immunofluorescence, anti-neurofilament immunofluorescence, or R-phal staining to detect MTs, NFs, and MFs, respectively. Sympathetic neurons grown on an adhesive substratum for 3–4 d in the continuous presence of cytochalasin E (Marsh and Letourneau, 1984) extended neurites which completely lacked MFs but contained MTs along their entire lengths (Fig. 1). NFs in these cells were disrupted as well, appearing sparse and fragmented compared to control cells where they are extensive and continuous throughout all processes (data not shown). Neurons grown for 1 d on adhesive substrata in the continuous presence of either vinblastine or nocodazole (Lamoureux et al., 1990) extended neurites which appeared completely devoid of MTs but contained MFs along their entire lengths as shown in Fig. 1. NFs in vinblastine-treated cells were also disrupted, being either entirely absent or confined to the cell body where they colocalized with the few MTs present in these cells. The distribution of mitochondria in the cytochalasin-treated vs. vinblastine-treated processes was strikingly different. Mitochondria, detected with R123 (Johnson et al., 1981), were distributed throughout MT-containing neurites grown in cytochalasin (Fig. 2) where they moved bidirectionally, often at rates faster than 1 μm/s. In contrast, in neurons grown in vinblastine, mitochondria were entirely confined to the cell bodies and did not even enter into the MF-containing neurites of these cells (Fig. 2).

Mitochondrial Behavior in Established Neuronal Cultures after MF and/or MT Depolymerization

The inability of mitochondria to move from the cell body into neurites that lacked MTs suggested that at least anterograde mitochondrial transport could not occur along MFs alone. In order to investigate whether mitochondria were capable of retrograde transport along MFs, we first grew neurons under normal conditions for 1 d, allowing them to produce long axons that contained MTs, MFs (Fig. 3), and NFs (Fig. 5) and exhibited abundant mitochondrial transport (Morris and Hollenbeck, 1993). We then treated the cultures with either cytochalasin E to disrupt MFs or vinblastine to depolymerize MTs, or both drugs to disrupt MFs and MTs simultaneously. As shown by R-phal staining, a 30-min exposure to 20 μg/ml cytochalasin E profoundly disrupted the normal actin cytoskeleton leaving only a few small and scattered foci of staining in the axons and cell bodies, while the MT staining remained unaffected (Fig. 3). Conversely, treating established neuronal cultures with 1 μM vinblastine for 60 min virtually eliminated MT immunofluorescent staining while the MFs remained intact (Fig. 3). Although a high vinblastine level was necessary in order to depolymerize the stabilized MTs in established cultures, the MTs disappeared in a consistent time-dependent pattern starting in the region of the growth cone and progressing back toward the cell body. After 60 min of vinblastine exposure, 82% of 11,410 μm of axon screened was devoid of anti-tubulin staining, and the remaining 18% where residual anti-tubulin staining was present was adjacent to the cell bodies within slightly thicker segments of axon. Whether MFs or MTs were disrupted individually, or both arrays were completely disrupted by simultaneous treatment with 20 μg/ml cytochalasin and 1 μM vinblastine, the NF array looked indistinguishable from control cells when assayed by indirect immunofluorescence (Fig. 5). We confirmed the absence of MTs in vinblastine-treated axons by thin section electron microscopy. While control axons displayed many MT profiles (mean ± SEM = 11.0 ± 1.1 per cross section, n = 44 axons) examination of both longitudinal and cross sections from hundreds of vinblastine-treated axons failed to reveal a single unambiguous MT profile (Fig. 4, *A* and *B*), except for a single MT in a single axon hillock. Quantitative morphometry of a large number of cross-sections confirmed the modest decrease in diameter of vinblastine-treated cells (Fig. 4, *C* and *D*) that had been observed at the light microscope level, and demonstrated that despite the elimination of MTs, the vinblastine-treated axons retained the same number of neurofilaments as control cells (Fig. 4, *E* and *F*). Nonetheless, to ensure that the observations of mitochondrial behavior described below were made in regions completely devoid of MTs, all observations were made after ≥75 min of drug treatment and were made far from the cell body and in regions where axons were of uniform diameter.

Mitochondria exhibited movement in both the anterograde and retrograde directions in control, cytochalasin-treated, and vinblastine-treated cultures, but with dramatically different patterns of motility (Figs. 6 and 7). As previously shown (Morris and Hollenbeck, 1993), mitochondria in control cells spent the majority of their time stationary with some mitochondria exhibiting bidirectional transport, often at velocities greater than 1 μm/s. Similarly, in the cytochalasin-treated cultures, many mitochondria remained stationary while others moved bidirectionally at fast axonal transport rates of 1 μm/s or faster. In contrast, while mitochondria in vinblastine-treated cells

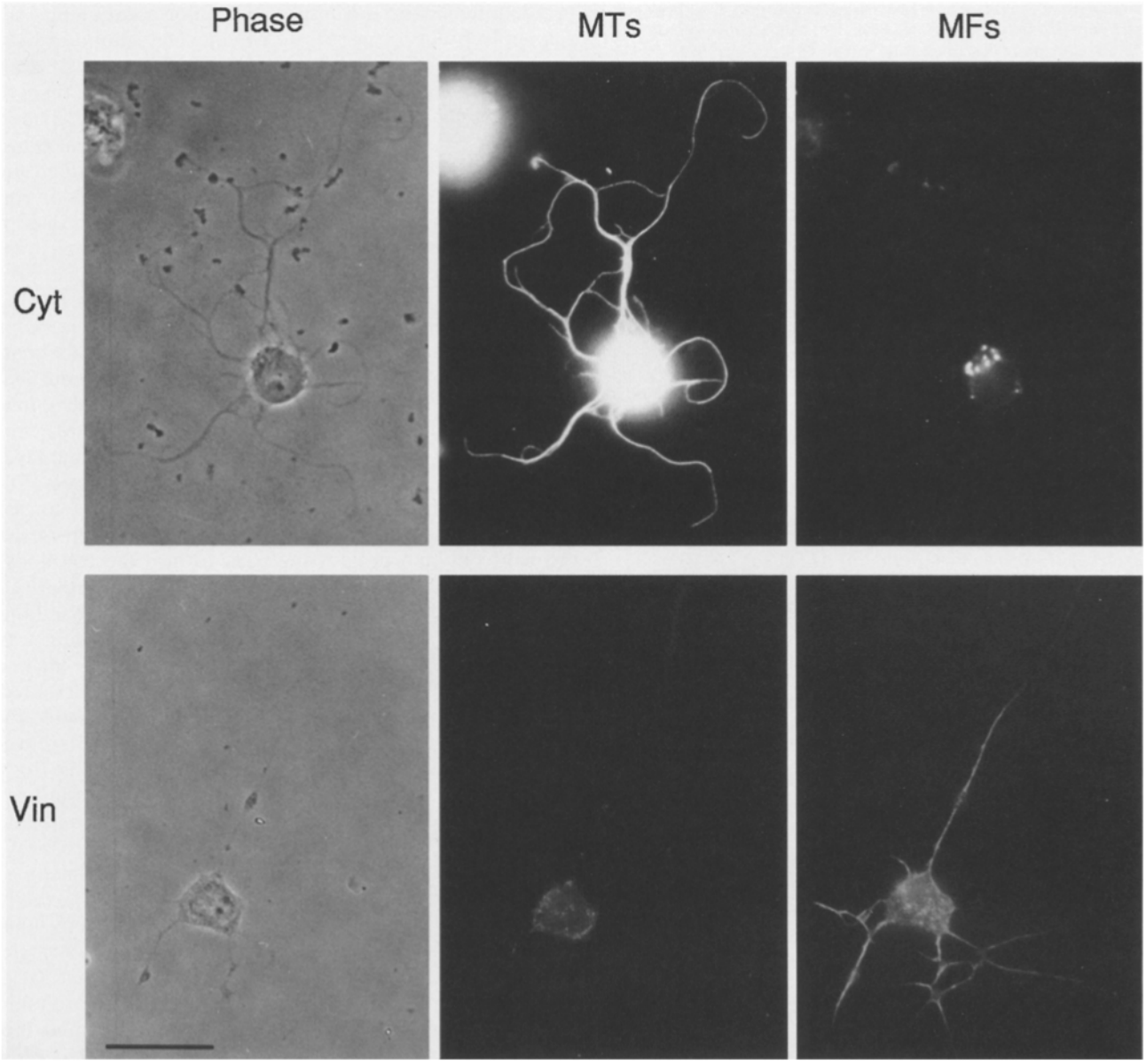

Figure 1. Neurons grown in the absence of MTs or the absence of MFs. Phase contrast micrographs (*Phase*), immunofluorescent detection of MTs, and R-phal detection of MFs in sympathetic neurons that were treated with drugs from the time of plating reveal the state of their cytoskeleton. (*Top*) Cells grown in the continuous presence of 20 μg/ml cytochalasin E for 3 d (*Cyt*) have a normal MT density, but lack a coherent MF array. (*Bottom*) Cells grown in the continuous presence of 100 nM vinblastine for 1 d (*Vin*) lack MTs but have an essentially normal MF density. The latter result was also obtained by growth in the continuous presence of nocodazole for 1 d (not shown). Bar, 20 μm.

also clearly moved bidirectionally, the movements were consistently slower than in control or cytochalasin-treated cultures. Finally, in cultures treated with both cytochalasin and vinblastine simultaneously, causing both MFs and MTs to be disrupted while NFs remained intact (Fig. 5), all axonal mitochondria remained completely stationary during the entire observation period (56 mitochondria/min). In these cells, video-enhanced phase contrast microscopy revealed that some organelle movement continued within the cell body (data not shown), where residual MTs remained (Fig. 3). To further analyze these differences in transport velocities, mitochondrial movements were quantified in detail for each drug treatment.

A comparison of the distribution of velocities which occur in control, cytochalasin-treated, and vinblastine-treated cells (Fig. 7) confirmed that in normal growing axons, mitochondria spent a large fraction of their movement time at velocities ≤ 0.3 μm/s yet continued to display a broad range of fast movements ≥ 0.3 μm/s with nearly equal frequencies. The velocities of mitochondria in cytochalasin-treated axons were substantially different from controls: they moved with similar frequencies over a broad range of velocities with 70% of their movement time spent at ≥ 0.3 μm/s. As a result, their mean velocity in both directions was nearly double that in control axons. In contrast, moving mitochondria in vinblastine-treated axons spent most

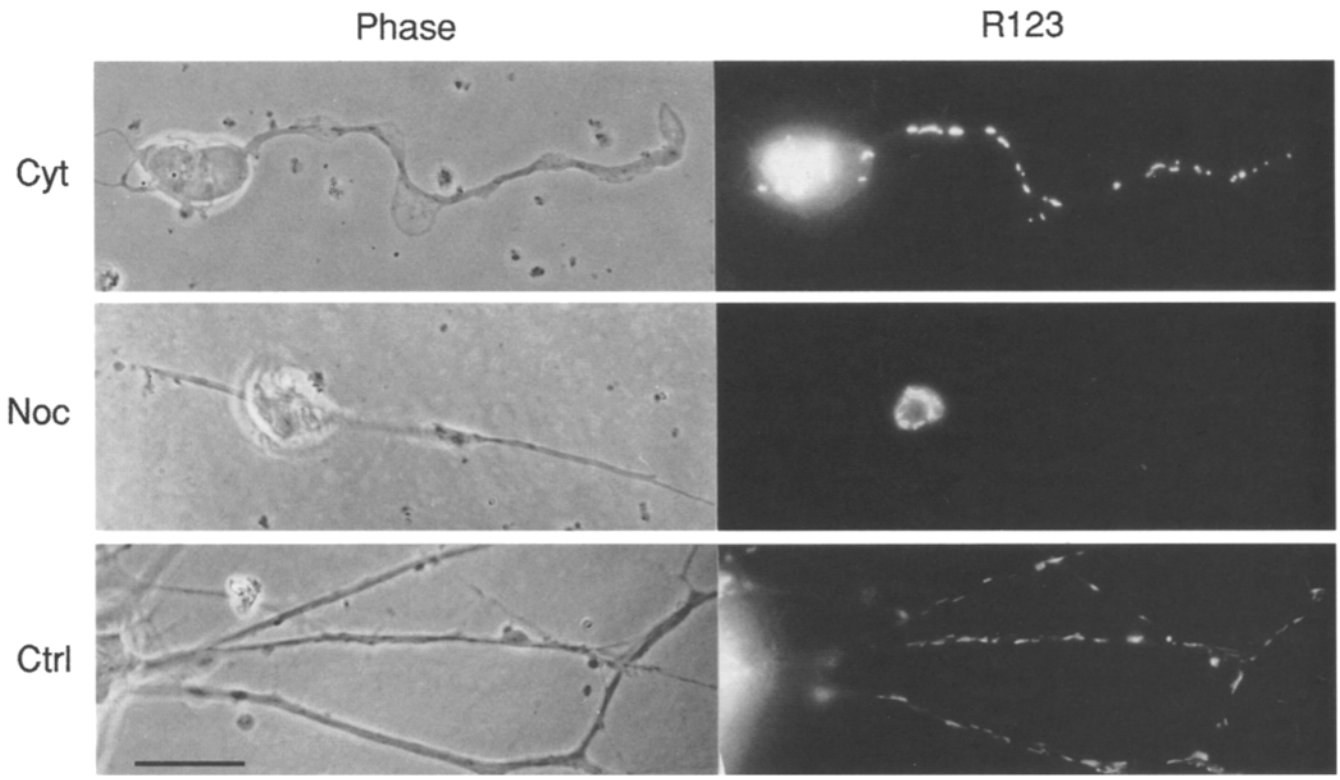

Figure 2. Mitochondrial distribution in live neurons grown in the absence of MFs or MTs. Neurons were grown in drugs from the time of plating, and phase contrast micrographs (*Phase*) and epifluorescent images of mitochondria stained with R123 in living neurons (*R123*) were compared to determine the distribution of mitochondria. (*Top*) In cells grown in the continuous presence of cytochalasin E for 3 d (*Cyt*), mitochondria have entered the neurite and become distributed along its length. (*Middle*) In cells grown in the continuous presence of nocodazole for 1 d (*Noc*), mitochondria have failed to enter the neurite and are restricted to the cell body. (*Bottom*) Cells grown in the absence of drugs (*Ctrl*) show the typical distribution of motile mitochondria. Bar, 20 μm.

of their time (average 79%) at velocities <0.3 μm/s (similar to controls) but displayed very few faster movements. When mean velocities of mitochondrial movement were calculated from pooled anterograde and pooled retrograde data for each drug treatment, the mean velocity in control cells fell between the faster mean velocities in cytochalasin-treated cells and the slower mean velocities in vinblastine-treated cells (Table I).

To ascertain why mitochondria were distributed throughout neurites grown continuously in cytochalasin yet absent from neurites grown continuously in vinblastine (Fig. 2), anterograde and retrograde mitochondrial movements in cytochalasin-treated and vinblastine-treated cultures were used to calculate net transport rates for mitochondria in each drug condition (Table I). As found previously (Morris and Hollenbeck, 1993), the bidirectional movements of mitochondria in control growing cells produced a net transport rate for the entire population of 1.1 μm/min in the anterograde direction, and thus the mitochondria advanced within the elongating control axons. After cytochalasin treatment, even though mean mitochondrial velocities in both directions increased, the net transport rate was slowed to one eighth of the control rate while remaining net anterograde. In contrast, treatment with vinblastine, while reducing the mean velocities in both directions, shifted the balance between anterograde and retrograde movements so as to produce rapid net retrograde transport (Table I). This retrograde mitochondrial flow could, over time, produce axons entirely drained of mitochondria (Fig. 2), and did, within the duration of vinblastine-treatment of these established cultures, produce long regions of distal neurite devoid of mitochondria (data not shown).

Analysis of mitochondrial motile duty cycles (percent of time spent moving or stationary) revealed how the net transport rates were being modulated (Table I). The population of mitochondria in control cells underwent net anterograde movement because, although they spent most of their time stationary, more of their movement time was spent moving anterograde than retrograde. The net transport of mitochondria in cytochalasin-treated cells was reduced from the rate in control cells by both a small increase in time spent stationary, and by a reduction of greater than one third in the anterograde duty cycle, from 11% of time in control cells to 7% in cytochalasin-treated cells, while the retrograde duty cycle remained almost unchanged. It is notable that this is similar to the way axonal mitochondria shift their net transport from anterograde to retrograde when axonal growth is blocked by reducing their anterograde duty cycle without altering their retrograde duty cycle (Morris and Hollenbeck, 1993).

Yet this is markedly different from the way net transport direction was reversed to retrograde in vinblastine-treated cells. There, mitochondrial net transport was reversed not only by a reduction in the anterograde duty cycle, but also

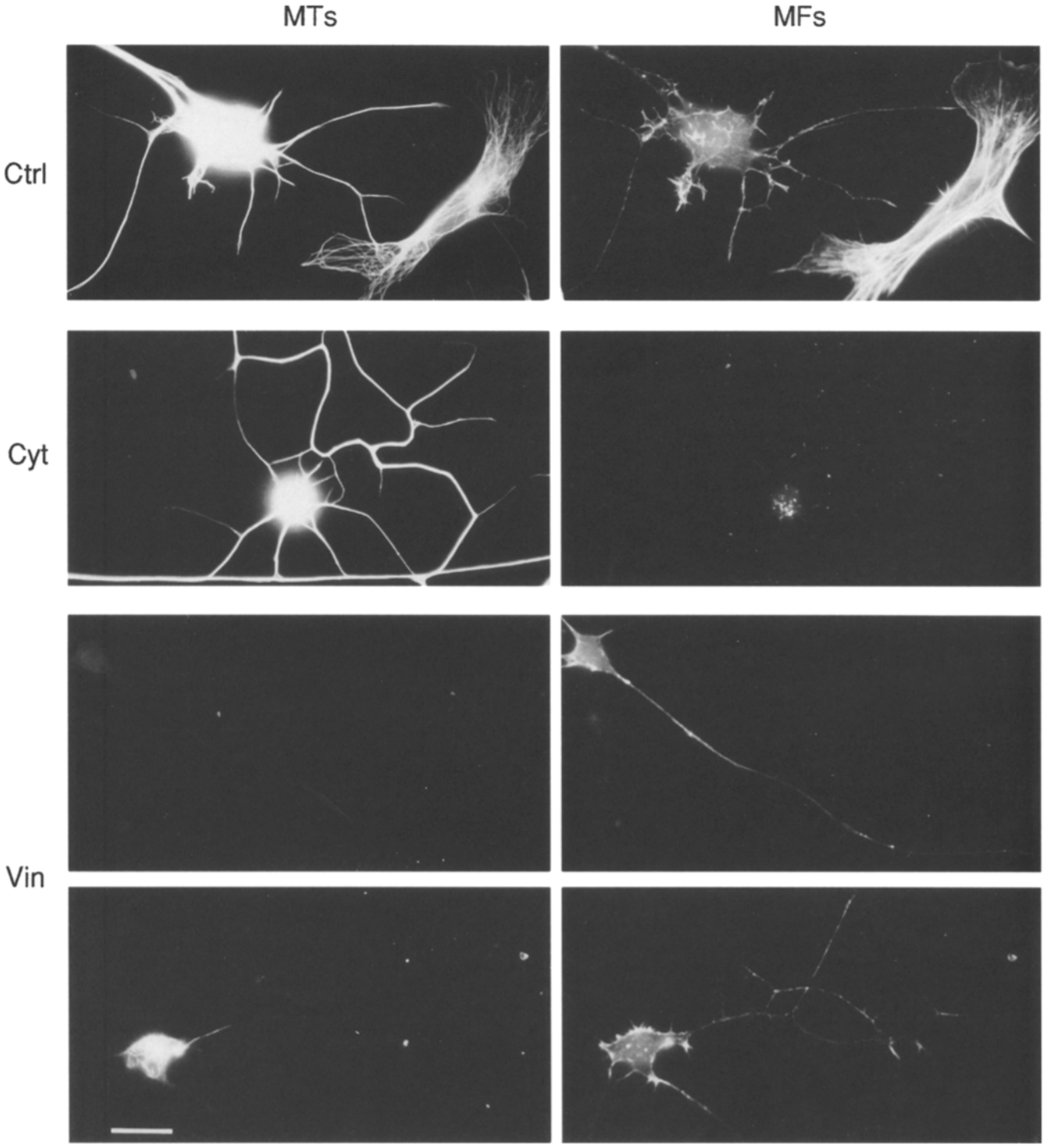

Figure 3. MFs and MTs in established cultures after cytochalasin or vinblastine treatment. In neurons grown under normal conditions for 1 d and then incubated with or without drugs for 1 h prior to fixation, immunofluorescent detection of MTs and R-phal detection of MFs reveals the state of the cytoskeleton. (*Top*) Cells fixed without drug treatment (*Ctrl*) have robust MT and MF arrays. (*Second row*) Cells treated with cytochalasin E (*Cyt*) retain dense MTs but lack MFs. (*Third* and *fourth rows*) Cells treated with vinblastine (*Vin*) vary from retaining virtually no MTs to retaining MTs in the cell body and a short proximal segment of the neurite, while their MF arrays appear essentially identical to controls. Bar, 20 μm.

by a threefold increase in the retrograde duty cycle, from 7% of time in control cells to 22% of time after vinblastine treatment. This is the first circumstance we have found in which the retrograde duty cycle was modulated to change the net rate or net direction of mitochondrial transport. In addition, mitochondria in vinblastine-treated cells spent a larger fraction of their time moving, totaling 29% of time observed, compared to both control cells (20% of ob-

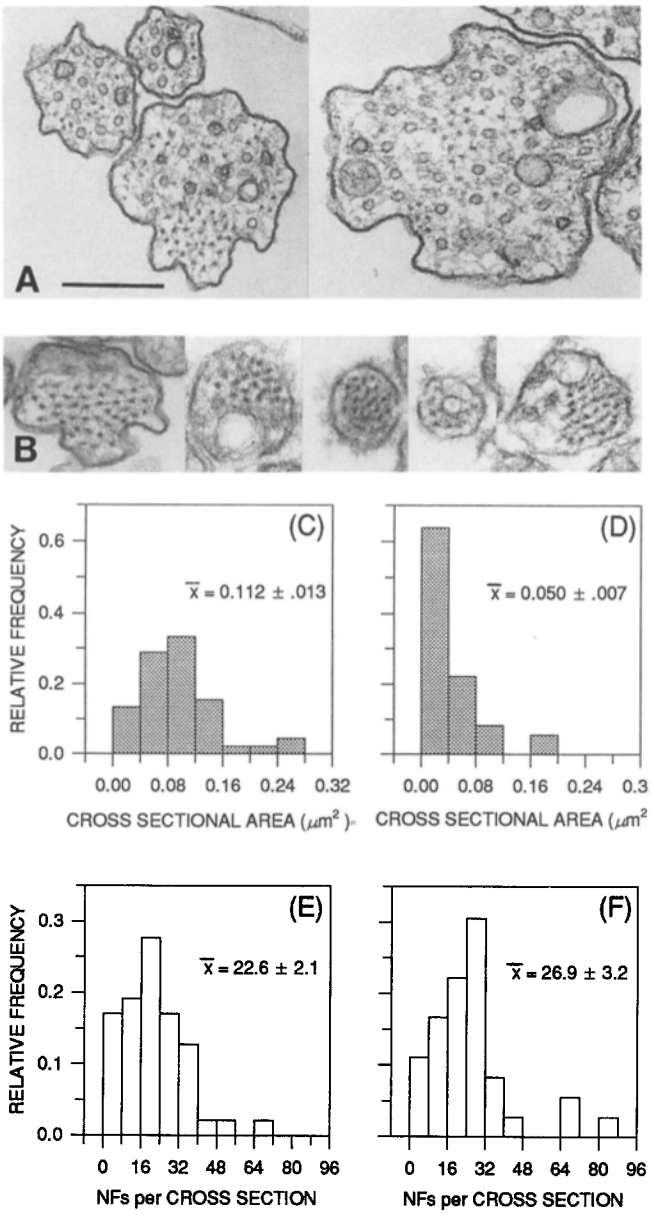

Figure 4. Ultrastructural confirmation of the elimination of MTs but lack of general cytoskeletal disruption in vinblastine-treated axons. (*A*) Four typical axonal cross-sections from control cultures show abundant round microtubule profiles interspersed with dot-like neurofilament cross sections and membranous organelles. (*B*) Five typical axonal cross sections from vinblastine-treated cultures reveal the absence of MTs but the presence of numerous neurofilament profiles, along with membranous organelles. Quantitative morphometry (*C–F*) shows that while the cross sectional area of vinblastine-treated axons (*D*) is less than that of control axons (*C*) ($t_{[1],n-1} < .005$), the number of neurofilaments per cross section is not significantly different ($t_{[2],n-1} > 0.2$) between control axons containing MTs (*E*) and vinblastine-treated axons lacking MTs (*F*). The mean of each distribution ±SEM is shown within each histogram. For (*C*) and (*E*), $n = 44$ axons; for (*D*) and (*F*), $n = 33$ axons. Bar: (*A* and *B*) 200 nm.

served time), and cytochalasin-treated cells (13% of observed time). Thus, although instantaneous rates of motility were slower in vinblastine-treated cells, overall mitochondrial movement was much more persistent in its direction.

A final feature distinguishing mitochondrial motility in control and vinblastine-treated cultures was the length of mitochondrial excursions between stops. First, the mean excursion length decreased significantly in vinblastine-treated vs. control cultures, from 1.3 ± 0.20 μm ($n = 299$) in controls to 0.37 ± 0.03 μm ($n = 243$) in vinblastine. In addition, the maximum excursion length, which is more likely to reflect the length of the track for movement in highly saltatory organelles such as mitochondria, decreased by an even greater degree under vinblastine treatment (Table 1).

Discussion

Mitochondria Move along Both MTs and F-Actin But Not along NFs in Neuronal Processes

In this study, we have demonstrated that mitochondria can move bidirectionally along both MTs and MFs in vertebrate axons by manipulating the cytoskeletons of neuronal processes and monitoring mitochondrial movements within the affected neurites. First, we used well-characterized techniques to grow neurites either lacking MFs or lacking MTs. Neurites grown in the continuous presence of cytochalasin (Marsh and Letourneau, 1984) were curled and devoid of MFs, yet they contained MTs (Fig. 1) and mitochondria (Fig. 2) along their entire lengths. Individual mitochondria in these neurites moved bidirectionally at velocities up to 2 μm/s. In contrast, growing neurites on adhesive substrata in the continuous presence of vinblastine or nocodazole produced straight, thin neurites which contained MFs along their lengths but which contained neither MTs (Lamoureux et al., 1990; Fig. 1) nor mitochondria (Fig. 2). The latter result suggested that MTs were necessary and sufficient for the transport of mitochondria in axons. Although the net direction of mitochondrial transport can be affected by neuronal process growth (Morris and Hollenbeck, 1993), it seemed unlikely that mitochondria were failing to enter the MT-deficient neurites because of the slowed axonal growth produced by vinblastine or nocodazole, since cytochalasin also slowed growth, but did not prevent anterograde mitochondrial movements. It seemed more likely that mitochondrial motility on MFs in neurites was either absent or intrinsically net retrograde.

To test this hypothesis, we used a second approach to perturbing the cytoskeleton: depolymerizing the MTs or MFs in established neuronal cultures in which the axons already contained normal cytoskeletal elements and large numbers of mitochondria. Using culture conditions in which we could depolymerize virtually all MFs as detected by R-phal or all MTs as detected by immunofluorescence protocols (Osborn et al., 1978; Fig. 3) and by electron microscopy (Fig. 4), we monitored the mitochondria for movement on the remaining cytoskeletal filament system. We found that bidirectional mitochondrial transport continued in both cytochalasin-treated cells which lacked a coherent MF array but contained normal MT and NF arrays, and in vinblastine-treated cultures which lacked MTs but contained normal MF and NF arrays. Yet, when both MFs and MTs were depolymerized by simultaneous cytochalasin and vinblastine treatments, leaving only an intact

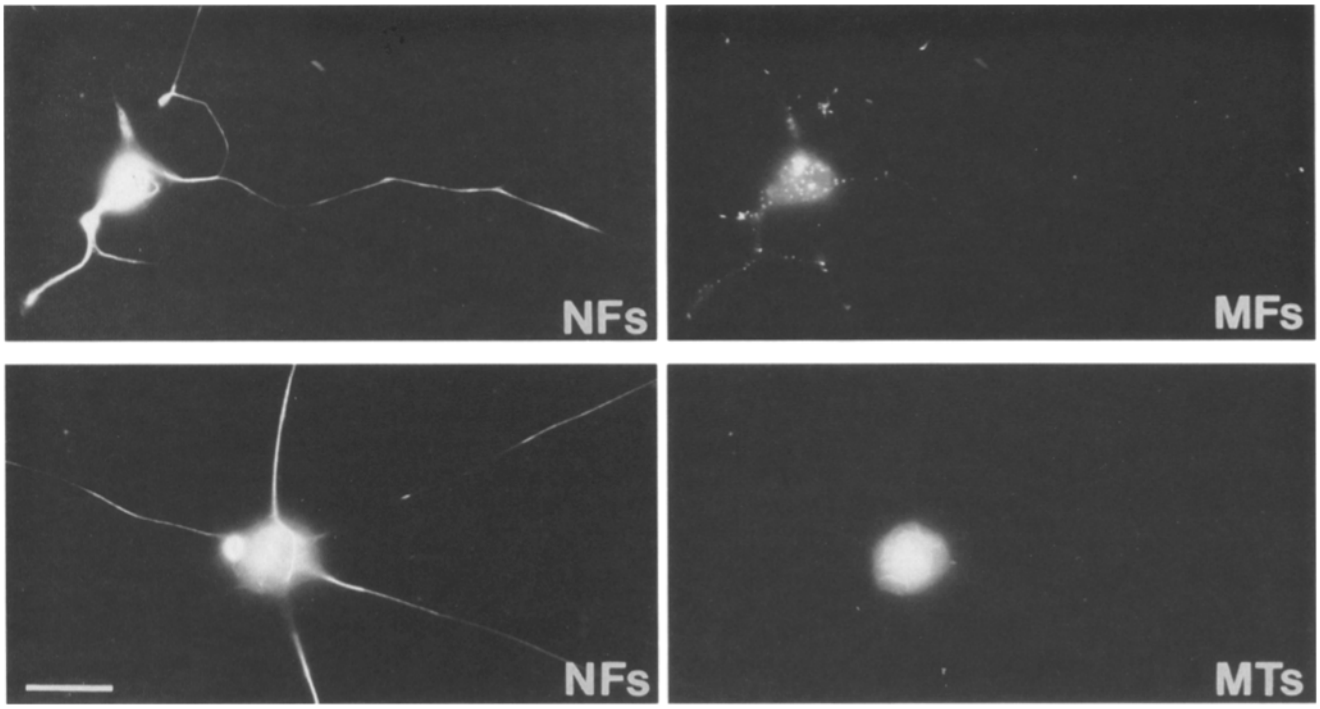

Figure 5. NFs, MFs, and MTs in established cultures after simultaneous cytochalasin and vinblastine treatment. Cytoskeletal elements are revealed by double staining with antibody against NFs and R-phal detection of MFs (*top cell*) or double immunofluorescent detection of NFs and MTs (*bottom cell*) in cultures grown under normal conditions for 1 d then incubated in vinblastine for 45 min followed by vinblastine plus cytochalasin for another 45 min prior to fixation and staining. In these cultures both MTs and MFs are depolymerized, while NF arrays remain extensive and essentially indistinguishable from controls (not shown). Bar, 20 μm.

NF skeleton, mitochondrial transport ceased completely. Thus, either MTs or MFs are sufficient for transport of mitochondria in embryonic chick sympathetic axons. In contrast, because the discontinuity of NFs in neurites grown in cytochalasin did not preclude mitochondrial transport there (Fig. 2), and because the presence of NFs in combined cytochalasin- and vinblastine-treated cultures did not support mitochondrial movements, NFs are neither necessary nor sufficient to support axonal transport of mitochondria. These results demonstrate that axonal transport of organelles can occur along actin filaments in intact vertebrate cells, consistent with results obtained in vitro (Kuznetsov et al., 1992; Langford et al., 1994; Kuznetsov et al., 1994). Indeed, Kuznetsov et al. (1992) described organelles that appeared to be mitochondria that were seen to move along both MTs and MFs in vitro.

Mitochondria Display Dramatically Different Patterns of Behavior on MTs and MFs

Detailed quantification showed that mitochondrial behaviors on the two cytoskeletal tracks were strikingly different in four ways (Fig. 7, Table I). First, the mean velocities of mitochondrial movements on MTs alone were nearly three times greater than those on MFs alone (Table I), consistent with the in vitro observations of Kuznetsov and coworkers (1992). Second, although mitochondria had slower mean velocities on MFs than on MTs, differences in motile duty cycle produced an 11-fold higher net transport rate on MFs than on MTs (Table I). On MFs, mitochondria tripled their retrograde duty cycle and reduced their anterograde duty cycle relative to control cells, while on MTs, they only reduced their anterograde duty cycle. As a result, mitochondria spent more than twice as much of their time moving when on MFs alone as when on MTs alone. Third, mitochondrial net transport on MFs alone was retrograde (reversed from the anterograde net transport exhibited on MTs alone or in control cells) (Morris and Hollenbeck, 1993; Table I). Fourth, the maximum excursion lengths of mitochondria in control cells were much greater than those on MFs alone (Table I), consistent with the demonstrated lengths of axonal MTs (Bray and Bunge, 1981) and MFs (Bearer, E. L., and T. S. Reese. 1994. *Mol. Biol. Cell.* 5:415a.).

The efficient net retrograde transport of mitochondria along MFs seems likely to explain their failure to enter neurites which contain only MFs (Fig. 2). The data from established, vinblastine-, or nocodazole-treated neurons indicate that the mitochondria are in fact capable of anterograde movement on MFs, but because they spend the vast majority of their time moving retrogradely, they do not achieve anterograde translocation into the MF-containing neurites. This observation of retrograde mitochondrial net transport on axonal MFs has interesting implications for the molecular components underlying MF-based axonal transport. First, if MFs in axons have, like MTs, a uniform polarity orientation, then the presence of pointed-end–directed MF-dependent motor proteins would be necessary to produce the bidirectional movements seen in MT-deficient neurites (Fig. 6, Table I). But since no pointed end-directed MF-dependent motor proteins have yet been identified in any system (Cheney et al., 1993*b*), we favor

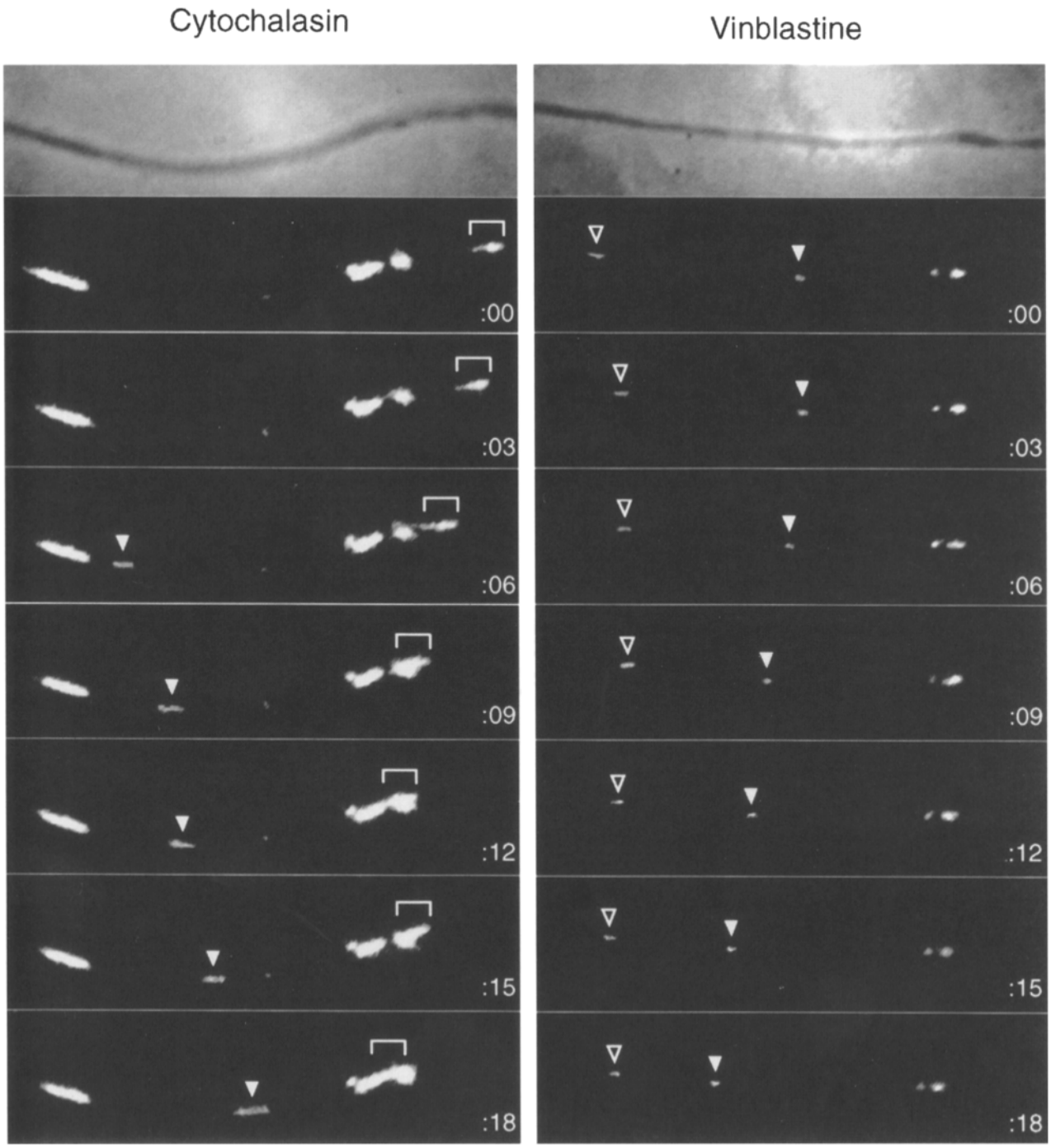

Figure 6. Examples of bidirectional mitochondrial movements in established cultures after cytoskeletal disruptions. With the growth cones out of view to the right, these series of typical low-light level video images show R123-labeled fluorescent mitochondria moving in axons of 1-d-old cultures treated with cytochalasin E for 1 h (*left*) to disrupt MFs, or with vinblastine for 2 h 15 min (*right*) to disrupt MTs. Images in each series show the same 25-μm segment of axon at 3 s intervals (as indicated in the lower right of each panel); the top panel in each is a phase contrast image of the axon taken just before the epifluorescent sequence. In *vinblastine*-treated cultures (*Vinblastine*), one mitochondrion (*open arrowhead*) moves anterograde (0–3 s), is stationary (3–9 s), moves retrograde (9–15 s), and then back anterograde (15–18 s); while another mitochondrion (*closed arrowhead*) is briefly stationary (0–3 s) before moving rapidly retrograde (3–18 s); and two other mitochondria (*right*) move steadily and slowly retrograde. In *cytochalasin*-treated cultures (*Cytochalasin*), one mitochondrion (*bracket*) moves rapidly retrograde (0–9 s), slowly retrograde (9–12 s), briefly anterograde (12–15 s), then retrograde (15–18 s); while another mitochondrion (*arrowhead*) enters the field at 6 s and moves rapidly anterograde.

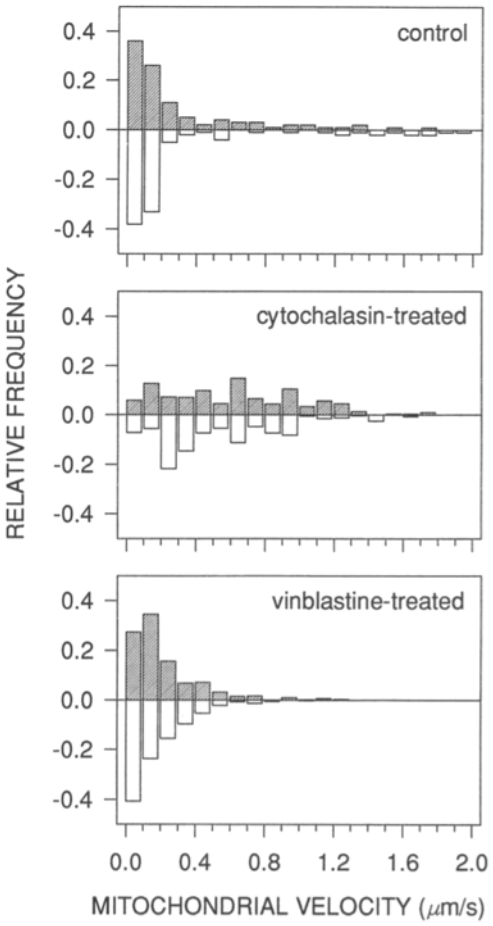

Figure 7. Relative frequency of occurrence of different mitochondrial velocities in established cultures. Velocities were determined from the distance and duration of individual mitochondrial movements. Relative frequencies of movement at each velocity were calculated by pooling all velocity values for each direction in each drug condition, summing the time spent moving at each velocity in one direction and dividing by the total time spent moving at any velocity in that direction. Anterograde velocities are designated as positive (*cross-hatched bars*) and retrograde velocities as negative (*white bars*). Data are derived from analysis of 72 mitochondria from 3 cells observed for 5,419 s in control cells, 16 mitochondria from 5 cells observed for 1,248 s in cytochalasin-treated cells, and 16 mitochondria from 6 cells observed for 1,604 s in vinblastine-treated cells.

Table I. Motile Behavior of Mitochondria in Drug-treated Established Axons

	Duty cycle	Mean velocity	Net velocity	Maximum excursion length
	%	μm/s	μm/min	μm
Control				
Anterograde	12	0.32	1.13	25
Retrograde	7	0.38	—	15
Stationary	81	—	—	—
Cytochalasin-treated				
Anterograde	7	0.62	0.14	6.7
Retrograde	6	0.54	—	42
Stationary	87	—	—	—
Vinblastine-treated				
Anterograde	7	0.23	—	1.1
Retrograde	22	0.18	1.52	5.3
Stationary	71	—	—	—

Velocities in the anterograde direction are designated as positive and in the retrograde direction as negative. Individual mitochondrial movements were quantified and pooled for all cells in each drug condition, and duty cycle (percent of time) was calculated from these pooled data. Individual distances moved in the anterograde direction were summed within these pools and divided by total time mitochondria spent moving anterograde to determine mean velocity in the anterograde direction, and the same calculations were performed for retrograde movements. Net velocity was determined by summing single measurements of every mitochondrion's net displacement over an entire observation period and dividing this by the duration of those single observation periods. Duty cycles were calculated from the same data used to generate velocity distribution histograms in Fig. 5. Data for net velocity were derived from analysis of 63 mitochondria from 3 cells observed for 4,434 s in control cells, 16 mitochondria from 5 cells observed for 1,401 s in cytochalasin-treated cells, and 16 mitochondria from 6 cells observed for 1,597 s in vinblastine-treated cells. Maximum excursion lengths were derived from analysis of 299 individual excursions for control cells, 146 for cytochalasin-treated cells, and 343 for vinblastine-treated cells.

the possibility that the 3:1 retrograde to anterograde duty cycle of mitochondria on MFs reflects a mixed orientation of MFs in axons such that the majority of plus- or barbed-ends of actin filaments are oriented toward the cell body with minus- or pointed-ends oriented outward toward the growth cone. It is possible that there may be subdomains of the axonal MFs which have uniform polarity, such as the cortical actin underlying the axolemma, while more centrally-located actin may have mixed polarity. Although it seems unlikely, we cannot rule out the possibility that axons in which the MT array is eliminated may develop different arrangements of MFs than control cells, rendering MF-based transport different there than in normal axons. However, the retention of normal neurofilament densities in vinblastine-treated axons (Fig. 4) argues against a gross reorganization of the cytoskeleton under MT-depolymerizing conditions.

Roles of Actin-based Movement in Axonal Transport

One interpretation of these data would be to imagine axonal organelle transport as a weighted mean of the MT- and MF-based behaviors shown in Fig. 7, with MF-based transport serving as a separate carrier for organelles and contributing significantly in its own right to the bidirectional flow of material within the axon. Yet in myriad studies, MT-based transport alone has been demonstrated both in vitro and in vivo to be an effective system for the transport of membrane-bound organelles within axons. In addition, due to the predominantly retrograde movements of mitochondria on MFs, it seems unlikely that this form of transport contributes significantly to net anterograde movement. Indeed, the question arises of why another transport system should be required at all in neurons. We think it likely that movements along MFs provide a localized transport system within axons to either cluster and localize organelles in one region or to move organelles which have become dissociated from MTs back onto MTs for continued transport over long distances in either direction. This would improve the efficiency of axonal transport in regions of the cell where MTs are few or absent (such as axonal branch points and growth cones) or could return organelles back to MTs after having become dissociated along a MT's length or at the end of an MT. Since single MTs do not extend for the entire length of longer axons (Bray and Bunge, 1981), and since the capacity of organelles to diffuse within cytoplasm is extremely limited

(Hou et al., 1990; Provance et al., 1993), providing a local mechanism for organelle motility could significantly improve the efficiency of transport by reducing the dwell times of organelles in these transport "dead zones".

Taken together, this body of data suggests that within vertebrate axons in vivo, mitochondria in particular, and perhaps organelles in general, utilize MT- and MF-dependent motor proteins to move along both MTs and MFs. Consistent with this, immunoblotting of 100-fold purified mitochondrial fractions from brain revealed reactivity with both MT- and MF-based motor proteins (data not shown). Furthermore, the intrinsic and unique transport properties of the two cytoskeletal filament systems may be balanced to produce a coordinated net transport of organelles which meets the cell's regional and physiological needs. It should be possible, by manipulating the intracellular signalling pathways within neurons with modified cytoskeletons, to elucidate the mechanisms by which MT- and MF-based organelle transport are regulated and coordinately used within the cell.

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