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Actin in Emerging Neurites Is Recruited from a Monomer Pool

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Contents

Abstract

Does actin in the emerging axons of regenerating neurons arise from the assembled or unassembled actin pool in the cell soma? We investigated this question by loading neurons with one of two fluorescently labeled molecules: rhodamine actin (r-actin) and rhodamine phalloidin (r-phalloidin). The assembly behavior of r-actin in vitro was identical to unlabeled actin. R-phalloidin binds tightly only to the filamentous form of actin (F-actin) and stabilizes filaments against disassembly. Hence, r-phalloidin-tagged filaments should be less likely to disassemble than r-actin-tagged filaments. Neurons of 10-d-old chick embryos were loaded with r-actin or r-phalloidin by triturating trypsinized dorsal root ganglia in isotonic sucrose containing the fluorescently tagged molecule. Isolated neurons were plated on glass coverslips in modified L15 medium containing nerve growth factor. Video images of the live cells on a thermoregulated stage were acquired with a computer imaging system.

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After 24 h in culture, the fluorescence distribution of r-phalloidin and r-actin was examined in live neurons of comparable morphology, neurite outgrowth, and intensity of somal fluorescence. Greater than 90% of the neurons labeled with r-actin ($n = 81$) contained detectable levels of fluorescence in emerging neurite fibers, often extending to the tip of the growing process. Less than 10% of the neurons labeled with r-phalloidin ($n = 53$) contained any fluorescence in the neurite fibers. In those that did contain fluorescence, the r-phalloidin usually was confined to the proximal segment of the neurite, and in no case was it found at the growing tip. Confocal microscopy and cooled CCD imaging of fixed neurons showed that all structures that incorporated r-actin or r-phalloidin also stained with bodipy phallacidin. This colocalization confirms the association of rhodamine-tagged species with F-actin. Our data support a model in which actin, needed in early stages of neurite outgrowth, arises from a pool in the soma that is capable of disassembly.

Index Entries: Actin; actin transport; dorsal root ganglia; neurite development; neuronal cytoskeleton; axonal transport; rhodamine actin; rhodamine phalloidin.

Introduction

The question of how cytoskeletal proteins are transported from their site of synthesis in the soma to very distant nerve terminals has attracted much attention over many years (Vallee and Bloom, 1991; Mitchison and Kirschner, 1988). We are focusing on one aspect of this transport problem. In what form is actin recruited from the soma when neurites first emerge? Two possibilities appear most likely: (1) either preexistent somal filaments are moved into the neurites, or (2) neurite formation depends upon disassembly of somal actin and its reassembly at new sites.

One method of both fluorescently labeling and stabilizing F-actin in cells is the introduction of fluorescently tagged phalloidin. Phalloidin is a cyclic heptapeptide, from the mushroom *Amanita phalloides*, that binds with high affinity to only the filamentous form of actin (Wieland and Faulstich, 1978; Cooper, 1987; Faulstich et al., 1988). Some fluorescent derivatives of phalloidin bind with even higher affinity to F-actin (Faulstich et al., 1983). At high concentration, phalloidin induces actin assembly (Wieland and Faulstich, 1978). At lower concentrations, it merely binds to preexisting F-actin and inhibits disassembly normally induced by a wide variety of agents (Wulf et al., 1979; Bernstein and Bamburg, 1989), including actin depolymerizing factor (ADF; Bamburg et al., 1980; Giuliano et al., 1988; Giuliano and Bamburg, unpublished result). It binds with 1:1 stoichiometry to F-actin subunits, stabilizing the filament. The effectiveness of the stabilization is a function of the percentage of subunits with bound phalloidin: a 0.2 molar ratio of phalloidin to actin confers >80% protection against disassembly by KI (Dancker et al., 1975). Phalloidin appears to reduce the dissociation rate constant for monomers in equilibrium with polymers (Coluccio and Tilney, 1984) by inhibiting the release of inorganic phosphate from hydrolyzed ATP that is bound to the actin subunits (Dancker and Hess, 1990). Phalloidin is not membrane permeable and can be introduced by either membrane permeabilization or mechanical loading (microinjection, scrape loading, and so on).

Low levels of microinjected phalloidin, covalently bound to the rhodamine fluorophor, were used to analyze actin filament translocation in Swiss 3T3 cells (Wang, 1987). Previous studies using Chinese hamster ovary cells showed large changes in the ratio of G-/F-actin in cells undergoing substratum attachment (Heacock and Bamburg, 1983a) and small changes in this ratio in synchronous cells undergoing cytokinesis (Heacock and Bamburg, 1983b). These results suggest that lamellipodia formation involves actin assembly, but contractile ring formation does not. Experiments with r-phalloidin confirmed these findings: formation of actin structures in lamellipodia required actin polymerization (Wang, 1985; Wehland and Weber, 1980), whereas actin filaments in the

contractile ring of dividing cells appear to be generated by translocation of preexisting filaments (Wang, 1987; Cao and Wang, 1990).

Mitchison and coworkers have microinjected r-actin in NIH-3T3 cells, fixed them, and stained them with fluorescein-phalloidin to show that actin is initially incorporated in the lamellipodial tips and then moved toward the base of the lamellipodia. Inhibition of r-actin incorporation by incubation of permeabilized cells with an actin-capping protein led to the conclusion that (+) end capping proteins partially regulate sites of assembly (Symons and Mitchison, 1991). Theriot and Mitchison (1991) used a photoactivatable form of fluorescently tagged actin to observe a remarkably rapid rate of turnover in epithelial keratocytes, a cell type more rapidly motile than fibroblasts. Keratocytes lack the focal adhesion contacts typical of fibroblasts and do not demonstrate the type of actin treadmilling observed at the leading edge of fibroblasts (Wang, 1985). An activated spot of F-actin remains fixed with respect to the substratum, whereas rapid assembly in the leading edge accomplishes forward movement of the keratocyte.

Okabe and Hirokawa (1990) reported that actin polymers in axons are not static structures, transported toward the growth cone, but rather dynamic structures that undergo assembly/disassembly along the entire length of the axon. Additionally, it is known from pulse-chase experiments that actin rapidly exchanges between soluble and sedimentable forms in neuronal soma (Clark et al., 1983). However, the question of how actin is moved into newly emerging neurites remains. Are preexisting filaments recruited from the soma as occurs in the case of contractile ring formation, or are monomers assembled from the soma as occurs in extending lamellipodia? To address this question, we have used r-phalloidin to stabilize and fluorescently tag actin filaments in the soma of neurons at the time the neurons are dissociated. We have then compared the transport of stabilized actin to the transport of fluorescently modified actin, still capable of undergoing normal assembly and disassembly.

Materials and Methods

Materials

Rhodamine X maleimide, bodipy phallacidin, and rhodamine phalloidin were obtained from Molecular Probes (Eugene, OR). Bovine pancreatic DNase I, trypsin, L15 culture medium, glucose, glutamine, penicillin/streptomycin/amphotericin, and dithiothreitol (DTT) were obtained from Sigma Chemicals (St. Louis, MO). Calf thymus DNA was obtained from Worthington Biochemical Corp. (Freehold, NJ). Methocel E4M Premium was obtained from Dow Chemical Co. (Midland, MI). Actin was purified from rabbit muscle, according to the method of Spudich and Watt (1971). Actin, in G-actin (mono-meric actin) buffer (2 m*M* Tris, pH 8.0, 0.2 m*M* $CaCl_2$) containing 0.5 m*M* ATP, was quick-frozen in liquid N_2 and stored at –70°C. The cloned cDNA, encoding actin depolymerizing factor (ADF), was expressed in *E. coli*, and ADF was purified from that source (Adams et al., 1990). Nerve growth factor (NGF) was purified from the submaxillary glands of adult male Swiss Webster mice (Varon et al., 1967).

Labeling of Actin and Biochemical Characterization of Labeled Actin

Actin was labeled with rhodamine X maleimide by a modification of the method of Detmers et al. (1981). Rhodamine X maleimide was chosen for this purpose for two reasons: (1) the fluorophor has unusual sensitivity and stability, and (2) the assembly properties of actin, modified on cys 374 with sulfhydryl reagents, have been well characterized (Cooper et al., 1983). F-actin (3 mg/mL) was incubated at 4°C for 18 h in F-actin stabilizing buffer (10 m*M* imidazole, pH 7.0, 0.1*M* KCl, 4 m*M* $MgCl_2$, 3 m*M* NaN_3) containing 0.3 m*M* rhodamine X maleimide and 0.2 m*M* DTT. Labeling was terminated by dilution into 1 m*M* DTT in G-actin buffer. Unreacted rhodamine X maleimide was removed by repeated dilution into

G-actin buffer and concentration of the labeled protein on Centricon 10 filters (10 kDa cutoff; Amicon, Danvers, MA). From the molar extinction coefficient of rhodamine (E_{568nm} = 82,000M^{-1} cm^{-1}; Haugland, 1989) and G-actin (E_{290nm} = 26,600M^{-1} cm^{-1}; Houk and Ue, 1974), the molar ratio of dye to protein was determined to be 0.78 and 0.95 in the two preparations used in these experiments. The ratio of 290 nm/568 nm absorbance for rhodamine was 0.24. The A_{290nm} of rhodamine-actin (r-actin) was corrected for rhodamine contribution following the procedure of Cooper et al. (1983) by subtracting (0.24)A_{568nm}. Rhodamine-G-actin (100-µL aliquots in G-actin buffer containing 0.5 m*M* ATP) was frozen in liquid N_2.

The self-assembly properties of r-actin were compared to unlabeled actin by monitoring the change in A_{232nm} following addition of KCl and $MgCl_2$ to 100 and 2 m*M*, respectively (Higashi and Oosawa, 1965). The morphologies of these actin filaments were compared by electron microscopy of negatively stained samples (Huxley, 1963). The DNase I inhibition assay (Blikstad et al., 1978; Harris et al., 1982) was used to compare the interaction of bacterially expressed ADF (Bamburg et al., 1991) with polymerized r-actin and polymerized unlabeled actin.

Preparation and Culture of Neurons Loaded with R-Actin and R-Phalloidin

Fluorescently labeled actin and phalloidin were loaded by trituration into dorsal root ganglion neurons by a modification of the method described by Borasio et al. (1989). Dorsal root ganglia from 10-d-old chick embryos were treated with trypsin in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution. The dorsal root ganglia were washed free of trypsin by resuspending them three times in 1 mL Dulbecco's Modified Essential Medium plus 10% heat-inactivated horse serum. Control ganglia were resuspended and triturated in 50 µL 0.32*M* sucrose. Experimental ganglia were triturated in 50 µL 0.32*M* sucrose, containing either 100 µg (48 µ*M*) monomeric r-actin or 24 U of r-phalloidin. Trituration with several lesser or greater concentrations of r-phalloidin resulted in either fluorescence or neuronal survival being unacceptably low. An isotonic sucrose solution was used to maintain actin in its unassembled state. Cells were washed free of unincorporated fluorescent molecules by three repetitive resuspension/centrifugation steps in 1 mL Hank's balanced salt solution. Immediately following trituration and washing, the dissociated neurons were plated on glass as described by Bray (1991).

Microscopy

Images of live neurons on a thermoregulated stage of a Nikon Diaphot microscope were acquired with a 100×, 1.4-na, oil immersion, Plan Apo Nikon objective, a 0.85-na condenser, a Quantex intensified CCD camera (for fluorescence), an MTI 70 series dage camera (for phase), and a Quantex QX-7 computer imaging system using QFM software (Quantex, Sunnyvale, CA). Each fluorescent image represents an average of 90 frames ($30s^{-1}$) from which a background image has been subtracted. Comparison of the first and third images of a single cell so acquired showed no photobleaching. Images were printed on a Sony 5000 video printer.

Cultures of dorsal root ganglia dissociated neurons that were loaded with either r-actin or r-phalloidin and grown for 24 h on glass coverslips were then fixed with 0.25% glutaraldehyde in phosphate buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS containing 10 mg/mL $NaBH_4$, and stained with 1/40 dilution of bodipy phallacidin (final concentration of 7.5 U/mL) in PBS. After coverslips were washed with PBS and deionized water, they were mounted on glass slides in the presence of the photoprotectant, DABCO (Sigma Chemical Co.). A Molecular Dynamics (Sunnyvale, CA) Sarastro confocal laser scanning microscope was used to detect dual fluorescence emission from neurons stained with bodipy phallacidin, and loaded with either r-actin or r-phalloidin. Samples were excited at 488 nm. The emission was split by a 565-nm

Table 1
Percentage of Neurons with Detectable Fluorescence in Neurites[a]

	Most intensely stained soma		Moderately stained soma		Elongated soma		Soma with incipient neurites	
	RA	RP	RA	RP	RA	RP	RA	RP
(+)	8	0	25	0	15	4	23	0
(+/–)	0	0	2	3	0	2	1	0
(–)	2	8	2	22	2	14	1	9
%	80	0	86	0	88	20	92	0

[a]Neurons of comparable morphology, comparable axonal outgrowth, and comparable levels of intensity in fluorescence staining have been compared. "+" Indicates detectable fluorescence in a process, "–" absence of fluorescence, and "+/–" borderline case, as demonstrated in Figs. 1 and 2. RA = r-actin; RP = r-phalloidin.

dichroic mirror before being filtered by a 530-nm narrow bandpass filter for bodipy detection and a 600-nm cutoff filter for rhodamine detection. The confocal microscope was equipped with the same 100× objective used with live cell cultures. Projections, constructed on Silicon Graphics computer from the optical sections so acquired, were photographed on the monitor with T-Max 100 film. The same Nikon objective was used to study these samples with a Photometrics cooled CCD camera. Images so acquired were printed on a Sony 5000 video printer.

Results

Distribution of R-Actin and R-Phalloidin in Differentiating Neurons

To determine whether the stabilization of microfilaments by r-phalloidin affects the recruitment of actin from the soma to emerging neurites, we examined, after 1 d in culture, the fluorescence in neurons loaded with r-actin or r-phalloidin. We compared the fluorescence in neurites of similar maturity that had emerged from morphologically similar somas with comparable fluorescent intensity. The data of Table 1 and Fig. 1 show that no neurites in the earliest stage of outgrowth from soma loaded with r-phalloidin had detectable fluorescence, but 23 out of 25 emerging neurites from r-actin-loaded soma had detectable fluorescence. Likewise, more mature neurites of spherically and elliptically shaped neurons with high levels and moderate levels of soma fluorescence showed far greater probability of fluorescence in their neurites if the neurons were loaded with r-actin than if they were loaded with r-phalloidin (Fig. 2; Table 1). During the first few hours in culture, both r-actin- and r-phalloidin-loaded neurons had discrete, small regions of intense fluorescence that appeared gradually during the first day to spread over the soma (Fig. 3f).

To address the question of whether r-actin and r-phalloidin, loaded (separately) in live neurons, associate with F-actin structures in the cell body, we first loaded neurons with these reagents. The loaded neurons were then cultured for 1 d, and displayed outgrowth similar to those of Figs. 1 and 2. They were then fixed and stained with bodipy phallacidin for study with both confocal microscopy and cooled CCD imaging. Optical sectioning and CCD imaging, with dual detection of bodipy and rhodamine in fixed cells, showed all structures, which incorporated r-actin or r-phalloidin, also stained with bodipy phallacidin (Fig. 3). The punctate appearance of r-actin in the neurites, seen in living cells (Figs. 1a and 2a,c), is also apparent in fixed cells (Fig. 3h) and corresponds precisely with bodipy phallacidin staining in these neurites

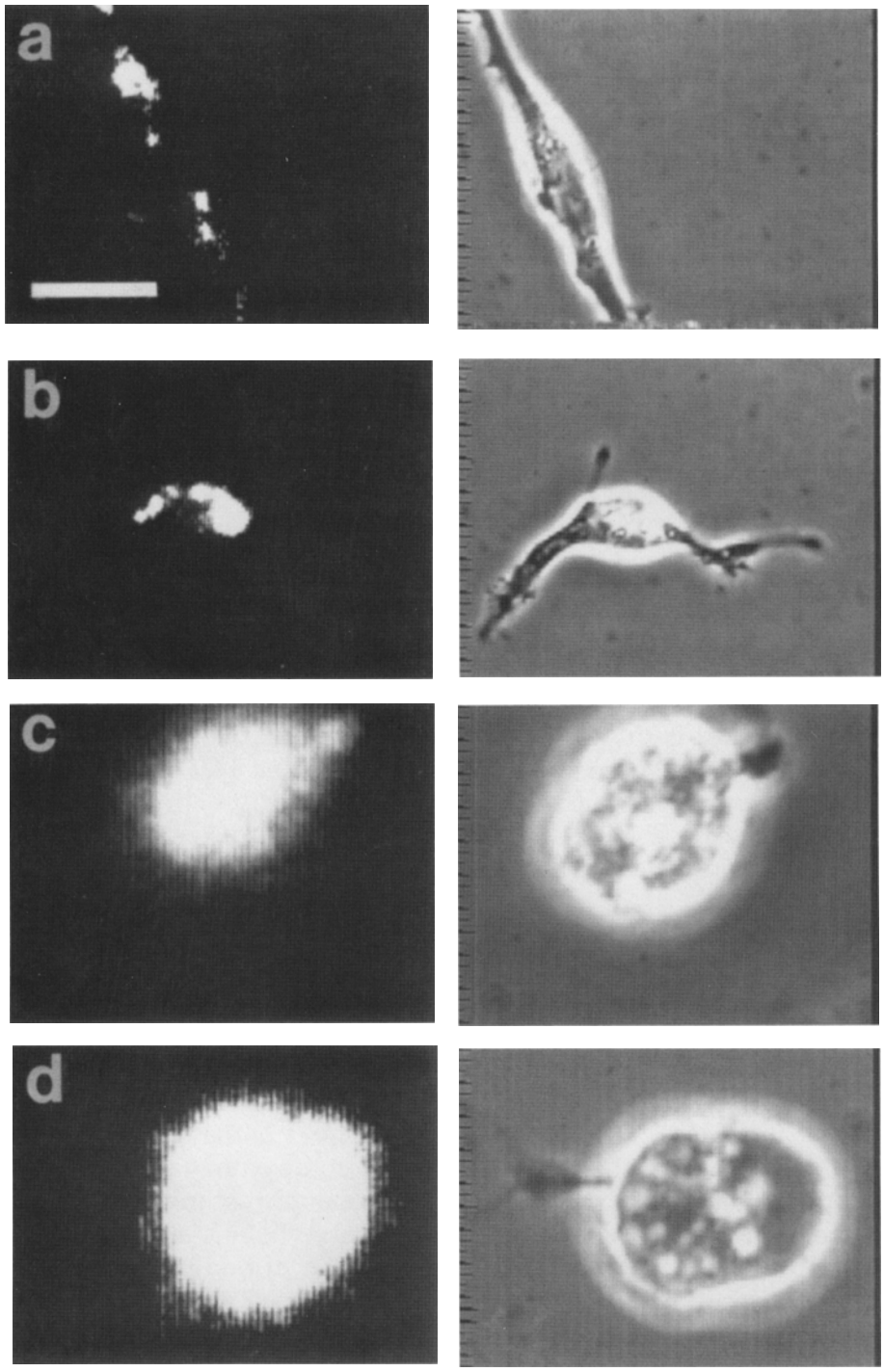

Fig. 1. Stabilization of actin filaments inhibits the transport of actin into neurites extending from neurons of different morphologies. Dissociated dorsal root ganglia neurons were loaded by trituration with either r-actin (a,c) or r-phalloidin (b,d). This figure shows neurons with elongated soma (a,b) or with incipient neurites (c,d). Micrographs are pairs of fluorescence (left) and corresponding phase images (right). Bar = 10 μm.

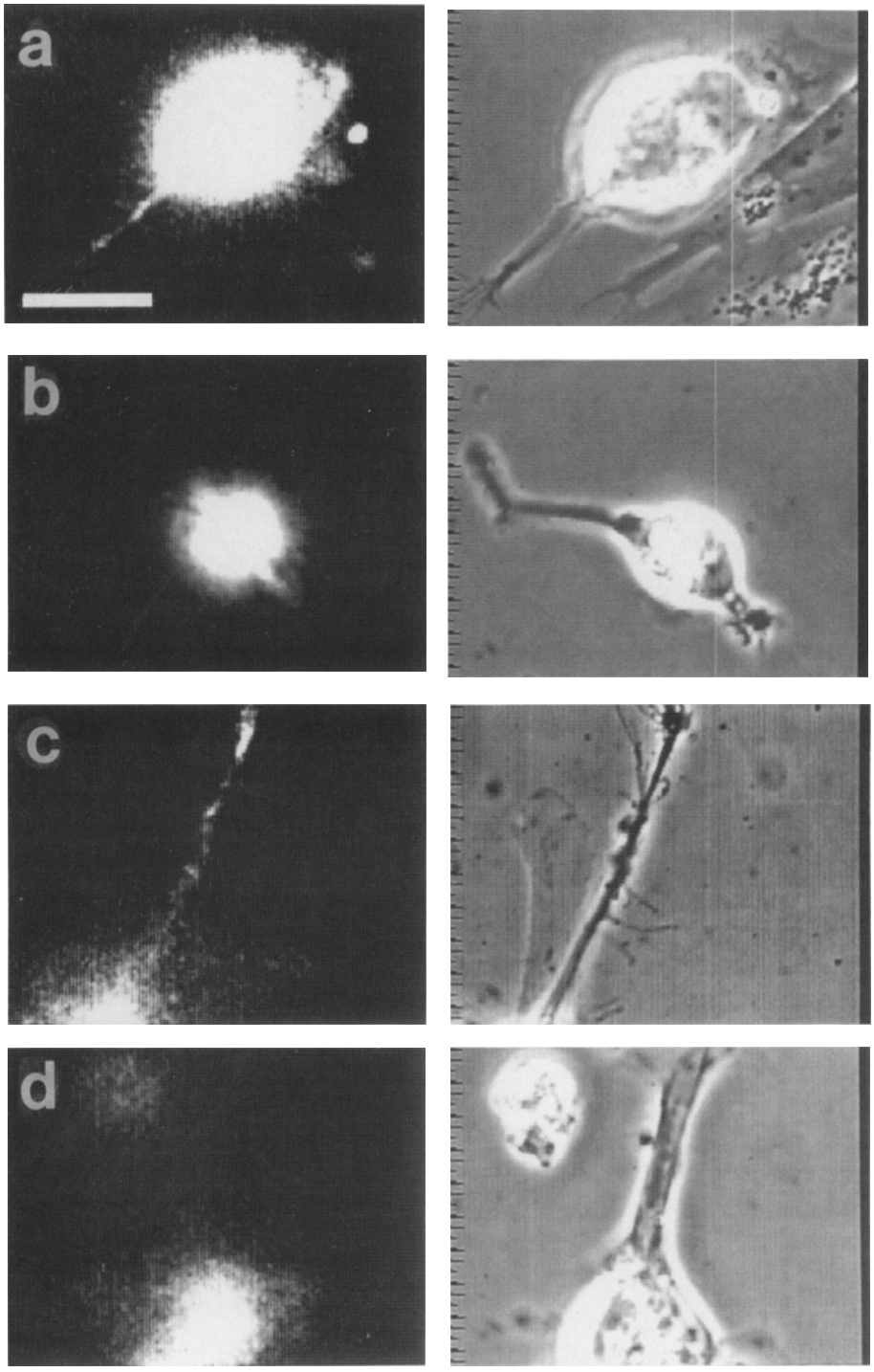

Fig. 2. Stabilization of actin filaments inhibits the transport of actin into neurites extending from neurons containing different amounts of fluorescent label. Dissociated dorsal root ganglia neurons were loaded by trituration with either r-actin (a,c) or r-phalloidin (b,d). Neurons in this figure are examples of the most intensely stained soma (a,b) and moderately stained soma (c,d). Micrographs are pairs of fluorescence (left) and corresponding phase images (right). Bar = 10 μm.

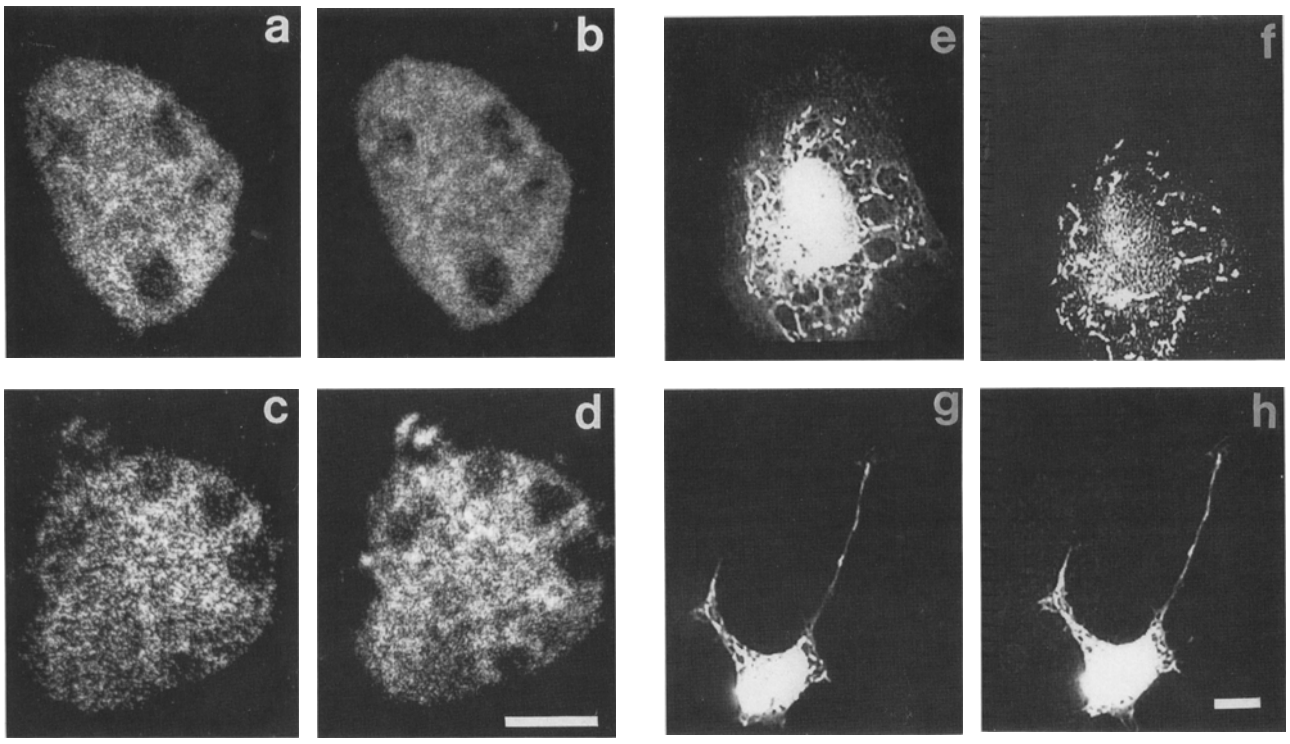

Fig. 3. R-actin and r-phalloidin, loaded into living neurons, co-localize with bodipy phallacidin fluorescence in fixed cells. F-actin was stained with bodipy phallacidin after fixation. Images (a–d) are the "see-through" projections of three sections taken with the confocal laser scanning microscope; the projections were computer constructed. Images (a) and (b) are of a neuron loaded with r-phalloidin; image (a) is bodipy phallacidin fluorescence, whereas image (b) is r-phalloidin fluorescence. Images (c) and (d) are of a neuron loaded with r-actin; image (c) is bodipy phallacidin fluorescence, whereas image (d) is r-actin fluorescence. Bar = 5 μm. Images (e–h) were acquired with a cooled CCD camera. Images (e) and (f) are of a neuron loaded with r-phalloidin, and extending a lamellipodium that encircles the soma and precedes neurite formation; (e) shows bodipy phallacidin fluorescence, whereas (f) shows r-phalloidin fluorescence. R-phalloidin fluorescence was not observed in the leading edges of the lamellipodia in this early stage of differentiation. Images (g) and (h) are of a neuron loaded with r-actin; image (f) shows bodipy phallacidin fluorescence, whereas image (h) shows r-actin fluorescence. Bar = 5 μm.

(Fig. 3g). Since bodipy phallacidin staining identifies F-actin, the overlap in rhodamine and bodipy signals indicates that (separately) loaded r-actin and r-phalloidin associated with cellular F-actin structures.

Assembly of R-Actin

Figure 4 shows the increase in 232-nm absorbance observed for labeled and unlabeled G-actin that occurred after salt addition. The absorbance of an identical aliquot of each actin species was constant over the same period of time when maintained in G-actin buffer, and these values have been subtracted from the assembly curves of the two actin species. Electron micrographs of negatively stained samples of labeled and unlabeled assembled actin showed identical, normal-looking microfilaments (data not shown). Derivatizing the actin with rhodamine had no apparent effect on the rate or extent of assembly.

Interaction of R-Actin with ADF

The DNase I inhibition assay is based on the fact that G-actin, but not F-actin, inhibits the enzymatic activity of DNase I (Blikstad et al., 1978). The assay has been adapted to measure actin depolymerization by regulatory proteins (Harris et al., 1982). Because G-actin complexed and

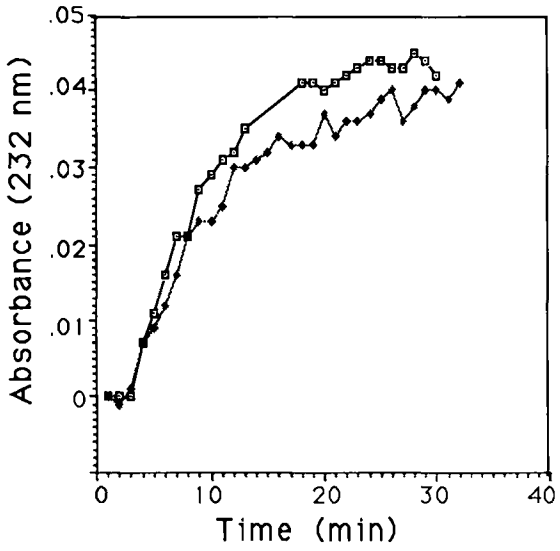

Fig. 4. Labeling of actin with rhodamine X maleimide does not affect assembly. Skeletal muscle was labeled to a molar ratio of rhodamine/actin of 0.8. Monomeric samples (10 μ*M*) were assembled by raising KCl to 100 m*M* and $MgCl_2$ to 2 m*M* at time zero. Starting absorbance values were set to zero before the initiation of assembly. Maximum absorbance did not exceed 2.0 absorbance units compared to a H_2O blank. The increase in absorption at 232 nm indicates assembly (Higashi and Oosawa, 1965). Unlabeled actin (⊡); r-actin (◆).

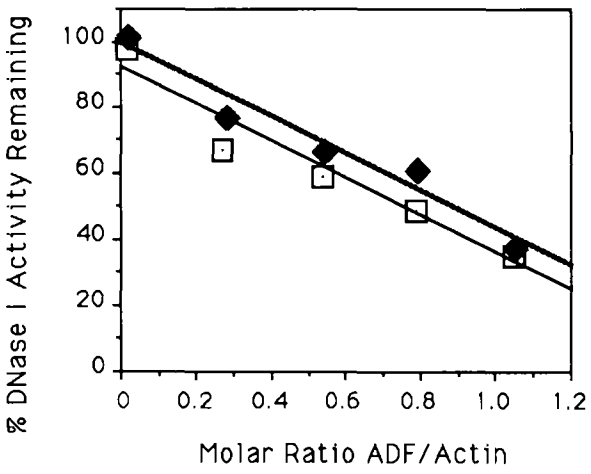

Fig. 5. Interactions of rhodamine-labeled and unlabeled F-actin with actin depolymerizing factor are indistinguishable. Each test sample in the DNase I assay contained 2.5 μg of F-actin (either rhodamine-labeled or unlabeled), 3 μg DNase I, an increasing amount of actin depolymerizing factor (ADF) in a constant total volume, and a constant amount of DNA. Each symbol represents the average of at least three assays. Standard deviations are less than symbol size. Unlabeled actin (⊡); r-actin (◆).

crosslinked with ADF inhibits DNase I as effectively as native G-actin (Daoud et al., 1988), the inhibition of DNase I by a mixture of ADF and F-actin can be used to determine quantitatively the extent of F-actin depolymerization induced by ADF. Figure 5 shows the increase in inhibition of DNase I observed when increasing concentrations of ADF were added to a fixed amount of either r-actin or unlabeled actin (Bamburg et al., 1991). Both preparations of rhodamine-modified actin showed an inhibition of DNase I similar to that of unmodified actin, indicating that ADF depolymerizes r-actin and unlabeled actin equally effectively.

Discussion

It is clear from the data presented that r-actin is far more likely to appear in the newly formed processes of regenerating neurites than is r-phalloidin. This observation supports the idea that filaments forming in growth cones and axons are derived from a dynamic actin pool, and not from the translocation of stable actin filaments. The neurites herein described were regenerating only in the sense that they emerged from cells that had probably differentiated before they were cultured. However, the neurites were not regenerating in the sense of merely extending growth from a stable axon existent before the introduction of fluorescently tagged species.

Interpretation of our results depends on: (1) the r-actin maintaining normal actin characteristics and exchange with endogenous actin, and (2) the r-phalloidin binding to F-actin and still allowing the F-actin to interact normally with the myriad of known actin-binding proteins in the neuron (Bamburg and Bernstein, 1991). We have demonstrated that our preparation of r-actin is capable of normal, salt-induced self-assembly and normal interaction with the only neuronal actin-binding protein we tested, ADF. Wang (1985) modified actin with another sulfhydryl

reactive reagent, iodoacetamidotetramethylrhodamine. That modified actin was incorporated into stress fibers of a fibroma cell (IMR-33), suggesting retention of normal interaction of the modified actin with actin-binding proteins of stress fibers. Phalloidin binding to actin does not inhibit cytoplasmic streaming in amoeba, which is actomyosin-based (Nothnagel et al., 1981), nor does it interfere with actomyosin interaction in vitro (Yanagida et al., 1984; Kron and Spudich, 1986). Interaction with tropomyosin, filamin, and α-actinin (Dancker et al., 1975; Wehland et al., 1980; Koteliansky et al., 1983) also appears unperturbed by phalloidin/actin binding. Furthermore, trace amounts of r-phalloidin can be used to study actin filament dynamics in live cells (Wang, 1987).

We think that during trituration, r-phalloidin, entering the cytoplasm of neurons through transient disruptions in the plasma membrane, binds rapidly to the first cortical F-actin it encounters. Because the phalloidin binding to F-actin is quite stable in living cells (Wang, 1987) and because initially the binding is probably at high molar ratio, the phalloidin most likely remains attached to those filaments and stabilizes them (Dancker et al., 1975). Within the first 3 h after plating, we observed prominent, fluorescent, bright spots in r-phalloidin-loaded (and r-actin-loaded) cells that diminished with time. At increasing distances from the site of phalloidin entry, one would expect the molar ratio of phalloidin to actin subunits to diminish and for the stabilization of F-actin to diminish accordingly. With greater time in culture, filaments with lower ratios of r-phalloidin/actin could at least partially disassemble (Dancker et al., 1975), and r-phalloidin, itself, given sufficient time, should eventually reequilibrate with F-actin throughout the cell. We think this explains why after longer periods of neuronal culture (1–3 d), r-phalloidin was more frequently observed in axons and terminals.

Our observations suggest that stabilization of filaments retards the transport of actin from the soma to the regenerating neurites. In essence, r-actin-loaded neurons show fluorescence in the most distal regions of neurites in early stages of regeneration, whereas r-phalloidin-loaded neurons do not. A possible, but we think not probable, source of the delay in appearance of phalloidin fluorescence in neurites could be owing to phalloidin entering the cells through endocytosis, somehow induced by the trituration, and then following an endosomal pathway. The data showing bodipy phallacidin-stained structures overlapping with entrapped r-actin and r-phalloidin fluorescence (confocal microscopy and cooled CCD imaging) support the idea that: (1) r-phalloidin binds to F-actin in the soma, and (2) transport of r-phalloidin into neurites is not delayed by r-phalloidin deposition into endoso-mal structures. Borasio et al. (1989) have shown that *H-ras* protein, similarly loaded into neurons of dorsal root ganglia by trituration, had the marked biological effect of inducing neurite outgrowth, suggesting that the exogenous protein was not degraded. Moreover, r-actin and r-phalloidin enter neurons only during trituration. In a control experiment, we added the fluorescent species after trituration and found no fluorescence in the plated neurons. The fluorescent species do not seem to be adsorbed to the cell surface and enter through endocytosis.

This recruitment of actin from the neuronal soma for formation of axonal filaments in emerging neurites resembles the delivery mechanism observed in the leading edge of lamellipodia (Wang, 1987; Theriot and Mitchison, 1991; Symons and Mitchison, 1991), rather than the translocation of actin filaments observed in the formation of the contractile ring (Cao and Wang, 1990). We and others have observed that a lamellipodial veil surrounds the entire neuronal soma before a growth cone and its associated neurite first form. As an early step in neurite formation, actin is apparently supplied to this veil (and to the leading edge of highly motile cells) in an unassembled form. The nature of the delivery system for this unassembled actin is unknown, but one likely candidate is actin depolymerizing factor (ADF), a major component of neuronal growth cones (Bamburg and Bray, 1987). ADF has

recently been shown to be transported along with actin in the slow component of axonal transport in chicken sciatic nerve (Bray et al., 1992).

Acknowledgments

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Note Added in Proof

Following submission of this manuscript, a paper by M. C. Sanders and Y.-L. Wang entitled "Assembly of actin-containing cortex occurs at distal regions of growing neurites in PC12 cells" appeared in *J. Cell Sci.* **100,** 771–780 (1991). The findings in this paper are consistent with those of our study.

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