

Analysis of the mechanism of fast axonal transport by intracellular injection of potentially inhibitory macromolecules: Evidence for a possible role of actin filaments

(actin polymerization/DNase I/serum factor/serotonergic neuron/*Aplysia*)

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ABSTRACT Although actin is thought to participate in several types of cell motility other than muscle contraction, no direct evidence has linked it to the force-generating mechanism for fast axonal transport. We have obtained evidence for the involvement of actin by microinjecting, into the serotonergic giant cerebral neuron of *Aplysia*, two preparations that have been shown to depolymerize actin filaments. One is a fraction of rabbit serum containing a heat-labile gamma globulin that affects actin polymerization in a manner similar to that of cytochalasin and several proteins that are thought to regulate the length of actin filaments. The other is bovine pancreatic DNase I which binds to actin stoichiometrically. Both preparations substantially decreased the transport of storage vesicles containing [³H]serotonin. Phalloidin, a toxic fungal peptide that binds to actin filaments but stabilizes rather than depolymerizes them, did not inhibit transport. We have not yet determined whether the inhibition of transport occurs during export of [³H]serotonin from the cell body into the axon or during translocation along the axon. Nevertheless, these observations provide a promising experimental indication that actin is involved in fast axonal transport.

The macroscopic properties of fast axonal transport have been extensively described (1, 2), but the subcellular structures involved in generating movement and the molecular details of their interaction are not understood. It seems reasonable to suspect that actin participates in the mechanism of transport because, besides its well-documented role in muscle contraction, actin has been implicated in several other types of non-muscle-cell motility (3, 4).

Progress has been slow toward obtaining reliable evidence that actin is involved in fast transport, however. Available drugs have not been selective enough to probe actin's role. Cytochalasin B, which was found to inhibit fast transport in some experiments (5) but not in others (6), interferes with cellular processes (4, 7) in addition to the polymerization of actin (8-10). Moreover, because complex nerves containing many small fibers traditionally have been used to study transport, pharmacological agents necessarily have been applied externally; thus, only drugs able to penetrate the cell membrane to a significant degree could be used.

We have been studying axonal transport of serotonergic vesicles in the giant cerebral neuron (GCN) in the central nervous system of the marine gastropod *Aplysia*. This neuron is large enough to allow injection of materials directly into the cell body (11-13). We describe here the use of two preparations that depolymerize actin filaments; both contain large proteins that would not be expected to penetrate into the cell. When injected into the GCN cell body, they block fast transport of

serotonergic storage vesicles. These experiments provide a promising indication for the involvement of actin in fast transport and establish the usefulness of approaching the mechanism of transport by delivering pharmacological agents directly into the cell.

Preliminary accounts of some of these experiments have been published (14, 15). While this manuscript was in preparation, it was reported that transport of labeled protein in the giant Retzius cell of the leech is inhibited after intrasomatic injection of DNase I (16).

MATERIALS AND METHODS

Animals and Intracellular Injections. *Aplysia californica* weighing 125-350 g were supplied by Pacific Biomarine Supply (Venice, CA) and Marine Specimens Unlimited (Pacific Palisades, CA) and kept at 15°C in aerated aquaria of Instant Ocean (Aquarium Systems, Eastlake, OH). Cerebral and buccal ganglia were removed from animals and the connective tissue sheath was partially dissected from the ganglion to facilitate impalement. The cell body of one member of the pair of GCNs was injected with the preparation to be tested by emptying the contents of a glass micropipette with the application of air pressure. We calibrated the volume injected by using an optical micrometer to measure the length of the fluid column within the micropipette. The relationship of column length to volume had been previously determined for the micropipette by using a solution of known concentration of radioactivity (13). After an appropriate interval of time, both cell bodies were injected with [³H]serotonin (11 Ci/mmol; Amersham/Searle, Arlington Heights, IL; 1 Ci = 3.7 × 10¹⁰ becquerels). Preparation of [³H]serotonin and the technique of intracellular injection have been described (17). Ganglia were bathed at room temperature (21-23°C) in an artificial sea water supplemented with amino acids and vitamins (17). The resting potentials of injected GCNs generally ranged from -50 to -65 mV; cells with resting potentials less than -42 mV were discarded.

As a measure of serotonin transport, we determined the partitioning of [³H]serotonin between ganglion and nerves. It has been shown that all of the radioactivity in the tissue is restricted to GCN (12). Three hours after injection of [³H]serotonin, the cerebrobuccal connective and posterior lip nerve, which contain the two major axonal branches of GCN, were cut at their points of attachment to either side of the cerebral ganglion. (The initial 1-2 mm segment of the GCN axon lies within the ganglion and thus is included with the cell body in this assay.) The ganglion was cut in half in cold 70% ethylene glycol (18) to separate the two cell bodies. Ganglion halves and

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Abbreviations: GCN, giant cerebral neuron; C1, first component of the complement system; ADF, actin-depolymerizing factor.

nerves were separately homogenized at 0°C in 0.5 ml of 0.2 M perchloric acid in order to extract the [³H]serotonin for assay by liquid scintillation counting.

Serum Proteins. Blood was collected from New Zealand White rabbits and allowed to clot. Pooled serum was supplied by Pocono Rabbit Farms (Canadensis, PA). Lyophilized guinea pig serum was obtained from Becton, Dickinson (Cockeysville, MD). The activity of human thrombin of greatest purity (Sigma) was measured by a standard clotting time assay (19). The first component of the complement system (C1) was obtained from Cordis (Miami, FL).

Fractionation with DEAE-Cellulose (20). Serum (20 ml) was applied to a column (60 cm × 2.5 cm) of Whatman DE-52 equilibrated at room temperature with 10 mM Na phosphate (pH 8.0). Eluate fractions (5 ml) with $A_{280} > 0.5$ were combined, and an equal volume of saturated (NH₄)₂SO₄ was added. After 16 hr, the protein precipitate was collected by centrifugation. Precipitation in 50% (NH₄)₂SO₄ was repeated, and the protein was dissolved in a minimal volume of water and dialyzed at 4°C against 0.15 M NaCl.

Gel Filtration. Samples (20 ml) of serum were fractionated on a column (5 cm × 90 cm) of Sephadex G-200 exactly as described (21) and concentrated by (NH₄)₂SO₄ precipitation.

Other materials. Bovine pancreatic DNase I (Schwarz/Mann, specific activity, 3000–4000 units/mg) was dissolved in 5 mM Na acetate (pH 5.0) at concentrations of 3–30 mg/ml. Nuclease activity was assayed at 25°C in 3 ml by the method of Kunitz (22). One unit of activity corresponds to a change of 0.001 A_{260} /min per ml. This specific activity is one-half to two-thirds of that reported for DNase of maximal purity (23, 24).

Rabbit actin was purified from skeletal muscle (25). *Aplysia* actin was purified from body wall muscle and used to immunize rabbits (ref. 26; unpublished data). Protein was determined by using Coomassie blue (27).

Phalloidin was obtained from Boehringer Mannheim and dissolved in 5% dimethyl sulfoxide to a concentration of 13 mM.

RESULTS

Inhibition of Fast Axonal Transport by a Gamma Globulin. Our original strategy was to inject anti-actin antibody as a specific intracellular probe to determine whether microfilaments participate in fast transport. In all experiments, we tested for inhibition of transport in one member of a pair of GCNs in the isolated nervous system and used the contralateral GCN as a control. Whole serum blocked fast transport, but we found no difference between sera from unimmunized rabbits and rabbits immunized with actin purified from *Aplysia* body wall muscle (Table 1). This result did not exclude antibody as the inhibitor of transport because it had recently been shown that sera from unimmunized animals contain anti-actin antibody (28). In an attempt to test whether the inhibitor was an anti-actin antibody, we fractionated serum on DEAE-cellulose by a standard procedure for preparing IgG (20). These preparations were found to inhibit fast transport at a lower protein concentration than did unfractionated serum. Nevertheless, the extent of the inhibition was somewhat variable. Although transport was almost completely inhibited in every experiment with some preparations, we found considerable variation between experiments with other less-potent preparations. Injections of large amounts of protein (as much as 85 ng per cell) did not affect the electrophysiological properties of GCN (resting membrane potential and antidromic spike amplitude).

The idea that the inhibitor of fast transport is an immune globulin directed against actin was abandoned when we de-

Table 1. Inhibition of fast axonal transport by a gamma globulin in rabbit serum

Preparation	Protein injected, ng	Inhibition*
Serum fractions from animals immunized with <i>Aplysia</i> actin		
Whole serum	85	64 ± 10 (4)
Fraction from DEAE column	38	95 ± 0.2 (3)
	43	87 ± 3 (6)
	45	50 ± 14 (3)
	17–46	49 ± 12 (4)
Serum fractions from unimmunized animals		
Whole serum	70	58 ± 9 (3)
Fraction from DEAE column	46	74 ± 1 (3)
	32	74 ± 4 (3)
	42	61 ± 10 (8)
	Heated DEAE fraction	
	24	−9 ± 12 (3) [†]
	42	25 ± 4 (4)
	Sephadex G-200 fractions	
ADF	18	83 ± 2 (6)
IgG	18	23 ± 8 (5)
	Serum proteins	
Albumin	40	10 ± 3 (3)
Thrombin	35 [‡]	14 (1)
C1	10 [§]	1 (1)

Proteins were injected into the cell body of one GCN 4 hr before injection of [³H]serotonin into the cell bodies of both GCNs. Transport was assayed 3 hr after the injection of [³H]serotonin. Inhibition, the difference between transport in the control cell and transport in the cell injected with protein, is expressed as a percentage of the value of the control. The data for different batches of the fraction from the DEAE column are listed individually.

* Mean (±SEM) % decrease in transport in the injected neuron. Number of experiments is in parentheses.

[†] The negative value indicates an increase in transport in the experimental cell.

[‡] Microunits.

[§] Milliunits.

veloped methods of assaying anti-actin antibody (26) and failed to show any correlation between titer of anti-actin antibody and activity in inhibiting fast transport. A further indication that an immune globulin is not involved was the observation that mild heat treatment (56°C for 0.5 hr) markedly decreased the inhibitory activity (Table 1).

Norberg *et al.* (21) partially purified a gamma globulin from animal sera that appears to depolymerize actin fibers catalytically or substoichiometrically; they named it actin-depolymerizing factor (ADF). We think that the inhibitor of fast transport may be ADF because the properties of the two are similar. In addition to being a gamma globulin, ADF is inactivated by mild heating. ADF was purified by gel filtration on Sephadex G-200 and was found to be a protein with a molecular size slightly greater than that of albumin. In order to provide further evidence that the inhibitor of transport is ADF, we tested the ADF-containing fractions prepared by gel filtration (21) and found them to inhibit transport of [³H]serotonin strongly (Table 1). The fractions containing IgG from the same column were much less active.

In order to confirm the presence in serum of a factor that depolymerizes actin, we developed a viscometric assay to determine the effect of the factor on the salt-induced polymerization of actin. We chose to assay polymerization because we have found it to be a more reliable and sensitive measure than

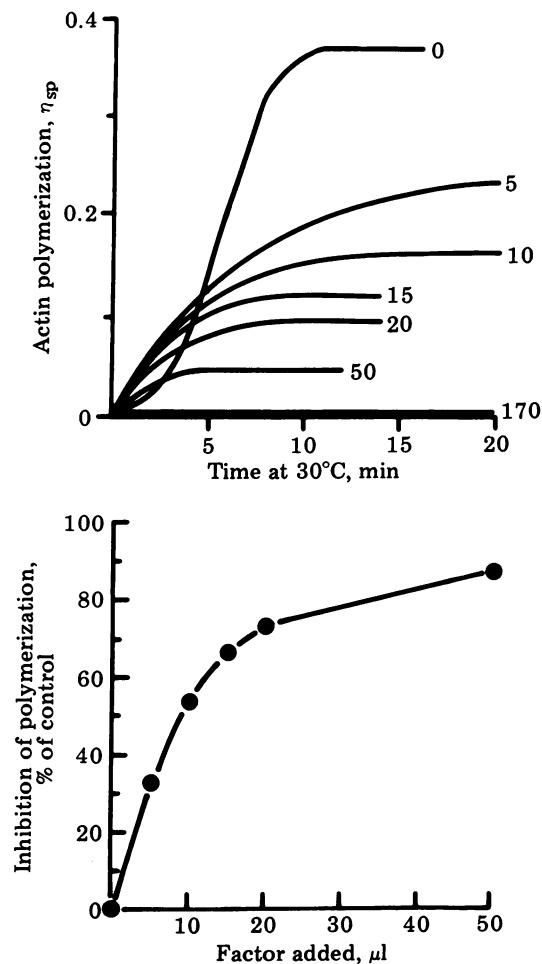


FIG. 1. Inhibition of actin polymerization by ADF. Polymerization was monitored in a Cannon-Manning type 100 semi-microviscometer at 30°C in 0.5 ml containing various amounts (in μ l) of ADF [23 mg/ml, purified as described (21)] in 5 mM CaCl_2 /5 mM ATP/0.1 M NaCl/0.6 mM Tris-HCl, pH 8.0. Polymerization was initiated by the addition of 0.43 mg of rabbit G-actin. (Upper) Viscometry readings were made every 2 min. Specific viscosity was calculated as (flow time at t /flow time at t_0) - 1. (Lower) The plateau value of viscosity attained after 20 min is expressed as a percentage of the viscosity reached in the absence of ADF (curve marked 0 in Upper). When added in amounts greater than 50 μ l, ADF completely suppressed polymerization. After heating at 60°C for 15 min, 170 μ l of ADF reduced the plateau value of polymerization only 16%.

depolymerization. The inhibitor of fast transport profoundly reduced the final extent of polymerization, although it increased initial rate and shortened the normal lag period (Fig. 1). The plateau viscosity depended upon the amount of inhibitor added; polymerization could be totally prevented by the addition of a sufficiently large amount. These kinetic features of the inhibition are also characteristic of polymerization in the presence of β -actinin (29-31) and the cytochalasins (8). Inhibition of polymerization was abolished by mild heat treatment.

Because ADF has not yet been completely purified, the inhibitor of fast transport might be another serum protein. Two candidates, thrombin and C1, have been eliminated (Table 1). Thrombin is a gamma globulin and a protease that cleaves actin (32) as well as fibrin. The ADF purified by gel filtration was assayed and shown to have thrombin activity. We then tested the same amount of pure thrombin and found it to be ineffective against fast transport. C1 injected in the amount present in serum was also found to be inactive. Because we used guinea

Fig. C1, we also compared the fractions prepared from guinea pig serum by DEAE-cellulose chromatography to those from rabbit sera; both were equally effective inhibitors of fast transport (data not shown).

Inhibition of Fast Axonal Transport by DNase I. DNase I, which has been found to bind to G-actin stoichiometrically and to depolymerize F-actin (33, 34), also inhibited fast transport of [^3H]serotonin when injected into the cell body of GCN. The inhibition depended upon the amount of the nuclease injected (Fig. 2). Maximal inhibition occurred with injections of DNase greater than 10 ng, an amount corresponding to approximately 1% of the total protein present in the cell body.

Consistent with the idea that the inhibition involves specific interaction between DNase and actin in the neuron, treatment of the enzyme with actin purified from rabbit skeletal muscle decreased the extent of inhibition obtained when the mixture of DNase and rabbit actin was subsequently injected into GCN. Samples of DNase that inhibited transport by 55% were capable of only 29% inhibition after addition of an equimolar amount of actin before injection. Incomplete removal of inhibition was not surprising because the nuclease activity of these samples was decreased by only 33%. Lazarides and Lindberg (33) also found that DNase activity was not completely inhibited by equimolar amounts of actin and suggested that some actin molecules are incapable of binding to DNase.

Although not thoroughly investigated, the inhibition of fast transport also appeared to depend on the time of exposure to DNase. The results shown in Fig. 2 were obtained when DNase was injected 2 hr before the injection of [^3H]serotonin used to test transport. Preliminary experiments in which the time interval between the injections of enzyme and of [^3H]serotonin was prolonged to 4 hr resulted in greater inhibition (data not shown).

Failure of Phalloidin to Inhibit Fast Transport. Phalloidin, a toxic peptide of the poisonous mushroom *Amanita phalloides*, binds specifically to F-actin. Unlike DNase and ADF, however, it does not depolymerize actin filaments; in fact, it stabilizes them (35). Intracellular injection of phalloidin into cells in culture (36) or into amoebas (37) has been shown to block cell locomotion. In six experiments, 13-26 pmol of phalloidin was injected into GCN. We found that transport of [^3H]serotonin

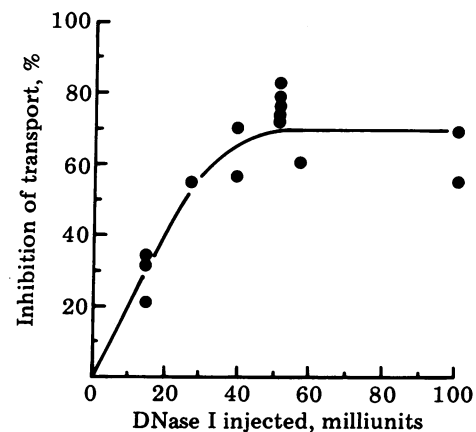


FIG. 2. Inhibition of [^3H]serotonin transport by injection of DNase I. Injection of DNase into one GCN of a pair was followed 2 hr later by injection of [^3H]serotonin into both cells. After an additional 3 hr, transport was assayed. Inhibition, the difference between transport in the control cell and transport in the cell injected with DNase, is expressed as a percentage of the value in the control. The specific activity of DNase was 3-4 milliunits/ng of protein. The smooth curve was fitted by eye to the data points, each of which represents a single experiment.

was not significantly affected (increased by $5 \pm 11\%$). On a molar basis, the amount of phalloidin injected was 40–80 times greater than the amount of DNase required to inhibit maximally.

DISCUSSION

Intracellular injection of two proteins that have been shown to interfere with the polymerization of actin blocked fast axonal transport. In contrast, injection of phalloidin, a peptide that binds to F-actin and stabilizes the filamentous state, was found to be ineffective. These experiments provide a direct approach for probing the role of actin in transport. There are three kinds of questions that need to be answered, however, before it can be concluded that actin participates in generating the force required to move materials along the axon. As with other pharmacological agents, we first need information about the specificity of DNase I and the serum factor. Second, we must determine whether these proteins actually inhibit translocation along the axon rather than interfere with some somatic process preliminary to transport. And finally, we need to find out whether the effects of DNase and the serum factor are primarily on the mechanism of axonal transport or are secondary to a general deterioration of the neuron's cytoskeleton.

Specificity of the Serum Factor and DNase I. The serum factor that decreased movement of [³H]serotonin in our experiments shares several characteristics with the ADF described by Norberg *et al.* (21). It is a gamma globulin that emerges from a Sephadex G-200 column in fractions between those containing albumin ($M_r = 68,000$) and IgG ($M_r = 150,000$) and is inactivated by mild heat treatment. We have evidence (unpublished data) that ADF affects polymerization by acting on the growing ends of actin filaments like the cytochalasins (8–10), β -actinin from muscle (29–31), and two recently described factors from other sources (31, 38, 39).

Although the activity of ADF can explain the inhibition of transport observed, it is possible that some other activity in serum is responsible. We have purified ADF 50-fold from serum by $(\text{NH}_4)_2\text{SO}_4$ purification and gel filtration, but many unknown serum proteins remain. Thrombin and C1 have been eliminated, however (Table 1). The factor must be purified further before we can be sure that inhibition of transport is due to ADF.

DNase I has been shown to bind avidly to G-actin (33, 34). Because F-actin is in equilibrium with G-actin, this stoichiometric binding results in the depolymerization of actin filaments (34). Injection of DNase into amoebas results in the local disappearance of actin filaments and the cessation of normal amoeboid movement (40). Nevertheless, in addition to nuclease activity, the DNase preparation may have activities (24) besides the depolymerization of actin filaments within the *Aplysia* neuron. DNase can be used in affinity chromatography to remove actin selectively from cell homogenates (33), however, and we have shown that treatment with rabbit skeletal muscle actin partially reverses the inhibition brought about by the injected enzyme.

It may be questioned why IgG raised against *Aplysia* body wall actin, which has been shown to crossreact with *Aplysia* neuronal actin (26), did not decrease the movement of [³H]serotonin. Although several explanations could be offered to account for the ineffectiveness of the anti-actin antibody, the most likely is that we did not inject a sufficient amount into the neuron. Although the amount of actin in GCN has not been measured, it can be estimated from the neuron's volume [14 nl (11)] and from the concentration of actin in other cells [5–10% (41)] to be approximately 100 ng. If a substantial fraction of this actin must be bound for the movement of [³H]serotonin to be

blocked, then a large amount of any agent that acts by binding stoichiometrically must be administered to be effective. For example, maximal inhibition was achieved by injection of 7.5–10 ng of DNase (Fig. 2), which would be expected to bind an equimolar amount (34) of actin (10–13 ng). In contrast, far less than equimolar amounts of the anti-actin antibody were injected because the anti-actin comprises only a small percentage of the total IgG (26). On the other hand, ADF, which we also injected in relatively small amounts, is effective probably because it depolymerizes actin catalytically or substoichiometrically (ref. 21; unpublished data).

Site of Inhibition within the Neuron. The experiments reported here do not show definitively that DNase I and the serum factor disrupt the actual translocation of [³H]serotonin along the axon. Reduction in amount of [³H]serotonin appearing in the cerebrobuccal connective and lip nerve could have resulted from a decrease in the amount of [³H]serotonin exported from the cell body into the axon. Export presumably involves a series of steps not usually considered to be part of the transport process. Preliminary experiments suggest that neither ADF nor DNase interferes with the metabolism of serotonin in the injected neuron. We found that degradation of [³H]serotonin was not increased, as might be expected if the injected proteins had inhibited the packaging of the transmitter into storage vesicles (42). Furthermore, we also found that the appearance in the axon of newly synthesized membrane glycoproteins that had incorporated ³H-labeled sugar (43) was also blocked, indicating that the inhibition affected export of organelles other than serotonergic vesicles. Nevertheless, we have not yet shown that translocation itself is impaired by DNase or the serum factor. This could be determined directly by injecting the inhibitors into the axon. Intra-axonal injection, however, has not yet been successful with the narrow axons of GCN; it has been accomplished with the larger axon of R2, another identified *Aplysia* neuron (44).

Is the Action of the Inhibitors Selective? ADF and DNase I are likely to inhibit transport by means of their ability to depolymerize actin filaments. Even when the specificity of the inhibition is firmly established, caution must still be used in the interpretation of these experiments as well as of all previous studies with colchicine and other agents that result specifically in the depolymerization of microtubules (1). Depolymerization of either microfilaments or microtubules can disrupt the architecture of the axon, and the resulting blockage of fast transport may be merely a secondary manifestation of the deterioration of the cytoskeleton. Because actin filaments can be expected to participate in cellular processes other than axonal transport, these pharmacological experiments are insufficient to identify conclusively the molecular components responsible for fast axonal transport. Nevertheless, they provide a direct experimental indication that actin filaments are involved.

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