Microinjection into an identified axon to study the mechanism of fast axonal transport

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ABSTRACT Microinjection into an axon of an identified invertebrate neuron is shown to be a useful technique for analyzing the mechanism of fast axonal transport. It permits direct assessment of the effect of agents that cannot permeate the plasma membrane on the translocation of material in the axon. The actin filament depolymerizer DNase I, when injected into the axon of the Aplysia neuron R2, caused a local block of fast transport of [3H]glicoprotein. Two agents that should interfere with the functioning of actin filaments without causing extensive depolymerization, the N-ethylmaleimide-modified nuclease S1 fragment of microtubule (injected) and dihydrocytochalasin B (applied externally), had no effect. Together these results suggest that actin plays a structural role in the axonal cytoskeleton rather than a role in transport force generation, the effect of DNase I being mediated by structural disorganization of the axoplasm. Experiments were also done with inhibitors of dynein, the microtubule-associated ATPase. erythroid-9-[3-(2-Hydroxynonyl)jadenine blocked transport but vanadate was ineffective.

The study of cell motility has been aided greatly by the use of preparations in which the experimenter has direct access to the cytoplasm and, thus, the motile machinery. There are three general types of such preparations: (i) isolated cytoplasm, as used in the study of ameboid movement (1); (ii) cells with plasma membranes that have been permeabilized by treatment with detergent, as used to study mitosis (2); and (iii) large cells into which materials are introduced by injection through an impaling micropipette, such as amebae (3) and egg cells (4). One advantage of these preparations is that they permit analysis of the mechanism of motility by using agents that interact specifically with suspected constituents of the motile apparatus but do not readily permeate an intact plasma membrane. Evaluation of the role of actin in a motile process should especially benefit from accessibility to the cytoplasm, because the small repertory of actin-specific agents has lately been augmented by the introduction of several nonpermeant agents.

Fast axonal transport is a motile process in neurons in which organelles, such as transmitter storage vesicles, travel from their somal birthplace to areas of utilization in the axon and synapses and lysosomes laden with cellular debris make the return trip (5). It may be a manifestation of the saltatory organelle movement that is seen in several other types of cells (6). The properties of fast transport are well described, but the molecular mechanism is not understood, although morphological and pharmacological data suggest a role for microtubules (5).

Recently, Isenberg et al. (7) and Goldberg et al. (8) reported the use of "direct access" preparations to analyze the mechanism of fast transport. Actin-specific agents were microinjected into the cell bodies of giant invertebrate neurons, and some were found to inhibit transport. Injection into the cell body does not, however, allow one to distinguish an actual impairment of organelle translocation in the axon caused by diffusion of the inhibitor into the initial segment from effects on somal processes such as assembly and mobilization of organelles for export. A resolution of this ambiguity is reported here with the microinjection of agents directly into the axon of an identified neuron.

A preliminary account of some of these experiments has been published (9).

MATERIALS AND METHODS

Animals and Intracellular Injections. Aplysia californica weighing 90–150 g were obtained from Pacific Biomarine Supply (Venice, CA) or Marine Specimens (Pacific Palisades, CA) and kept at 15°C in aquaria containing aerated Instant Ocean (Aquarium Systems, Eastlake, OH). The animals were fed seaweed and were used within a few weeks of receipt.

The abdominal ganglion and its associated nerves were removed from the animal and kept at room temperature (22 to 23°C) in an artificial sea water supplemented with amino acids and vitamins (10). Solutions were injected into the axon of the giant cell R2 essentially as described by Treistman and Schwartz (11). A short segment of the right pleuroabdominal connective, which contains the major axon of R2, was exposed briefly to a solution of trypsin to facilitate impalement of the axon of R2 with the double-barreled electrode used for microinjection. After trypsinization, the cell body of R2 in the abdominal ganglion was impaled with a double-barreled microelectrode and approximately 0.1–1.0 pmol of [3H]fucose was injected. Then, after the appropriate interval, the axon of R2 in the trypsinized segment of the right connective was impaled and 0.5 nl of the appropriate solution was injected into the axon. A precise volume could be injected because the injection electrode was calibrated before use (8). Preparations in which the resting potential recorded in the cell body was less negative than –38 mV or the resting potential recorded in the axon was less negative than –45 mV were discarded. Usually, the resting potential recorded in the cell body was –40 to –50 mV and the resting potential recorded in the axon was –50 to –60 mV.

Analysis of Fast Transport. After intrasomatic injection of [3H]fucose, radioactivity remains restricted to R2 (12, 13). [3H]Glycoprotein in the cell body and axon of R2 was measured as described (12, 13). The abdominal ganglion and right connective (or segments thereof) were homogenized at 0°C in 10% trichloracetic acid/1% phosphotungstic acid and the precip-

Abbreviations: BzSO4F, phenylmethanesulfonyl fluoride; Me2SO, dimethyl sulfoxide; MalNEt, N-ethylmaleimide; EHNA, erythroid-9-[3-(2-hydroxynonyl)jadenine, GCN, giant cerebral neuron.
iates were collected on pads of glass microfiber (GF/C). The pads were washed twice with 5% Cl₂COO⁻ and then repeatedly with ethanol/ether (1:2) and ether to remove [³H]glycolipid and residual Cl₂COO⁻, and radioactivity was measured with a liquid scintillation spectrometer.

In those experiments in which the spatial distribution of [³H]glycoprotein along the length of the connective was determined, the ganglion and extended connective were rapidly frozen with solid CO₂ on a metal plate and the connective was sectioned sequentially into 1-mm-long segments (13).

**Materials.** Trypsin (type XI; diphenylcarbamoyl chloride treated) was obtained from Sigma, dissolved in supplemented artificial sea water, and stored at −20°C in samples of a size sufficient for one experiment. Bovine serum albumin was also obtained from Sigma and was dissolved in 10 mM Tris base (pH 7.6) for use. [³H]Fucose (56 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was obtained from New England Nuclear. Bovine pancreatic DNase I (nominal specific activity, 2,812 units/mg) was obtained from Schwarz/Mann (Spring Valley, NY). It was dissolved in either 5 mM NaOAc (pH 5.0) or 50 mM Tris base, pH 7.6/0.5 mM phenylmethylsulfonyl fluoride (BzlSO₂F)/2.5% isopropanol. When the latter solvent was used the solution was, before storage at 4°C, maintained for 0.5 hr at room temperature to allow the BzlSO₂F to inhibit any contaminating proteases. DNase I activity was assayed by the method of Kunitz (14). Dihydrocytochalasin B was obtained from Aldrich Chemical (Metuchen, NJ) and stored as a 10 mM stock solution in 100% dimethyl sulfoxide (Me₂SO) and then diluted to the appropriate concentration with Instant Ocean just prior to use. Preparations were kept in the dark during exposure to dihydrocytochalasin B. N-Ethylmaleimide (MalNEt)-modified S1 fragment of rabbit skeletal myosin, the generous gift of W. Z. Cande, was dissolved in water and stored at 4°C. Solutions were stored for no longer than 10 days before use. erythro-9-[3-(2-Hydroxyxynonyl)]adenine (EHNA) was obtained from Burroughs Wellcome. Just prior to use, EHNA was dissolved in 100% Me₂SO and then diluted to final concentration with Instant Ocean. Sodium orthovanadate (Na₃VO₄) was obtained from Fisher Scientific (Springfield, NJ) and was stored as a 10 mM stock solution in water at pH 10 to prevent polymerization of the ionic species (15). Dilution to the appropriate concentration was done just prior to use by addition of 20 mM Tris base (pH 7.6). The final pH of the solution to be injected was 8.5.

**RESULTS**

**Effect of DNase I on Transport.** It had been shown that injection of DNase I into the cell body of the giant cerebral neuron (GCN) of *Aplysia* reduces the fast transport of serotonin (8). Material cannot be injected into either of the two main axons of the GCN because they are too thin to be impaled reliably. Material can be injected directly into the major axon of the *Aplysia* neuron R2 (11), however, because this axon is considerably larger (50–75 μm in diameter). Fast axonal transport in R2 is assayed by injecting [³H]fucose into the cell body in the abdominal ganglion. Some of the [³H]fucose is incorporated into glycoproteins in the membranes of organelles that are exported from the cell body and move by fast axonal transport in the major axon of R2 in the right connective (13).

DNase I produced a complete block of fast transport of [³H]glycoprotein when injected into the axon (Fig. 1C; Table 1). In the experimental protocol used, [³H]glycoprotein would have begun to arrive at the site of DNase I injection 1 to 2 hr after the injection and continued to arrive for 4 hr. The fact that there was an extremely large accumulation of [³H]glycoprotein just proximal to the site of injection indicates that fast transport in the axon segment between the cell body and the site of injection must have proceeded normally throughout the experiment; that is, material was free to move up to the region of the injection. So, DNase I did not diffuse back to the cell body and block export, a very unlikely possibility in any case considering the distance, the size of the DNase I molecule (M, 31,000), and the amount required to exert such an effect in the cell body relative to the amount injected into the axon. In fact, as much [³H]glycoprotein appeared in the right connective when DNase I was injected into the axon as when there was no axon injection (data not shown).

The block of transport caused by DNase I was not a nonspecific result of the injection of a substantial amount of protein into the axon, as injections of similar amounts of albumin were without effect (Fig. 1B; Table 1). Nor was the block caused by the trypsin or chymotrypsin that can contaminate preparations of pancreatic DNase I, because previous treatment of the nuclease with BzlSO₂F, which irreversibly inhibits the contaminating proteases (16), had no effect on the ability of DNase I to block transport.

**Effects of Other Actin-Specific Agents on Fast Transport.** Two other agents that interact specifically with actin, with distinct effects, did not block fast transport of [³H]glycoprotein in the axon of R2.
Table 1. Effects on fast axonal transport of \(^{[3]H}\)glycoprotein in R2 of actin-specific agents injected into the axon

<table>
<thead>
<tr>
<th>Agent injected</th>
<th>Amount injected, ng</th>
<th>% total axonal (^{[3]H})glycoprotein in distal part of connective</th>
<th>No. of experiments (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>29.7</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>10</td>
<td>21.9 ± 2.2</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>35.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DNase I</td>
<td>10</td>
<td>3.4 ± 2.1</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DNase I/BzlSO(_2)F</td>
<td>3</td>
<td>7.0 ± 5.4</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>1.7</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>1.1 ± 0.9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>MalNEt-modified S1 myosin fragment</td>
<td>6</td>
<td>46.4</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>37.6</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

\(^{[3]H}\)Fucose was injected into the cell body of R2 and, 1 to 2 hr later, the agent was injected into the axon of R2 at a site 9–12 mm from the abdominal ganglion. All but the most proximal 15 mm of the connective is designated the distal part, and thus all \(^{[3]H}\)glycoprotein in this part moved through the site of injection. Results are expressed as mean (n = 1 or 2) or as mean ± SEM (n ≥ 3).

Dihydrocytochalasin B. Cytochalasin B is a cyclic mold metabolite that prevents the polymerization of actin (17) and disrupts networks of actin filaments without causing extensive filament depolymerization (18, 19). Several motile processes thought to be actin-dependent are blocked by cytochalasin B (20), presumably as a result of either or both of those effects. Dihydrocytochalasin B is a recently developed derivative of cytochalasin B that binds to actin with high affinity without having the non-actin-related interaction with the plasma membrane also seen with cytochalasin B (21).

The cytochalasins readily pass through the plasma membrane (20, 21), so it was unnecessary to inject dihydrocytochalasin B into the axon. Rather, \(^{[3]H}\)fucose was injected into the cell body of R2 and \(^{[3]H}\)glycoprotein was allowed to migrate into the right connective in the absence of dihydrocytochalasin B. Only after a ligature was tied at the proximal end of the connective to prevent further export of \(^{[3]H}\)glycoprotein from the ganglion was dihydrocytochalasin B added to the bath. In this way, effects of the agent could be assigned with certainty to translocation of material in the axon rather than to export from the cell body. The presence in the bath of a large dose of dihydrocytochalasin B for a long period of time did not block transport (Fig. 2). An immediate cessation of transport due to the dihydrocytochalasin B would have restricted \(^{[3]H}\)glycoprotein to approximately the first 6 mm of the connective (22). That is not the case nor is there a retardation of the front of radioactivity as would be expected from a delayed or partial effect. Clearly, the right connective contains no unusual permeability barrier limiting the access of lipid soluble molecules to the axon of R2, as both EHNA (see below) and colchicine (11) block transport in the axon of R2 when applied externally. Also, injection of 200 \(\mu\)M (100 \(\mu\)g/ml) cytochalasin B into the cell body of the GCN has no effect on transport of \(^{[3]H}\)serotonin (unpublished data.).

MalNEt-modified S1 myosin fragment. Recently, Cande has shown that modification of the S1 fragment of myosin by treatment with MalNEt eliminates its ATPase activity while leaving it capable of binding to actin (2). Being unable to release from actin in the presence of ATP, this myosin fragment and MalNEt-heavy meromyosin irreversibly block the interaction of actin with endogenous myosin in a cell and have been shown, when given access to the cytoplasm by microinjection or plasma membrane permeabilization, to block cytokinesis, which is thought to be driven by actomyosin (2, 4). The MalNEt-modified S1 myosin fragment, then, inhibits the functioning of actin without altering its structure in the cell. It has not heretofore been used to analyze transport. Injection of up to 8 ng (0.8 nl of a 10 ng/ml solution) of the modified myosin fragment into the axon of R2 did not block fast transport of \(^{[3]H}\)glycoprotein (Table 1). Injection of the fragment 3 hr, rather than 1 to 2 hr, after intrasomal injection of \(^{[3]H}\)fucose, when \(^{[3]H}\)glycoprotein should just have begun to arrive at the axon injection site, also produced no block of transport (data not shown). Thus, the MalNEt-modified S1 myosin fragment did not produce a transient block of transport that, depending on its duration, might not have been revealed by the experiments summarized in Table 1.

Effects of Agents that Inhibit Dynein on Fast Transport.

EHNA. EHNA is an adenine derivative that inhibits dynein, thereby blocking sperm motility (23). It is effective when applied externally. A 3-mm segment of the right connective was isolated from the abdominal ganglion and the rest of the connective by being placed in a well sealed with petroleum jelly. EHNA was added to the Instant Ocean bathing the isolated segment 2.5 hr after intrasomal injection of \(^{[3]H}\)fucose (0.5 hr before the expected arrival of \(^{[3]H}\)glycoprotein at the segment) and was maintained at a concentration of 3 mM for the remaining 3.5 hr of the experiment. The spatial distribution of \(^{[3]H}\)glycoprotein in the connective shows that relatively little \(^{[3]H}\)glycoprotein was able to pass through the isolated segment (Fig. 3B). Thus, EHNA inhibited fast transport. This appeared to be approximately a threshold concentration for inhibition, as 2 mM EHNA inconsistently caused inhibition (data not shown).

Vanadate. Vanadium in the +5 oxidation state inhibits the
DISCUSSION

DNase I and Other Actin-Specific Agents. DNase I, a depolymerizer of actin filaments (3, 16, 26, 27), had previously been shown to block transport when injected into the cell body of either the Retzius cell of leech (7) or the GCN of Aplysia (8). In neither case could it be ascertained whether DNase I blocked the actual movement of organelles along the axon rather than their formation in, or exit from, the cell body. Such uncertainty is inherent in a procedure in which the blocking agent gains access to the axoplasm via the somal cytoplasm. The current experiments show that DNase I indeed blocks translocation of material in the axon, as its injection into the axon at a site far from the soma results in a local block of the movement of $^{3}$H]glycoprotein. The possibility should be considered that the block of transport is not due to an effect on actin filaments, either being caused by a contaminating protease, rather than DNase I, or resulting from an interaction of DNase I with a cellular constituent other than actin. It seems quite unlikely that the inhibitory factor is not DNase I since $\text{BzISO}_{2}\text{F}$, which eliminates any residual proteolytic activity (16), did not reduce the blocking potency. Also, addition of actin to DNase I injected into neuronal cell bodies does reduce the inhibition of transport (7, 8). (This experiment could not be included in the present series because addition of sufficient actin to reduce greatly the DNase I activity resulted in a solution too viscous to be injected into the axon.) DNase I binds to actin with considerable specificity—it can be used as an affinity ligand to purify actin from cell homogenates (28) — and has been shown to depolymerize cellular actin filaments (3, 16, 27). But there is a preliminary claim for the existence of a DNase-binding macromolecule other than actin in Tetrahymana cytoplasm (29), so a non actin-mediated effect of DNase I, while unlikely, cannot be discounted.

As previously suggested (8), the block of fast transport caused by DNase I could result from a loss of actin filaments normally engaged in force generation for transport or from disordering of the axoplasm caused by the loss of those filaments. The latter explanation implies a structural role for the actin filaments in the axon, stabilizing the architecture of the axoplasm, perhaps via direct or indirect links with neurofilaments and microtubules. It seems at this time the preferable explanation, at least insofar as one may judge from the lack of effect of two other actin-specific agents, dihydrocysteinosasolin B and the MalNEt-modified S1 fragment of myosin, on transport of $^{3}$H]glycoprotein in the axon. Perhaps the use of larger amounts of these agents might have revealed effects on transport. All that can be said on this point is that such amounts would be considerably in excess of what is required to block other types of motility. The concentration of dihydrocysteinosalin B used here was several-fold higher than needed to inhibit any other susceptible type of motility (20, 21), and that of the MalNEt-modified fragment was similar to, or somewhat greater than, that used in previous experiments (2, 4). What sets DNase I apart from these other agents that inhibit actin-dependent motility is its ability to depolymerize stable actin filaments. Thus, if DNase I caused extensive loss of actin filaments, as it has been shown to do when applied to other cells (3, 16, 27), its effect on axoplastic organization would be expected to be most drastic of all the agents.

Dynein Inhibitors. Microtubules are often suggested to be part of the fast transport machinery. Drugs that depolymerize microtubules block transport (5), and spatial associations between microtubules and materials moving by fast transport have been described (30, 31). It was thus of interest to determine whether a block of fast transport would result from the use of two recently described inhibitors of dynein, the ATPase that...
generates the force for the most clearly established example of microtubule-dependent motility, ciliary and flagellar movement (32). This determination was of particular importance because these inhibitors have recently been found to block other manifestations of intracellular organelle movement (33, 34).

EHNA blocked transport of [3H]glycoprotein at a concentration similar to that producing inhibition of the dynein-dependent process of sperm tail movement (23) and inhibition of pigment granule transport in fish scale erythropoieses (33). This result is consistent with the idea that a dynein-like ATPase supplies the energy for free transport, but other considerations leave the issue unsettled. EHNA can inhibit cellular enzymes—including ATPases—other than dynein (35), dynein has not been found in axons, and injection of high concentrations of the other dynein inhibitor, vanadate, into the axon of R2 had no dramatic effect on the transport of [3H]glycoprotein.

Utility of Axon Injection. The results reported here demonstrate the feasibility of using microinjection into the axon of an identified giant Aplysia neuron as a technique for gaining direct access to the axoplasm to study the mechanism of fast axonal transport. Macromolecules, in this case DNase I, can be injected into the axon of R2 in amounts sufficient to cause a complete local block of transport. Thus, this should be a technique suitable for assessing unambiguously the effects of hitherto unusable large probes, such as enzymes and antibodies, on transport within the axon.

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