GTPγS Inhibits Organelle Transport along Axonal Microtubules

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Abstract. Movements of membrane-bounded organelles through cytoplasm frequently occur along microtubules, as in the neuron-specific case of fast axonal transport. To shed light on how microtubule-based organelle motility is regulated, pharmacological probes for GTP-binding proteins, or protein kinases or phosphatases were perfused into axoplasm extruded from squid (*Loligo pealei*) giant axons, and effects on fast axonal transport were monitored by quantitative video-enhanced light microscopy. GTPγS caused concentration-dependent and time-dependent declines in organelle transport velocities. GDPβS was a less potent inhibitor. Excess GTP, but not GDP, masked the effects of coperfused GTPγS. The effects of GTPγS on transport were not mimicked by broad spectrum inhibitors of protein kinases (K-252a) or phosphatases (microcystin LR and okadaic acid), or as shown earlier, by ATPγS. Therefore, suppression of organelle motility by GTPγS was guanine nucleotide–specific and evidently did not involve irreversible transfer of thiophosphate groups to protein. Instead, the data imply that organelle transport in the axon is modulated by cycles of GTP hydrolysis and nucleotide exchange by one or more GTP-binding proteins. Fast axonal transport was not perturbed by AlF$_4^-$, indicating that the GTPγS-sensitive factors do not include heterotrimeric G-proteins. Potential axoplasmic targets of GTPγS include dynamin and multiple small GTP-binding proteins, which were shown to be present in squid axoplasm. These collective findings suggest a novel strategy for regulating microtubule-based organelle transport and a new role for GTP-binding proteins.

O NE of the principal functions of microtubules in interphase cells is to serve as tracks along which numerous types of membrane-bounded organelles travel through the cytoplasm. This class of intracellular motility is especially well developed in the axonal processes of neurons, where vesicles containing secretory products and axolemmal proteins are carried anterogradely, toward microtubule plus ends and the axon terminal, whereas those containing endocytosed materials travel in the opposite, or retrograde, direction (9, 18, 72, 76, 81). Organelles like mitochondria, which do not participate directly in exocytic or endocytic pathways, undergo bidirectional, microtubule-dependent motility in axons (49). Collectively, these organelle movements are known as fast axonal transport, and the motor proteins, kinesin and dynein, are thought to be responsible, at least in part, for the anterograde and retrograde components, respectively (5, 55, 79).

Although organelles frequently undergo lengthy, uninterrupted, unidirectional excursions, the cell must have mechanisms for regulating organelle traffic. In neurons, for example, newly formed vesicles that are earmarked for anterograde fast axonal transport must associate with appropriate microtubules and plus end-directed motors in or near the cell body.

Subsequently, these vesicles must travel through the axon, and be delivered to specific functional domains. There is evidence that the retrograde motor, dynein, is transported toward the axon terminal, presumably in an inactive form, by anterogradely moving vesicles (30). It has been speculated, therefore, that when such vesicles reach the axon terminal, dynein becomes activated to direct retrograde organelle traffic, and anterograde motors, such as kinesin, are decommissioned (30). To account for the complexity of fast axonal transport, the neuron must have mechanisms for starting and stopping the movement of organelles, determining their direction of travel relative to microtubule polarity, and turning motor molecules on and off.

The present study was undertaken to examine how neurons regulate fast axonal transport, a topic that previously had been virtually unexplored. Recently acquired evidence that GTP-binding proteins regulate membrane traffic at a variety of steps prompted us to explore whether microtubule-based organelle motility is also affected. This diverse group of factors encompasses numerous small (∼18–25 kD) polypeptides (4, 24, 26) and heterotrimeric G-proteins (4, 23), as well as a newly recognized family of proteins related to the microtubule-stimulated GTPase, dynamin (12, 54, 61, 80,

84). Several GTP-binding proteins are involved in regulating the budding of vesicles and tubules from donor membranes, and their targeting and fusion to specific acceptor membranes. Included among this collection of GTP-binding proteins are low molecular weight species, such as ARF (16, 68, 73) and several members of the rab group (11, 19, 20, 51, 58), as well as heterotrimeric G proteins, at least some of which are sensitive to pertussis toxin, and therefore belong to the G_i or G_o groups (2, 16, 38).

A second plausible mechanism for modulation of fast axonal transport is through protein kinases or protein phosphatases. Roles for kinases and phosphatases in the regulation of microtubule-based organelle motility have already been established in one cell type, fish scale chromatophores. In these cells, pigment granule dispersion along microtubules is coupled to cAMP-stimulated phosphorylation of a 57-kD protein, whereas pigment granule aggregation requires protein phosphatase 2B, a calcium-activated enzyme also known as calcineurin (46, 47, 62-64, 75). There is also reason to suspect that functional properties of microtubule motor proteins are regulated by cycles of phosphorylation and dephosphorylation, as both the heavy and light chains of kinesin can be phosphorylated in neurons (Elluru, R. G., K. K. Pfister, G. S. Bloom, and S. T. Brady, unpublished results; Hollenbeck, P. J., unpublished results) and nonneuronal cells (Buster, D., M. Lohka, and J. M. Scholey, unpublished results; Farshori, P. O., and D. Goode, unpublished results). Regulatory mechanisms based on GTP and protein phosphorylation are not mutually exclusive. For example, signal transduction by G-proteins can lead to downstream activation of protein kinases and phosphatases (14).

The experimental system used for the present study was axoplasm extruded from squid giant axons. When observed by video-enhanced light microscopy, extruded squid axoplasm can be seen to conduct vigorous, bidirectional, fast axonal transport (6). Because these preparations lack a surrounding plasma membrane, and a perfusion buffer that supports organelle motility along microtubules has been developed, it has been possible to define many biochemical requirements and pharmacological properties of fast axonal transport by systematically varying the composition of the buffer (7, 8, 42). The discovery of kinesin (5, 78), in fact, stemmed directly from perfusion studies of the nonhydrolyzable ATP analogue, 5'-adenylylimidodiphosphate, or AMP-PNP (41). In experiments described here, various probes for GTP-binding proteins, and broad spectrum inhibitors of protein kinases or phosphatases were added to the perfusion buffer, and the effects on organelle transport velocity were monitored by quantitative video microscopy. Prior studies have demonstrated the presence of such regulatory proteins in squid axoplasm (22, 37, 82). The results of the current studies imply that fast axonal transport is modulated by cycles of GTP hydrolysis and nucleotide exchange by one or more species of GTP-binding protein. Heterotrimeric G-proteins apparently are not involved in this form of regulation, but potential regulatory factors include dynamin and multiple small GTP-binding proteins, whose presence in axoplasm was demonstrated in blotting experiments. No evidence was found that the protein kinases and phosphatases affected by the inhibitors play significant roles in regulating microtubule-based organelle motility in the main body of the axon.

Materials and Methods

Axoplasm was extruded from giant axons that had been dissected out of squid (*Loligo pealei*) (7), which were supplied by the Marine Resources Department of the Marine Biological Laboratory (Woods Hole, MA). Extruded axoplasmic segments, which typically were ∼2 cm long and ∼0.55 mm in diam, were mounted in specimen chambers fabricated from a pair of no. 0 thickness coverslips (7). Preparations were observed by Nomarski differential interference contrast microscopy using a 100×, 1.3 N.A. planapochromatic objective on a Zeiss Axiomat (Carl Zeiss, Inc., Thornwood, NY) interfaced with a Hamamatsu C1966 AVEC video system. Preparations of extruded axoplasm were perfused with buffer X/2 (42), or with buffer X/2 supplemented with any of the following experimental probes: GTPγS, GDPβS (Boehringer Mannheim Biochemicals, Indianapolis, IN or Sigma Chemical Co., St. Louis, MO); GDP, GTP (Sigma Chemical Co.); AlF_4^- (a mixture of $AlCl_3$ [Mallinckrodt Inc., St. Louis, MO] and KF [Sigma Chemical Co.]); K-252a, microcystin LR (Calbiochem Corp., La Jolla, CA); PKI peptide, a synthetic peptide that specifically inhibits protein kinase A (product number P-3294; Sigma Chemical Co.); okadaic acid (gift from Dr. Philip Cohen, University of Dundee, Scotland). All perfusion buffers contained 1 mM ATP (Sigma Chemical Co.). 20 μl of experimental buffer was perfused into segments of extruded axoplasm, which typically had a volume of ∼5 μl. The concentrations of experimental probes cited throughout the text and figures represent those in the buffers before they were perfused into axoplasm. Final concentrations were ∼80% of the original values, as a result of dilution by the axoplasm. Before perfusion and for ∼40 min thereafter, velocity measurements were made for anterograde and retrograde transport. Postperfusion velocities were also determined for organelles traveling along isolated microtubules of unknown polarity located at axon peripheries. The velocity measurements were made in real time directly on the video monitor with the aid of a Hamamatsu C2117 Videomanipulator (42). The UTSTAT program (Academic Computing Services, University of Texas Southwestern Medical Center, Dallas, TX) was used to compute least square regression lines and F tests.

Western blotting of axoplasm was performed as described earlier (8) using an affinity-purified rabbit antidynamin antibody (gift from Drs. Chris Burgess and Richard B. Vallee, Worcester Foundation for Experimental Biology, Shrewsbury, MA) made against a glutathione S-transferase/rat brain dynamin fusion protein (12), and an alkaline-phosphatase labeled secondary antibody. To detect small GTP-binding proteins, isolated axoplasm was subjected to SDS-PAGE (8), and resolved proteins were transferred from the gel to a nitrocellulose filter, which was then incubated in [α-^{32}P]GTP in the presence of excess unlabeled GTP or ATP (40). A Molecular Dynamics phosphor imager interfaced with a Hewlett-Packard Laserjet III printer was used to visualize radiolabeled bands and print the results.

Results

As in our previous video studies of fast axonal transport in extruded squid axoplasm, three distinct types of organelle velocity measurements were made (8, 42). Within interior regions of axoplasm, where microtubules were aligned parallel to the long axes of the axons with their plus ends facing the distal ends of the segments (81), velocities were determined for both anterograde and retrograde fast axonal transport. These two forms of transport are distinguished from each other by the types of motile organelles that predominate (18, 72, 76), and the motor molecules that apparently are responsible for movement (56, 79). The third type of measurement was of organelles moving along microtubules located at axon peripheries. Having separated from the main axoplasmic mass after buffer perfusion, the polarities of these isolated microtubules could not be determined. Motility of organelles along peripheral microtubules are distinguished from microtubule-based movements in the axon interior, because the interior, but not the periphery, contains a dense meshwork of filaments and other structures that can impede microtubule-based organelle transport.

Effects of Guanine Nucleotides

Axoplasm was perfused with buffer supplemented with GTPγS or GDPβS, thiophosphorylated derivatives of GTP or GDP, respectively. Both modified nucleotides can bind to heterotrimeric and small GTP-binding proteins. Because GTPγS is essentially nonhydrolyzable, it can maintain these proteins in a condition analogous to the GTP-bound state (23, 26). GTPγS is also a potent inhibitor of the microtubule-stimulated GTPase activity of dynamin (70). Because of these properties, thiophosphorylated guanine nucleotides have become powerful tools for identifying intracellular phenomena that are regulated by GTP-binding proteins.

As shown in Fig. 1, dose-dependent inhibition of organelle transport rates was seen in extruded axoplasm over the course of a 40-min exposure to either GTPγS or GDPβS. In axons perfused with 0.25 mM GTPγS, average anterograde and retrograde organelle velocities decreased to $\sim$32% and $\sim$43% of control levels, respectively. Higher concentrations of GTPγS yielded similar effects (data not shown). A smaller, but statistically significant (P < 0.05) inhibition of anterograde and retrograde transport was induced by 0.1 mM GTPγS, but no significant effects were observed at 0.05 mM GTPγS. The effects of 0.8 mM GDPβS were similar to those of 0.1 mM GTPγS, but no inhibition was observed at lower GDPβS concentrations.

The extent to which organelle transport velocities were inhibited by the modified guanine nucleotides increased in a time-dependent manner. Fig. 2 illustrates the kinetics of inhibition by 0.25 mM GTPγS for anterograde and retrograde transport, and for organelle motility along isolated microtubules located at axon peripheries. Inhibition of peripheral transport was statistically significant (P < 0.05), but less pronounced than that observed for anterograde and retrograde motility. When transport rates were extrapolated to 40 min postperfusion, organelles in axons perfused with 0.25 mM GTPγS moved along peripheral microtubules at 59% of control velocities.

GTPγS and GDPβS are supplied commercially as powdered tetralithium and trilithium salts, respectively, and were reconstituted to 100 mM solutions in buffer X/2 or water, each containing 10% β-mercaptoethanol by volume. Consequently, experimental perfusion buffers had concentrations of lithium as high as 2.4 mM, and of β-mercaptoethanol as high as 0.08%. Perfusion buffers containing 4 mM lithium and 0.1% β-mercaptoethanol, but lacking GTPγS and GDPβS did not inhibit fast axonal transport (data not shown). The inhibition documented in Figs. 1–4 (below) was therefore induced by the thiophosphorylated guanine nucleotides.

The steady-state GTP concentration in squid axoplasm has been estimated to be $\sim$0.5 mM (53). Perfusion of buffer into extruded axoplasm dilutes all axoplasmic components not present in the buffer to approximately one fifth of their levels in the intact axon, or to $\sim$0.1 mM in the case of GTP. Presumably, the effects of GTPγS and GDPβS were caused by their having outcompeted the $\sim$0.1 mM endogenous GTP for binding to targets that normally hydrolyze the naturally present nucleotide. Consistent with this possibility, when extruded axoplasm was coperfused with 0.25 mM GTPγS plus either 1 mM GTP or GDP, the effects of GTPγS were masked completely by the excess GTP, but not by the equal
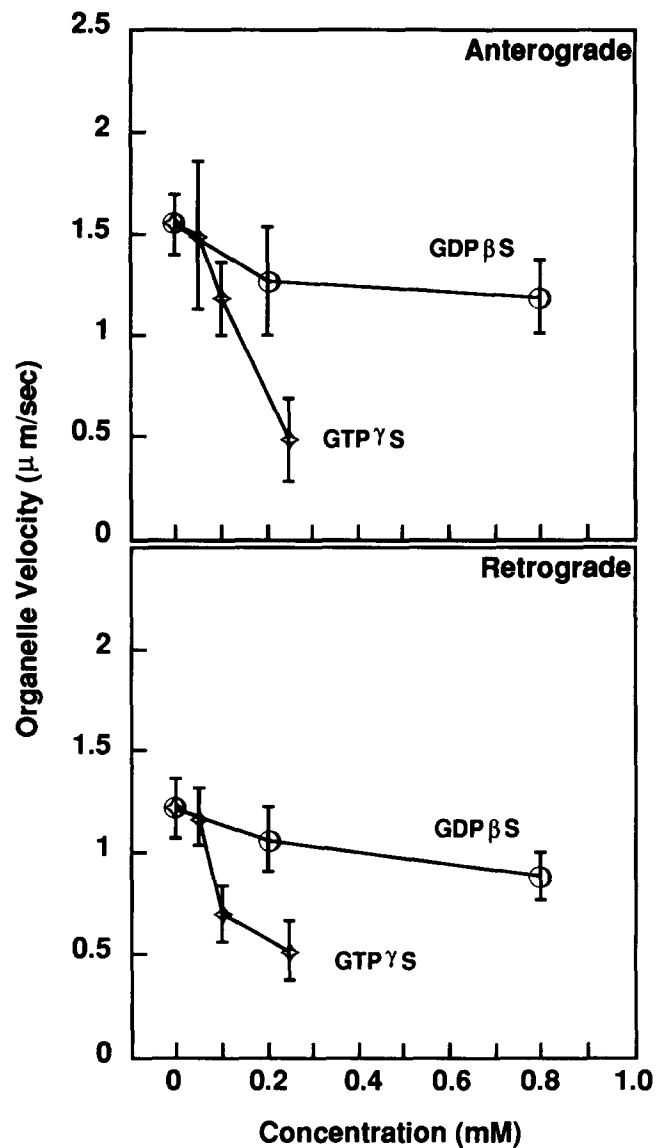

Figure 1. Dose-dependent inhibition of organelle motility by GTPγS and GDPβS. Organelle velocities were measured after perfusion of buffer X/2 alone, or buffer X/2 supplemented with various concentrations of GTPγS or GDPβS. Data points show mean organelle velocities for each condition at 40 min postperfusion (extrapolated from time course data, such as those shown in Fig. 2). For each condition, at least 15 observations from at least three different axoplasm preparations were used. Error bars indicate 95% confidence intervals of the means. Statistically significant inhibition (P < 0.05) was caused by 0.1 mM GTPγS, 0.25 mM GTPγS, and 0.8 mM GDPβS for anterograde and retrograde transport.

excess of GDP. As can be seen in Fig. 3, GTP blocked the effects of GTPγS for anterograde and retrograde transport, as well as for organelle motility along peripheral microtubules.

Perfusion of Protein Kinase and Protein Phosphatase Inhibitors

Two compounds that suppress the activities of protein kinases were tested, and the results are shown in Fig. 4. The microbial alkaloid, K-252a, which potently inhibits protein
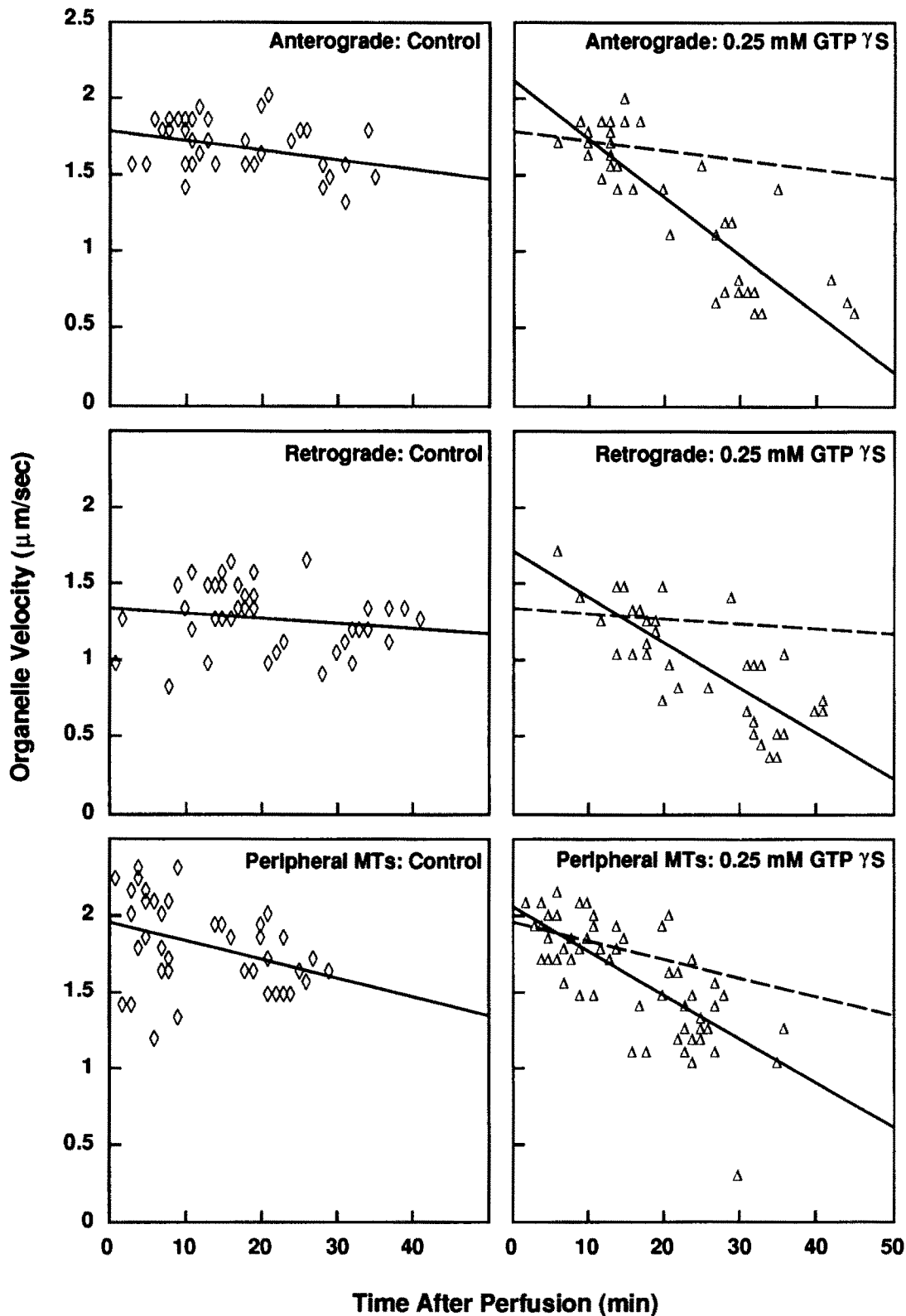
Anterograde: Control

Anterograde: 0.25 mM GTP γS

Retrograde: Control

Retrograde: 0.25 mM GTP γS

Peripheral MTs: Control

Peripheral MTs: 0.25 mM GTP γS

2.5
2
1.5
1
0.5
0

Organelle Velocity (μm/sec)

Time After Perfusion (min)

0   10   20   30   40   50

Figure 2. Kinetics of inhibition of fast axonal transport by 0.25 mM GTPγS. After perfusion of buffer X/2 supplemented with 0.25 mM GTPγS, time-dependent reductions in transport velocities were seen for organelles moving in axon interiors toward (*Anterograde*) and away from (*Retrograde*) synaptic terminals, and along isolated microtubules located at the edges of axoplasm (*Peripheral MTs*). Control axons were perfused with buffer X/2 alone. Each data point shown here represents a single measurement for either control or GTPγS-treated axons. Slopes were calculated using the lease squares regression formula, and graphs on the right allow direct comparison of control (*dotted line*) and GTPγS (*solid line*) data. Each graph shown here incorporates data obtained from at least six axoplasm preparations. F tests indicated that the slopes for control and GTPγS are significantly different ($P < 0.001$ for Anterograde and Retrograde; $P < 0.01$ for Peripheral MTs).
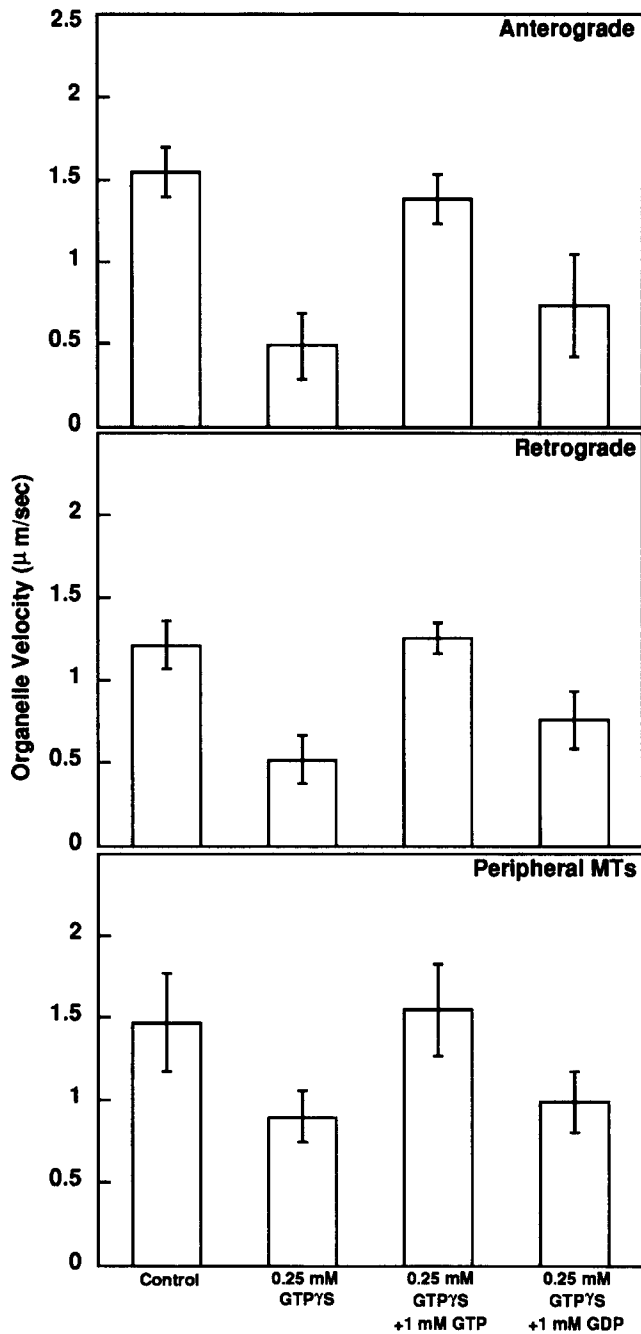
2.5
2
1.5
1
0.5
0

Anterograde

Retrograde

2

1.5

1

0.5

0

Peripheral MTs

2

1.5

1

0.5

0

Control | 0.25 mM GTPγS | 0.25 mM GTPγS +1 mM GTP | 0.25 mM GTPγS +1 mM GDP

Organelle Velocity (µm/sec)

Figure 3. Excess GTP, but not GDP, masks the inhibitory effects of GTPγS. Organelle velocities were measured following perfusion of buffer X/2 alone (*Control*), or buffer X/2 supplemented with the following: 0.25 mM GTPγS, 0.25 mM GTPγS + 1 mM GTP, or 0.25 mM GTPγS + mM GDP. Shown here are the means and 95% confidence intervals (*error bars*) for organelle velocities at 40 min postperfusion (extrapolated from time course data, such as those shown in Fig. 2). Coperfusion of GTPγS with a fourfold molar excess of GTP, but not GDP, protected organelle transport from the typical inhibitory effects of GTPγS. Extrapolated mean velocities for 0.25 mM GTPγS, and 0.25 mM GTPγS + 1 mM GDP were significantly different from the control ($P < 0.05$). Raw data for each condition include observations from at least three different axoplasm preparations.
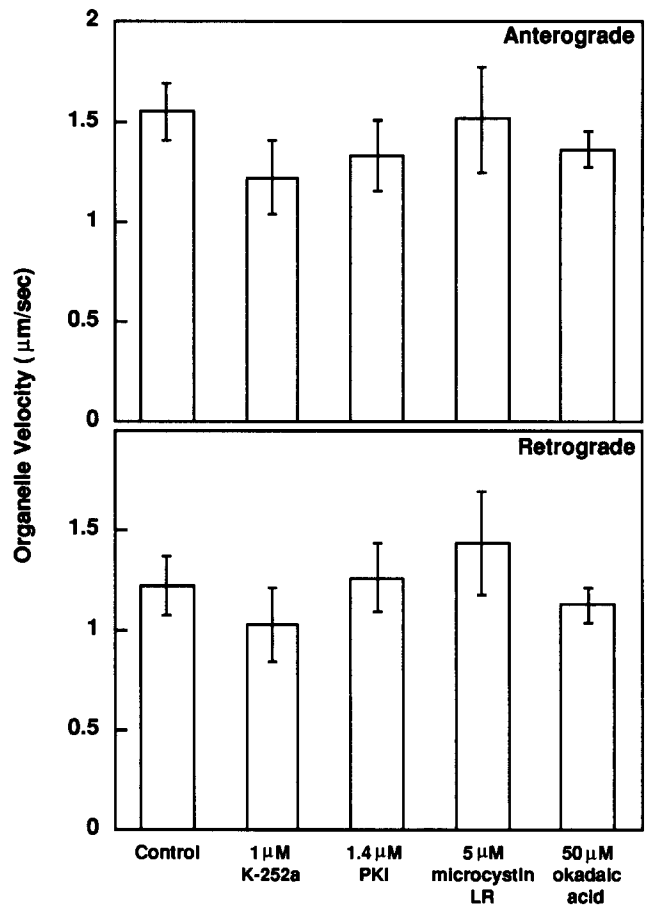
2

1.5

1

0.5

0

Anterograde

Retrograde

2

1.5

1

0.5

0

Control | 1 µM K-252a | 1.4 µM PKI | 5 µM microcystin LR | 50 µM okadaic acid

Organelle Velocity (µm/sec)

Figure 4. Effects of protein kinase and protein phosphatase inhibitors on anterograde and retrograde fast axonal transport. Organelle velocities were measured after perfusion of buffer X/2 alone (*Control*), or buffer X/2 supplemented with the following inhibitors: 1 µM K-252a, which inhibits protein kinases A, C, and G (36); 1.4 µM PKI peptide, a synthetic peptide which inhibits protein kinase A (13); and 5 µM microcystin-LR or 50 µM okadaic acid, chemically unrelated compounds which cause nearly complete inhibition in vitro of protein phosphatases 1 and 2A at 1–2 nM, and of protein phosphatase 2C at 1 µM (3, 14, 48). Shown here are the means and 95% confidence intervals (*error bars*) for organelle velocities at 40 min postperfusion (extrapolated from time course data, such as those shown in Fig. 2). No significant inhibition of organelle velocity was seen. Raw data for each condition included observations from at least three different axoplasm preparations.

kinases A, C, and G (36), completely blocks effects of these enzymes in cultured cells when present in tissue culture medium at 50 nM to 1 µM. Within this concentration range, K-252a has been reported to block NGF-dependent neurite outgrowth (27, 50) and protein phosphorylation (27), and to uncouple DNA synthesis from mitosis (77). In extruded axoplasm, however, organelle motility was unaffected by K-252a at concentrations up to 1 µM. In axons perfused with a 10-fold higher level of K-252a, anterograde transport velocities were reduced by ∼30% (not shown), but at such a high concentration, the specificity of K-252a for protein kinases must be questioned. 10 µM K-252a did not inhibit motility in the retrograde direction or along isolated, peripheral microtubules (not shown). PKI peptide, a 20-residue synthetic pep-

tide that inhibits protein kinase A with a K_i approximately eightfold lower than that of K-252a (13, 36) was also introduced into axoplasm, and failed to affect organelle motility at a concentration of 1.4 μM.

To test whether protein phosphatases are involved in regulation of fast axonal transport, two broad spectrum inhibitors of these enzymes, microcystin LR and okadaic acid, were perfused into extruded axons. Although chemically unrelated, these two compounds inhibit protein phosphatases 1 and 2A, and to a lesser extent, protein phosphatase 2B (3, 14, 48). Dramatic effects on protein phosphorylation (29), morphology of the Golgi apparatus (44), and ER to Golgi protein transport (15) have been noted in cultured cells treated with 0.1–1 μM okadaic acid. Like the protein kinase inhibitors, these protein phosphatase inhibitors had no apparent effect on organelle motility, even at concentrations as high as 5 μM for microcystin LR and 50 μM for okadaic acid (Fig. 4).

These collective results suggest that fast axonal transport is not regulated by cycles of phosphorylation and dephosphorylation mediated by protein kinases A, C, or G, and protein phosphatases 1, 2A, or 2B. Furthermore, they indicated that inhibition by GTPγS was not caused by irreversible transfer of the thiophosphate group to a component of the transport machinery. A previous study with ATPγS also indicated that protein thiophosphorylation does not alter fast axonal transport in squid axoplasm under comparable conditions (7).

Potential Targets for GTPγS

Proteins that are known to bind guanine nucleotides and have been implicated in regulation of membrane traffic include numerous heterotrimeric G-proteins and small GTP-binding proteins, as well as dynamin. Pertussis toxin-sensitive G-proteins are known to be present in squid axoplasm (82), and to test whether the effects of GTPγS on fast axonal transport might be due to these or other G-proteins, axons were perfused with AlF$_4^-$. This salt, which is formed by mixing low concentrations (1–100 μM) of AlCl$_3$ with higher concentrations (5–30 mM) of KF or NaF, mimics the effects of GTPγS on all known heterotrimeric G-proteins, but does not affect small GTP-binding proteins (34, 74). As shown in Fig. 5, AlF$_4^-$ did not inhibit fast axonal transport, implying that binding of GTPγS to heterotrimeric G-proteins did not underlie the inhibition of organelle motility by GTPγS.

Probes that uniquely affect the functions of small GTP-binding proteins or dynamin have not yet been developed, but methods for detecting these proteins are available. To determine whether dynamin or small GTP-binding proteins are present in squid axoplasm, nitrocellulose blots of electrophoretically resolved axoplasm were probed with an antibody to dynamin (12), and with [α-^{32}P]GTP, which binds on blots to small GTP-binding proteins, but not to heterotrimeric G-proteins (40). Fig. 6 illustrates that dynamin, and multiple, electrophoretically distinct, small GTP-binding proteins of $\sim$25 kD are present in extruded squid axoplasm, and therefore represent potential targets for GTPγS. The small GTP-binding proteins were labeled by [α-^{32}P]GTP in the presence of excess unlabeled ATP, but not in the presence of excess unlabeled GTP.
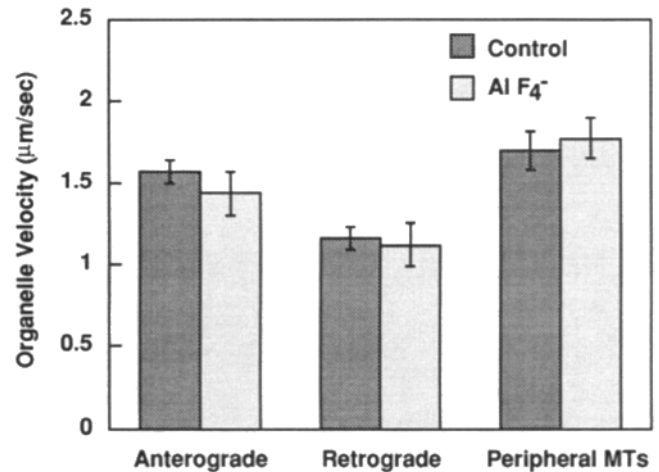

Figure 5. Aluminum fluoride does not inhibit fast axonal transport. Organelle velocities were measured after perfusion of buffer X/2 alone (*Control*), or buffer X/2 supplemented with AlF$_4^-$ (100 μM AlCl$_3$ plus 5 mM KF), which mimics the effects of GTPγS on heterotrimeric G-proteins, but not on other GTP-binding proteins (34, 74). Shown here are the means and 95% confidence intervals (*error bars*) for organelle velocities at 40 min postperfusion (extrapolated from time course data, such as those shown in Fig. 2). AlF$_4^-$ did not inhibit transport velocities, implying that heterotrimeric G-proteins were not the targets of GTPγS. Raw data for each condition included observations from at least four different axoplasm preparations.

Discussion

Heterotrimeric G-proteins have long been known to mediate signal transduction across the plasma membrane (4, 23). Along with numerous small GTP-binding proteins (11), they have been implicated more recently as important regulators of exocytic and endocytic membrane traffic (4, 24, 26, 60). Attention to these proteins in the context of membrane traffic has focused largely on their roles in controlling how vesicles bud from donor membranes, and are subsequently targeted and fused to acceptor membranes. To cite just a few of many currently known examples, ER to Golgi transport is apparently regulated by a heterotrimeric G$_i$ or G$_o$ protein (16, 38), and the small GTP-binding proteins, ARF (16, 68, 73) and rab1b (58), whereas there is evidence that rab3a regulates neurotransmitter secretion at axon terminals by a process that involves reversible association of this small GTP-binding protein with the outer surface of the synaptic vesicle (19, 20, 51). In this report, we present evidence for a new and distinct role for GTP-binding proteins in membrane traffic, modulation of fast axonal transport.

Fast axonal transport requires the coordinated action of several components, including transport tracks, molecular motors, and regulatory factors. Direct evidence that microtubules serve as the transport tracks came from video-enhanced light microscopic studies of squid axoplasm (1, 6, 66), similar to those used for the experiments described here. Subsequent studies using this approach led to the discovery of kinesin, the first known motor protein for microtubule-based organelle transport (5, 41, 78). Kinesin and another microtubule-stimulated ATPase, cytoplasmic dynein,
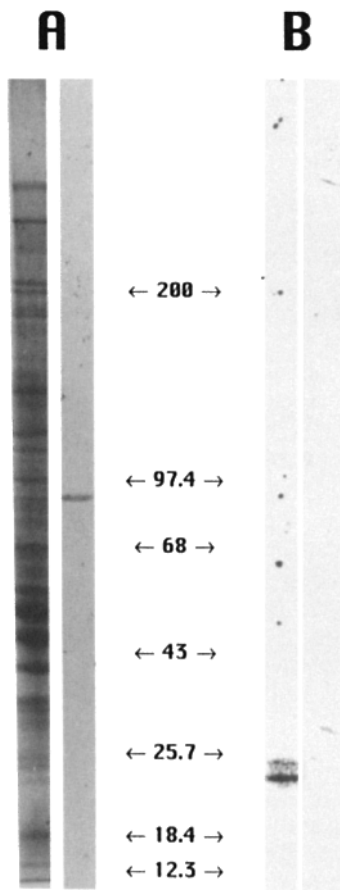
Figure 6. Small GTP-binding proteins and dynamin are present in squid axoplasm. Nitrocellulose blots of isolated axoplasm were probed with either antidynamin or [α-³²P]GTP. (A) Shown here are a Coomassie blue-stained gel lane of unfractionated axoplasmic proteins (left) and a corresponding Western blot stained with antidynamin (right). Note the single immunoreactive dynamin band. (B) Comparable blots were treated with [α-³²P]GTP, which detects small GTP-binding proteins (40). Multiple labeled bands of ~24 kD were seen when the incubation buffer was supplemented with an excess (10 μM) of unlabeled ATP (left), but the bands were not observed when 10 μM unlabeled GTP was used (right). Molecular weight markers (in kD) are indicated in the center of the figure.

have since been studied extensively, and are considered to move membranous structures toward microtubule plus and minus ends, respectively, in a broad variety of cell types (17, 30–32, 39, 43, 45, 55–57, 65, 67, 78, 79, 83).

In contrast to the properties of the transport tracks and motors, the regulation of fast axonal transport, and microtubule-based organelle motility in general, has been the subject of only a few studies. One such investigation revealed a potential regulatory role for synapsin I, which when perfused into squid axoplasm led to undetectable, partial, or complete inhibition of anterograde and retrograde transport, depending upon its phosphorylation state. Complete suppression of motility was caused only by fully dephosphorylated synapsin I. Inhibition of organelle motility in axon interiors was not accompanied by inhibition along peripheral microtubules, indicating that synapsin I was not directly affecting the transport machinery (52).

The only system in which transport regulation has been examined at greater length is fish scale chromatophores. These cells conduct hormone-coupled cycles of pigment aggregation and dispersal along radially arranged microtubules, a process that provides fish with the ability to regulate their coloration. Pigment granule movements depend upon a 57-kD protein, the phosphorylation state of which is controlled by protein kinase A, and protein phosphatase 2B (calcineurin). Granule dispersion occurs after phosphorylation of the 57-kD protein, while aggregation is linked to its dephosphorylation (46, 47, 62–64, 75).

The experiments described here indicate that regulation of fast axonal transport is quite different from regulation of pigment granule movement in chromatophore cells. Inhibitors of protein kinases or protein phosphatases implicated in pigment granule motility exerted little or no effect on microtubule-based organelle motility in squid giant axons. In contrast, GTPγS, and to a lesser extent, GDPβS, were found to inhibit fast axonal transport. Several mechanisms may be considered to explain the effects of the thiophosphorylated guanine nucleotides. Foremost among them are interference with the action of GTP-binding proteins, transfer of thiophosphate groups from nucleotide to protein, competitive inhibition of ATP binding to microtubule motor proteins, and metabolic poisoning. As described in the following paragraphs, our collective data from this and two prior studies (7, 42) favor the conclusion that the targets of the thiophosphorylated guanine nucleotides were GTP-binding proteins other than those of the heterotrimeric G-protein class. The mechanism of this inhibition does not appear to involve protein kinases A, C, or G, or protein phosphatases 1, 2A, or 2B acting downstream.

Organelle transport appears to be optimal when the relevant GTP-binding proteins can complete cycles of GTP hydrolysis and nucleotide exchange. Nonhydrolyzable guanine nucleotides, such as GTPγS or GDPβS, when perfused at concentrations sufficient to compete successfully with endogenous GTP in nucleotide exchange reactions, presumably arrested the GTP-binding proteins at various stages of a normal cycle. The effects of GTPγS appear competitive, because the lowest concentration that detectably inhibited transport (0.08 mM after perfusion) was estimated to be slightly less than equimolar to the diluted pool of endogenous GTP (~0.1 mM), and higher levels of GTPγS were much more effective at reducing the velocities of motile organelles (see Fig. 1). The observation that 1 mM GTP, but not GDP, prevented 0.25 mM GTPγS from inhibiting transport (see Fig. 3) indicates that GTPγS acted upon targets which require a hydrolyzable nucleotide to function properly. The hydrolyzable nucleotide in question must be GTP, moreover, because ATP was always present at 1 mM, and under those conditions ATPγS does not inhibit fast axonal transport at concentrations below 2 mM (7).

The fact that GDPβS was a much weaker inhibitor than GTPγS (see Fig. 1) suggests that triphosphates are normally preferred over diphosphates in exchange fractions, a characteristic of both heterotrimeric (21) and small GTP-binding proteins (33). The kinetics of inhibition by GTPγS imply that nucleotide exchange occurs slowly in isolated axoplasm, although this may not reflect physiological rates everywhere in the axon. Organelles often move continuously for long dis-

tances in isolated axoplasm (6, 7), raising the possibility that the primary sites at which GTP-binding proteins inhibit organelle motility are axonal extremities.

It is unlikely that the GTPγS effects were due to irreversible, inhibitory thiophosphorylation of proteins that are part of the organelle transport machinery. Thiophosphorylated proteins can be generated by protein kinases using ATPγS as a substrate, and protein-bound thiophosphate groups are highly resistant to removal by phosphatases (10, 25). Casein kinase II can use ATP, ATPγS, or GTP as a substrate (28), and might have been able to use GTPγS as a thiophosphate donor in the experiments described here. If that were possible, ATPγS should have been at least as effective as GTPγS at inhibiting fast axonal transport, but GTPγS inhibits at a 20-fold lower concentration than ATPγS (7). Moreover, thiophosphorylation of protein must have been very limited in the present experiments because ATP, the preferred substrate for protein kinases, was always present in substantial excess over GTPγS. The fact that several broad spectrum protein kinase or phosphatase inhibitors failed to affect organelle motility represents further evidence that protein phosphorylation and dephosphorylation mediated by enzymes sensitive to the inhibitors tested do not appreciably influence fast axonal transport (Fig. 4).

The GTPγS effects could not have resulted from competitive inhibition of ATP binding to microtubule motor molecules. Although the suitability of GTPγS as a substrate for kinesin or dynein has not been reported, both GTP and ATPγS have been examined. GTP can be hydrolyzed by kinesin (39) and cytoplasmic dynein (71), it promotes kinesin-dependent microtubule gliding (59), and it minimally supports bidirectional organelle motility in squid axoplasm (42). Nevertheless, ATP is clearly the optimal substrate for both enzymes, and was present in our perfusion buffers in a fourfold molar excess to a GTPγS concentration that caused maximum inhibition of organelle transport. ATPγS barely supports microtubule gliding mediated by kinesin or an axonemal dynein, though the latter can hydrolyze ATPγS with modest efficiency (69). As mentioned earlier, ATPγS appears to be relatively inert in the axon, neither supporting organelle motility on its own (42), nor inhibiting the ability of ATP to promote fast axonal transport, except when present in significant molar excess to ATP (7).

The possibility that GTPγS inhibited organelle motility by interfering with energy metabolism can also be eliminated. Prior studies demonstrated that 1 mM exogenous ATP sustains fast axonal transport, even when the ability of the axon to synthesize ATP is crippled by the addition of metabolic inhibitors, such as 2,6-dinitrophenol (7). The most plausible explanation for the results presented here is that GTP-binding proteins were the direct targets of GTPγS, and the mechanism of transport inhibition by GTPγs was independent of protein kinases A, C, or G, or protein phosphatases 1, 2A, or 2B.

It must be emphasized that the results of this study do not exclude other forms of regulation for fast axonal transport, and are not necessarily incompatible with results of the fish chromatophore studies (46, 47, 62, 63, 75). For example, protein phosphorylation or dephosphorylation may be important for initiating fast axonal transport at the origin of the axon, or halting motile organelles or reversing their direction of transport at specific axonal sites. Protein-bound phos-

phates that are relevant for organelle motility might normally turn over slowly through most of the length of the axon. If that were the case, our experiments with probes such as K-252a, microcystin-LR, and okadaic acid would not have revealed the importance of such posttranslational modifications. In addition, the inhibitors used did not affect all classes of kinases and phosphatases, so other types may be involved in regulating fast axonal transport.

The exact GTP-binding proteins which influence fast axonal transport remain to be determined. They are unlikely to include heterotrimeric G-proteins (4, 23), because AlF_4^-, a specific probe for proteins of this class (34, 74), did not affect microtubule-based transport in the axon (see Fig. 5). The most promising candidates include those which were detected on blots of axoplasm (see Fig. 6): small GTP-binding proteins (4, 24, 26) and the microtubule-stimulated GTPase, dynamin (54, 71). The small GTP binding proteins detected in axoplasm were ~25 kD, in the size range of some of the rab proteins, but generally higher in molecular weight than the ras or ARF proteins (35). In light of the evidence presented here, a basis for identifying the GTP-binding proteins that modulate organelle motility along microtubules, and characterizing their mechanisms of action has now been established.

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