Evidence That All Newly Synthesized Proteins Destined for Fast Axonal Transport Pass through the Golgi Apparatus

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ABSTRACT   Effects of the sodium ionophore, monensin, were examined on the passage from neuronal cell body to axon of materials undergoing fast intracellular transport. In vitro exposure of bullfrog dorsal root ganglia to concentrations of drug <1.0 μM led to a dose-dependent depression in the amount of fast-transported [^{3}H]leucine- or [^{3}H]glycerol-labeled material appearing in the nerve trunk. Incorporation of either precursor was unaffected. Exposure of a desheathed nerve trunk to similar concentrations of monensin, while ganglia were incubated in drug-free medium, had no effect on transport. With [^{3}H]fucose as precursor, fast transport of labeled glycoproteins was depressed to the same extent as with [^{3}H]leucine; synthesis, again, was unaffected. By contrast, with [^{3}H]galactose as precursor, an apparent reduction in transport of labeled glycoproteins was accounted for by a marked depression in incorporation. The inference from these findings, that monensin acts to block fast transport at the level of the Golgi apparatus, was supported by ultrastructural examination of the drug-treated neurons. An extensive and selective disruption of Golgi saccules was observed, accompanied by an accumulation of clumped smooth membranous cisternae.

Quantitative analyses of 48 individual fast-transported protein species, after separation by two-dimensional gel electrophoresis, revealed that monensin depresses all proteins to a similar extent. These results indicate that passage through the Golgi apparatus is an obligatory step in the intracellular routing of materials destined for fast axonal transport.

Passage through the Golgi apparatus (GA) is a highly conserved step in the intracellular transport of newly synthesized proteins that must reach the cell surface for membrane insertion or secretion (10, 19, 26, 38). By extending this generalization to neurons, it can be argued that proteins undergoing fast axonal transport also have been routed through the GA since their primary destinations are axonal and synaptic regions of the plasma membrane (11, 45). Evidence in support of this view is sparse but suggestive. Electron microscope autoradiographic studies provided the first indication that neuronal proteins synthesized in the rough endoplasmic reticulum (RER) pass through the GA before undergoing fast transport in the axon (5, 6). Further, several specific proteins known to be rapidly transported have been observed in association with GA. Acetylcholinesterase (AChE) activity reappears in neuronal RER and GA after inactivation of pre-existing AChE with diisopropylfluorophosphate (21, 29). Association between the transmitter-synthesizing enzymes, dopamine-β-hydroxylase and tyrosine hydroxylase, and GA has been demonstrated by immu-

nocytochemical techniques (3, 27). Finally, at least one group of fast-transported proteins, the glycoproteins, must pass through the GA (8, 16), since this intracellular site contains the glycosyltransferases for completing carbohydrate side chains (23, 31).

In non-neural cells, additional evidence for GA involvement in the intracellular routing of membrane-associated proteins has come from recent studies with the sodium ionophore, monensin. This compound has been found to inhibit secretion of a variety of proteins, including immunoglobulins (41, 43), amylase (42), AChE (33), and procollagen (42, 44). Similar effects were observed when monensin was directed against intracellular transport of surface rather than secretory proteins. Integral membrane proteins reached the GA but not the plasma membrane, as seen with surface antigens in lymph cells (40), virus membrane glycoproteins (20), acetylcholine receptors in muscle (30), and myelin proteins (28). At the ultrastructural level, striking alterations in the GA, including distended cisternae and dilated vacuoles, have been observed in drug-treated

cells. It is generally surmised that monensin prevents the formation of GA-derived vesicles that are normally required for delivery of proteins to the cell surface.

In this study, we used monensin as a probe to examine further the involvement of GA in the export of fast-transported proteins to the axon. In particular, we sought to determine whether passage through the GA is restricted to those proteins requiring post-translational glycosylation or represents a common step for all proteins destined for fast axonal transport. This study represents an extension of our interest in the initiation phase of fast axonal transport—the events by which selected proteins undergo centrifugal movement from their somal sites of synthesis to the fast transport system of the axon (13, 17). Parts of this work have been presented in preliminary form (15, 18).

MATERIALS AND METHODS

Conditions of In Vitro Axonal Transport

All experiments were carried out with the bullfrog *Rana catesbeiana* (Central Valley Biologicals, Clovis, CA). Dorsal root ganglia 8 and 9 were dissected in continuity with their respective spinal roots, spinal nerves, and sciatic nerve and placed in a multicompartment Lucite chamber that allows ganglia and nerve trunks to be selectively exposed to media of different compositions (4). In most experiments, each spinal nerve was ligated (6–0 silk thread) ~25 mm from the ganglion. Ganglia were pulse-labeled with a radioactive amino acid, sugar, or glycerol in a modified Ringer's solution (114 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 5.5 mM glucose, 20 mM HEPES, pH 7.4) that also contained 0.2 mM L-glutamine and a 1:50 dilution of other amino acids from Eagle's minimum essential medium (7). Conditions for pulse-labeling, incubation, termination, and processing of tissue were as previously described (35); details unique to each experiment are given in table or figure legends.

Stock solutions of 1 mM monensin in absolute ethanol were stored at −20°C. All working concentrations of monensin (0.02–1.0 µM in incubation medium) were 1% in absolute ethanol. This level of ethanol was routinely added to all control media even though preliminary studies showed that 1% ethanol had no marked effect on fast axonal transport of [³H]leucine-labeled proteins. In several experiments ganglia were exposed to monensin during a 1-h pre-incubation period as well as during the pulse-labeling period. These studies necessitated pulse-labeling of control and experimental ganglia in separate chambers. In all other experiments, the four ganglia from each animal were pulse-labeled in a common chamber, and the paired preparations were then transferred to control or experimental media.

Effects of monensin on fast axonal transport in desheathed spinal nerve trunks were examined after removal of ~20 mm of epineurium, from 10 mm below the ganglion to the junction of the eighth and ninth spinal nerves with the sciatic nerve (22).

Analysis of Fast-transported Proteins by Two-dimensional Gel Electrophoresis

After a 6-h pulse-labeling of ganglia with [³⁵S]methionine (100 µCi/400 µl medium) and a subsequent 18-h incubation, spinal nerves were desheathed, and the 4-mm nerve segment next to the ligature was prepared for electrophoresis as previously described in detail (35, 37). Briefly, nerve segments were homogenized in the presence of SDS, β-mercaptoethanol, Nonidet P-40 (NP-40), and urea (80 µl total volume). Samples were centrifuged and each total supernatant was subjected to two-dimensional gel electrophoresis (pI, ~4.5–8.5; ~20,000–200,000 mol wt). The positions of individual protein species on the gel were localized using the fluorographic pattern as a guide. Quantification of proteins was carried out by cutting selected spots from the gel and eluting labeled protein from each gel piece in scintillation fluid before counting (35, 46). Procedures of data analysis to determine the magnitude of monensin inhibition of individual fast-transported protein species have also been described (35).

Chemicals

Ultrastructural Analysis

Dorsal root ganglia were dissected from *R. catesbeiana* as described above and were incubated in a modified Ringer's solution for 1–12 h at 22°C. Basic incubation medium contained 114 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 11 mM glucose, and 20 mM HEPES at pH 7.4. All working concentrations of monensin (0.001–0.1 µM in the incubation medium) were in 0.25% ethanol. No morphological alterations were observed in ganglia incubated in media containing 0.25% ethanol without monensin.

After incubation, the roots and the spinal nerve were removed and the ganglia were prepared for electron microscopy as previously described (24). Briefly, the ganglia were fixed in 1% glutaraldehyde and 1% paraformaldehyde, postfixed in buffered 1% OsO₄, stained en bloc in 0.5% uranyl acetate, and embedded in Epon-Araldite. Ultrathin sections were stained with both uranyl acetate and lead citrate.

RESULTS

Effects of Monensin on the Amount and Rate of Materials Undergoing Fast Axonal Transport

The sensitivity of fast axonal transport to monensin was examined first by monitoring the accumulation of newly synthesized radiolabeled material next to a spinal nerve ligature. At 20 h after pulse-labeling of ganglia with [³H]leucine, the drug was seen to depress the amount of acid-insoluble radioactivity in a dose-dependent manner (Fig. 1 a). (During this time period, the only labeled materials reaching the ligature were those that had undergone rapid transport.) Similar experiments were carried out with [³H]glycerol as a test of proposals that lipids undergo fast-transport in a manner similar to that of protein (1, 12, 25, 32). Fig. 1 b shows a dose-dependent effect comparable to that seen with amino acid precursor. As also shown in Fig. 1, the effects of monensin were enhanced when nerve preparations were pre-incubated with the drug. No con-
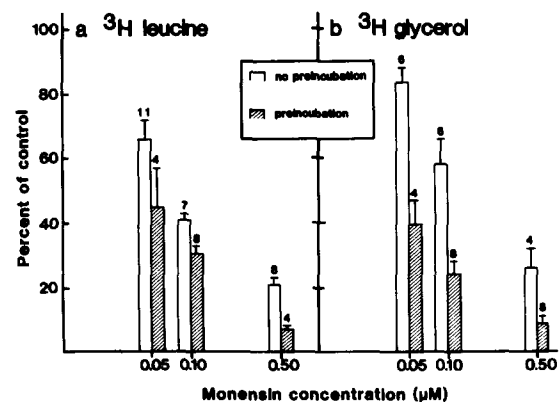

FIGURE 1   Effect of monensin on accumulation proximal to a nerve trunk ligature of fast-transported materials labeled with [³H]leucine (a) or [³H]glycerol (b). In one series of studies (open bars) ganglia of preparations from both sides of the animal were pulse-labeled for 1 h with either [³H]leucine or [³H]glycerol. One preparation was then transferred to medium containing 0.1 µM monensin while the contralateral preparation was transferred to drug-free medium. After 19 h at 18°C, the amount of radioactivity in the 3-mm nerve segment next to the ligature was compared in experimental and control preparations. Values were normalized to the total levels of incorporated precursor in the ganglia and nerve trunks of the twin preparations. In a second series of studies (hatched bars), the experimental preparations (ganglia and nerve trunks) were exposed to 0.1 µM monensin during a 1-h preincubation and a 1-h pulse-labeling period as well as during a 19-h incubation period. The percent of control transport (mean ± SE) is indicated for each concentration of monensin. The number of experiments for each set of conditions is given above the error bar.

sistent effect on incorporation of precursor was observed when the level of drug was kept below 1 μM (see Table I).

Incubation of nonligated nerve preparations in monensin generated transport profiles indicating a depressed amount of labeled material exported into the axon (Fig. 2). The shape of the profile also indicates that the onset of monensin action is rapid relative to the time for delivery of newly synthesized proteins to the axon. This is inferred from the magnitude of depression which appears similar for material appearing in the axon at early and late times. Little or no concomitant effect on the rate of transport was observed after either 9 or 18 h of incubation. Since the rate was unaffected, it seems unlikely that monensin inhibits ongoing transport within the axon. This was examined further by exposing desheathed spinal nerves to the drug while maintaining ganglia in drug-free medium (Fig. 3). No consistent differences in transport profiles were apparent between control and monensin-treated preparations.

The reversibility of monensin action was examined in studies where control and experimental ganglia of ligated nerve preparations were initially exposed to drug for 6 h, hour 2 of which was the pulse-labeling period. When experimental preparations were then returned to normal medium for 15 h, an increase of

labeled material in the 3-mm segment next to the ligature was observed (79.5 ± 27.9%; mean ± SE, n = 7) relative to the accumulation in control nerves maintained in monensin. Monensin effects on intracellular transport have also been shown to be reversible in studies on secretion of AChE (33) and immunoglobulins (39).

Figure 3 Profiles of [³H]leucine-labeled protein in desheathed spinal nerve selectively exposed for 12 h to control medium or to medium containing monensin. Epineurial sheaths were removed from spinal nerves of experimental (filled circles) and control (open circles) preparations. Nerve trunks of experimental preparations were exposed to medium containing 0.1 μM monensin during the 1-h pulse-labeling of ganglia and during the subsequent incubation period. Ganglia of experimental preparations, as well as ganglia and nerve trunks of control preparations, were maintained in drug-free medium during the postpulse incubation period. Each point (shown as mean ± SE) represents the radioactivity in the corresponding 3-mm nerve segment from four experiments.

TABLE I

Comparative Effects of 0.1 μM Monensin on Incorporation and Fast Axonal Transport Using [³H]Amino Acid or [³H]Sugar Precursors *

| | | Fast axonal transport§ | |
Precursor	Total incorporation‡	Uncorrected for incorporation	Corrected for incorporation
	cpm experimental/cpm control		
[³H]leucine (12)	1.07 ± 0.16	0.33 ± 0.05	0.31 ± 0.02
[³H]fucose (14)	0.99 ± 0.05	0.46 ± 0.03	0.47 ± 0.03
[³H]galactose (12)	0.45 ± 0.05	0.46 ± 0.06	1.00 ± 0.08

* Preparations were ligated, preincubated, pulse-labeled, and incubated under conditions similar to those in Fig. 1. Values are given as means ± SE; number of trials is shown in parentheses.

‡ Incorporation of precursor was monitored, after the incubation period, by summing acid-insoluble radioactivity in ganglia and successive 3-mm segments of nerve trunks.

§ Acid-insoluble radioactivity in the 3-mm nerve segment immediately next to a nerve ligature was used as the index of transport.
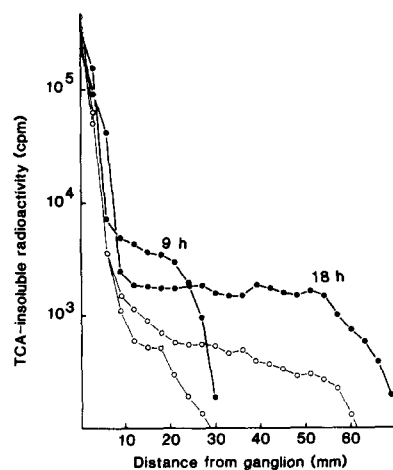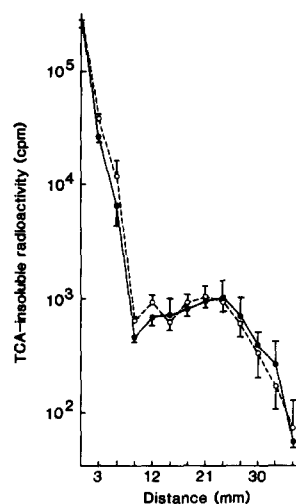
Figure 2 Profiles of [³H]leucine - labeled protein in spinal and sciatic nerve after 9 and 18 h of fast axonal transport in control medium and in medium containing 0.1 μM monensin. After pulse-labeling ganglia for 1 h at 22°C, each preparation was transferred to label-free incubation medium at 18°C for the duration of the experiment. Experimental preparations were preincubated with drug as described in legend to Fig. 1. TCA-insoluble [³H]protein was analyzed in successive 3-mm nerve segments as previously described (4). Experimental values (open circles) were depressed relative to control values (filled circles) to a similar extent at both time periods. Data are representative of four trials.
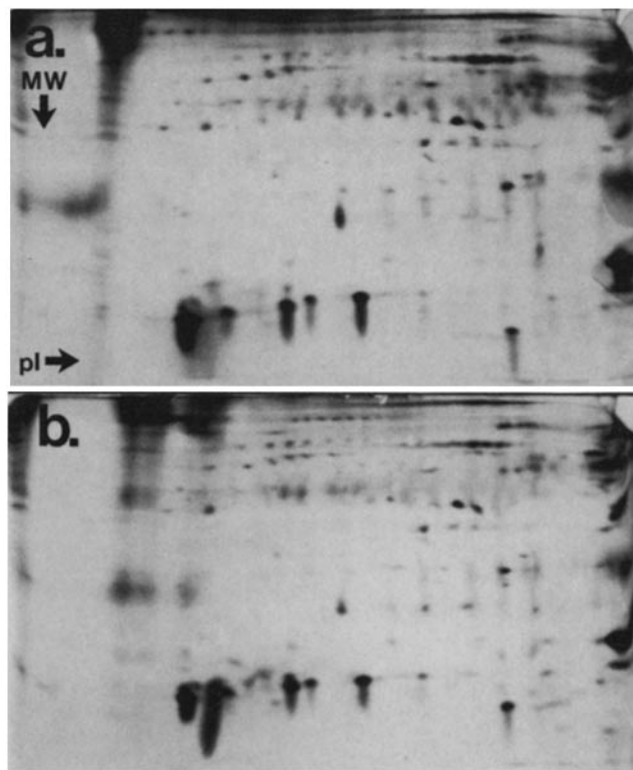
Figure 4 Fluorographic patterns of fast-transported proteins following two-dimensional gel electrophoresis. The patterns represent protein species transported in paired preparations exposed to control medium (a) or medium containing 0.1 μM monensin (b). Proteins were separated in the first dimension according to isoelectric point (pI); pH ranged from ~8.5 at the left of the gel to 4.5 at the right. Separation in the second dimension was according to molecular weight (MW) with an approximate range from 200,000 at the top of the gel to 20,000 at the bottom. See Fig. 5 for MW standards.
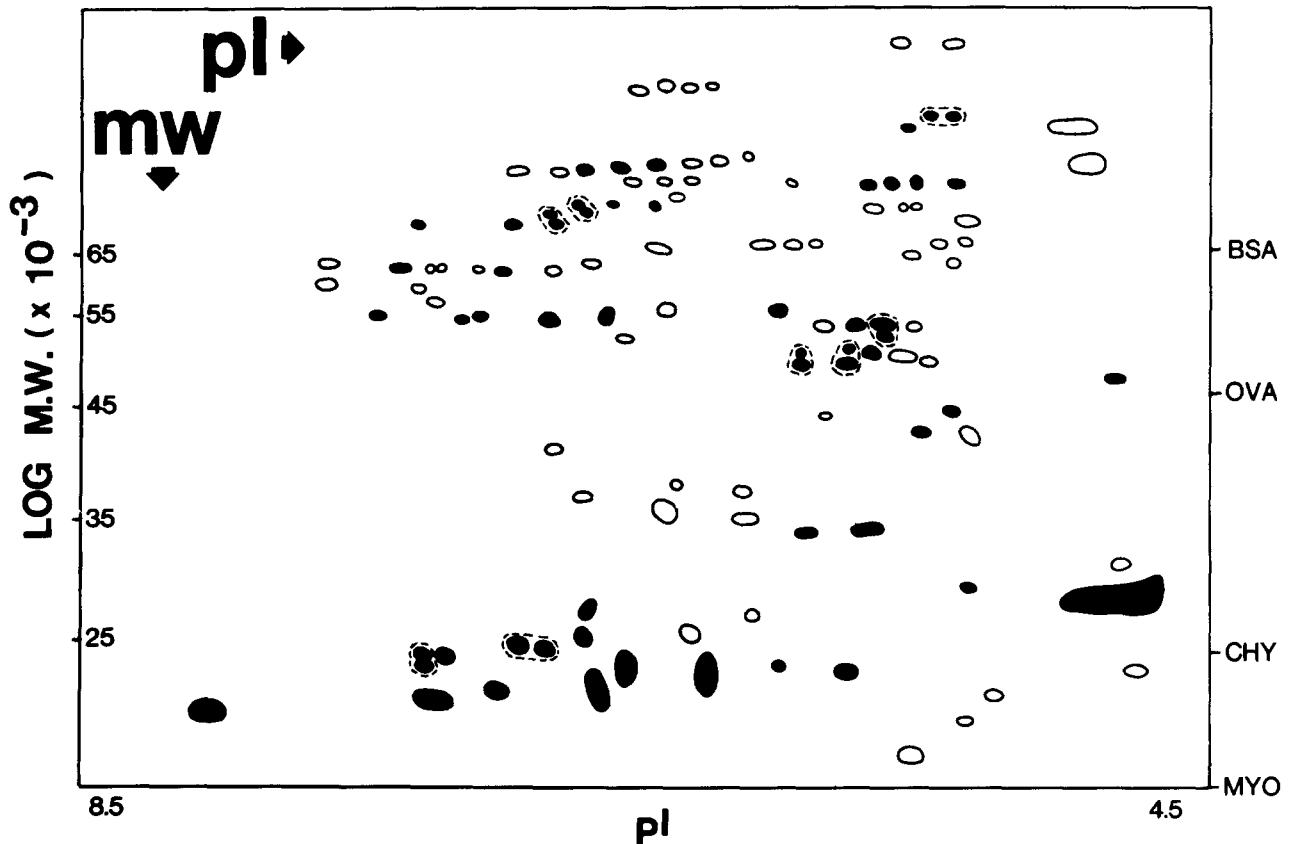
FIGURE 5 Reconstruction of a fluorographic pattern of fast-transported proteins. The pattern identifies the 48 most abundant transported species (filled symbols) that were chosen for quantitative analysis. Those spots outlined by dashes were analyzed together, since they could not be resolved on all gels as individual spots. Molecular weight estimations were made on the basis of distance migrated by bovine serum albumin (BSA), ovalbumin (OVA), chymotrypsinogen (CHY), and myoglobin (MYO). The indicated pI values are approximate.

Axonal transport of [^{3}H]fucose- or [^{3}H]galactose-labeled glycoproteins was next examined (Table I). Since addition of these sugars occurs in the GA (23, 31), effects of monensin on their incorporation and on the subsequent transport of labeled glycoproteins would help to localize the intracellular site of drug action. During the course of the pulse-labeling period there was no appreciable conversion of either fucose or galactose to amino sugars or amino acids (G. C. Stone and R. Hammerschlag, unpublished observations). With [^{3}H]fucose as precursor, monensin-treated neurons showed normal incorporation of the sugar, but the amount of fast-transported [^{3}H]fucose-labeled glycoprotein was markedly depressed. Thus, monensin affected [^{3}H]fucose- and [^{3}H]leucine-labeled proteins in a similar manner. By contrast, with [^{3}H]galactose as precursor, both incorporation of the sugar and the amount of fast-transported glycoprotein were depressed. Further, when the depression in transport was corrected for the concomitant reduction in galactose incorporation, monensin was found to have little or no net effect on transport.

Effects of Monensin on Individual Fast-transported Protein Species

Two plausible explanations for the observed actions of monensin are that all fast-transported proteins are depressed to a similar extent, and that subgroups of proteins are differentially affected. To examine these possibilities, proteins undergoing fast transport in preparations incubated in control medium or 0.1 μM monensin-containing medium were separated and com-
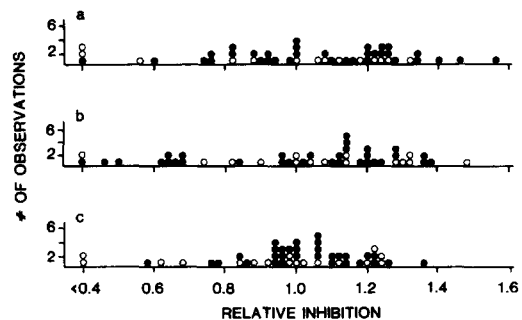

FIGURE 6 Frequency histograms of quantitatively analyzed gel spots. The 48 most abundant fluorographically revealed spots were localized on each gel, cut out, and their radioactivity levels determined by liquid scintillation counting. Relative inhibition values of these spots are shown for three representative gel pairs (a–c). Values either greater than or less than 1.0 reflect proteins that are respectively more sensitive or less sensitive to monensin than the average of all spots analyzed on that control/experimental gel pair. Proteins with molecular weights above (filled symbols) and below (open symbols) approximately 35,000 are also identified on each histogram since such an analysis had previously revealed subpopulations of proteins differentially sensitive to cobalt ions (35).

pared by two-dimensional gel electrophoresis (Fig. 4). No consistent qualitative differences were detected in the corresponding fluorographic patterns, suggesting that no fast-transported proteins are totally prevented from entering the axon. Quantitative comparisons were carried out on 48 of the most abundant species (Fig. 5). Normalization of raw cpm of the
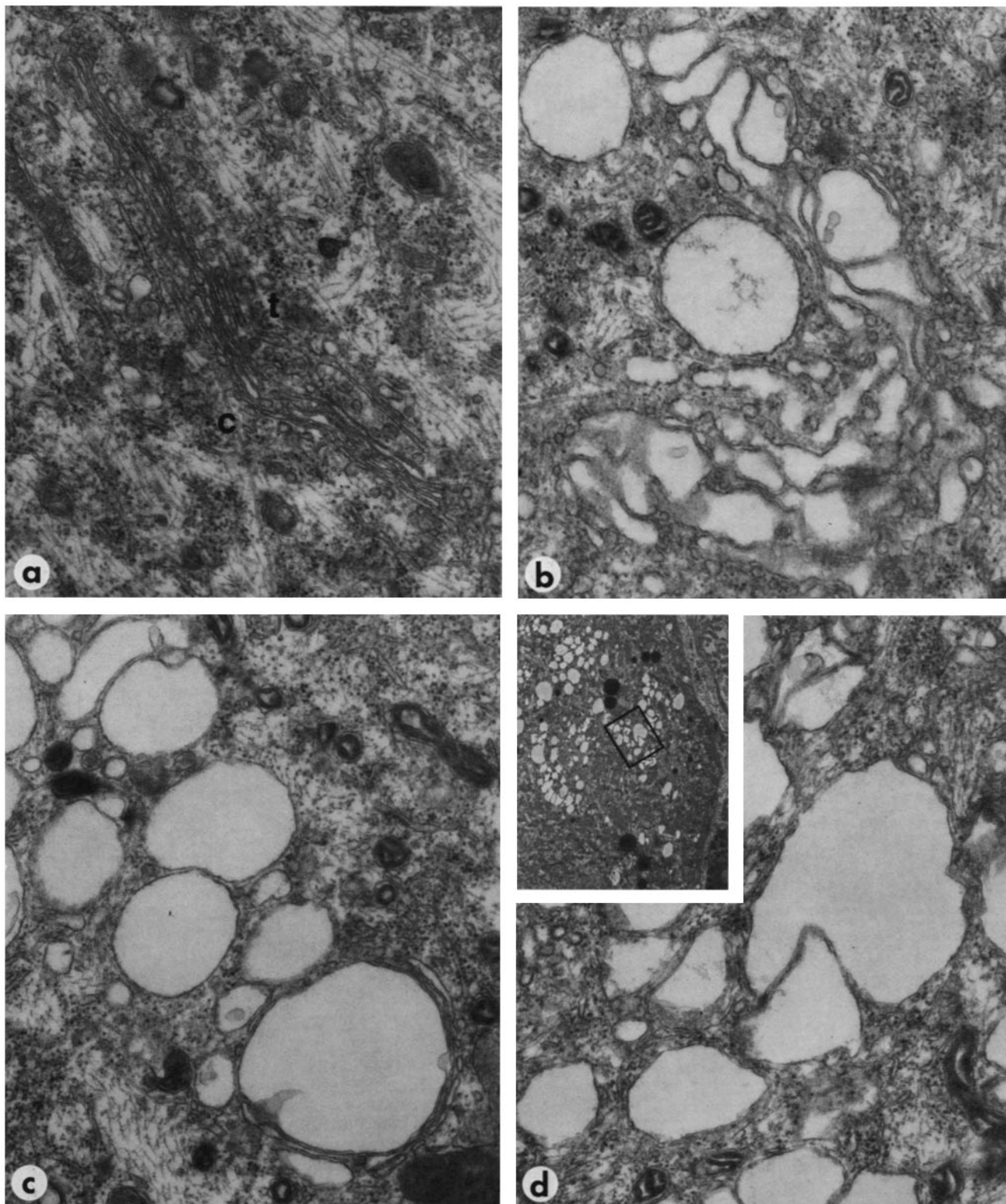
FIGURE 7   Golgi apparatus or its presumed equivalent in dorsal root ganglion neurons after incubation in control medium for 12 h (a) and in 10^{-7} M monensin for 1 (b), 3 (c), and 12 h (d). The cis (c) and trans (t) faces of the control Golgi apparatus are indicated. Disruption of the Golgi apparatus, which is well-progressed at 1 h, is accompanied by an accumulation of clumped rounded cisternae. By 12 h, these clumps have become quite extensive (see inset) and no trace of the Golgi apparatus saccules remains. × 30,000. Inset, × 2,000.

selected spots from six control/experimental gel pairs resulted in values grouped around a single mean; the extent of depression was $-55.2 \pm 4.0\%$ (mean ± SE).

Although direct quantitative comparisons suggest a common effect of monensin on all protein species, the data were examined further by an approach that had helped to define a relatively complex effect of cobalt ions on individual fast-transported species (35). Grouping protein species by molecular

weight (above and below 35,000 daltons) proved a useful criterion for defining two populations of proteins that are differentially affected by exposure of cell bodies to cobalt. Such an analysis of the present results, however, confirmed the impression that monensin affects all fast-transported proteins in a similar manner (Fig. 6). Values for both low and high molecular weight species appear grouped around a common mean. When specific proteins were examined in all three gel pairs, the extent of inhibition was consistent, similar to results in a previous study where such analyses were presented in detail (35).

Ultrastructural Analysis of Monensin-treated Cells

Within all experimental ganglia a decreasing gradient of cellular pathology was seen as cells deeper within the ganglia were examined. This gradient appeared to reflect a roughly 10-fold change in the concentration of monensin, i.e., cells near the center of ganglia incubated in 10^{-8} M monensin appeared similar to cells near the surface of ganglia incubated in 10^{-9} M monensin. Since most of the cell bodies lie near the surface of the ganglia, the following descriptions will refer to cells near but not at the surface of the ganglion.

Ganglia were examined following 1, 3, 6, and 12 h of incubation with 10^{-7} M monensin. After 1 h, the GA saccules were markedly swollen (Fig. 7). In addition, large round cisternae were often seen near the GA stacks. At 3 h (Fig. 7c), very little of the familiar GA structures remained. Instead, the cells were filled with many clumps of the rounded cisternae. Occasionally, flattened sacculelike structures were seen in close association with the rounded cisternae. Ganglia incubated for 12 h (Fig. 7d) appeared essentially the same as those incubated

for 6 h. Here, the extensive clumps of rounded cisternae appeared less defined than at 3 h. Furthermore, no evidence of the flattened sacculelike structures was seen in these neurons. At all times, the remaining cytoplasmic organelles—rough endoplasmic reticulum, cytoskeletal elements, mitochondria, and lysosomal and lipid bodies—appeared to be unaffected by the treatments.

At lower concentrations, the effect of monensin appeared less dramatic and more specific. After 12-h exposure to 10^{-9} M monensin, moderate swelling of the intermediate saccules was observed (Fig. 8a). Except for their displacement by the swollen intermediate saccules, the cis and trans face saccules appeared unchanged. Exposure for 12 h to 10^{-8} M monensin resulted in much more striking swelling of the intermediate GA saccules (Fig. 8b). In addition, these cells often contained small clumps of the rounded cisternae.

DISCUSSION

The present studies represent a further examination of the proposal that newly synthesized proteins destined for fast axonal transport are routed through the neuronal soma in a manner similar to that of proteins targeted for membrane insertion or secretion in non-neuronal cells (16, 17). In particular, the ionophore monensin—which is increasingly being recognized as a useful tool for blocking intracellular transport at the level of the GA (e.g., reference 42)—has been found to exert a potent inhibitory action on the transfer of fast-transported proteins from their sites of synthesis to the axon. The drug inhibits initiation of fast axonal transport in dorsal root ganglion neurons at concentrations similar to those effective in other secretory systems, and its major site of action in neurons is within the GA. At the ultrastructural level, an extensive and specific disruption of GA structure is observed in the neuronal
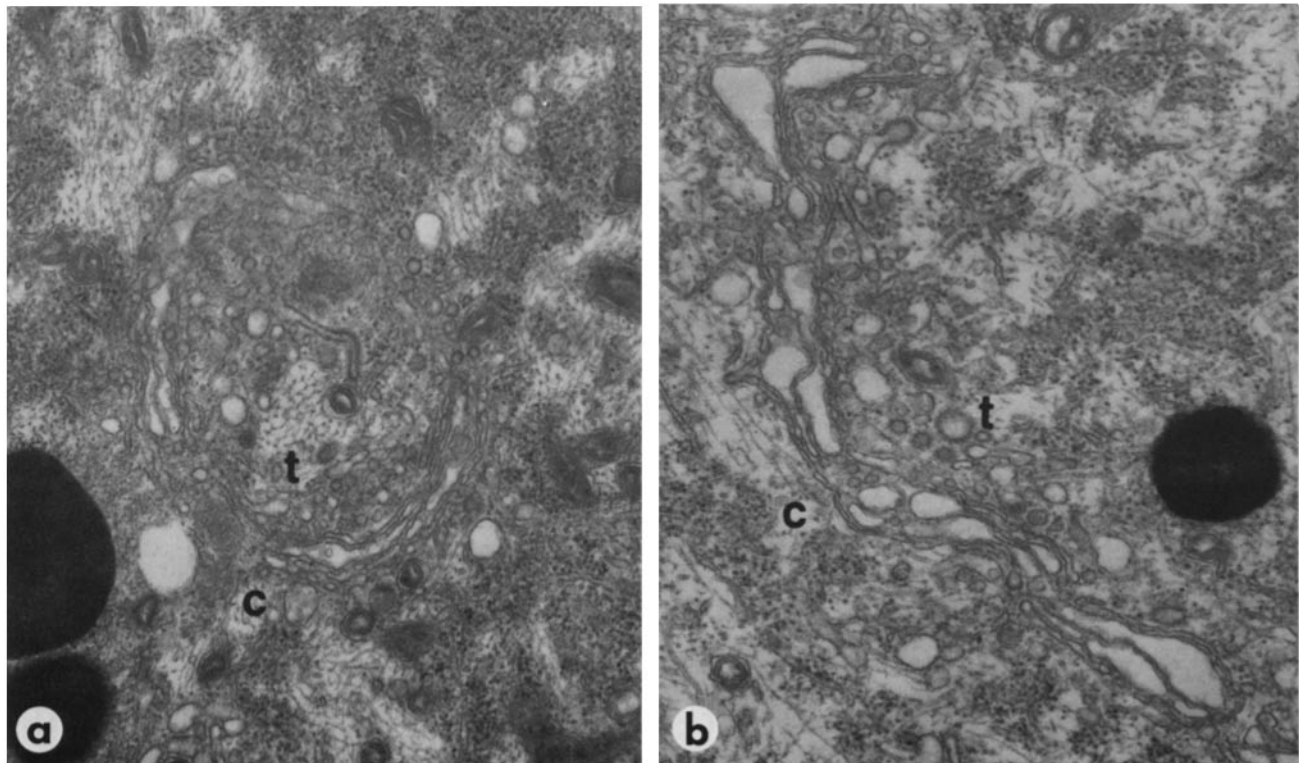

FIGURE 8   Golgi apparatus after 12-h incubation in 10^{-9} M monensin (a) and in 10^{-8} M monensin (b). Note that the intermediate saccules are especially sensitive to these treatments. × 30,000.

cell bodies of monensin-treated ganglia. Furthermore, there is a dramatic accumulation of clumped smooth cisternae. Functionally, the GA is implicated as the site of monensin action by the finding that incorporation of [³H]galactose into fast-transported proteins is depressed by the drug under conditions where [³H]leucine incorporation is unaffected. Thus, incorporation of galactose, which occurs predominantly in the GA, would seem to be reduced as a result of fewer substrate glycoprotein molecules gaining access to the sites of galactosyltransferase activity. Taken together, these findings clearly indicate that monensin disrupts normal GA organization and function.

Since the apparent reduction in amount of galactose-labeled proteins transported within the axon is accounted for by the depressed incorporation of the sugar, monensin has no further effect on transport once glycoproteins have exited the GA. Consistent with this conclusion is the lack of effect on ongoing fast transport when desheathed nerve trunks are exposed to the drug. Immunoglobulin secretion is affected by monensin in a similar manner. Once terminal sugar addition is completed, intracellular transport of these molecules to the cell surface is unaffected by the drug (43).

Our finding that monensin depresses incorporation of galactose but not fucose was unexpected since there is little evidence that terminal sugars are each added in a separate region of the GA (2, 43). That the drug may be directly affecting galactosyltransferase is not likely since many of the individual fast-transported proteins that are depressed by monensin do not contain either galactose or fucose (36).[1] It is also of interest that Co^{2+} exerts the same differential effect on sugar incorporation as does monensin (14). These findings are consistent with the possibility that fucose and galactose incorporation occur at spatially distinct sites such that monensin and Co^{2+} (quite likely by different mechanisms) can affect incorporation of one sugar and not the other. Current studies on the structure and maturation of the carbohydrate side-chains of fast-transported glycoproteins may help to resolve this question.

The present findings further clarify the initiation phase of fast axonal transport, the somal events involved in delivery of selected proteins to the axon. First, a central role for the GA is underscored. Since biochemical and morphological evidence suggest that monensin acts at this intracellular site, and since appearance in the axon of all fast-transported species is inhibited by the drug, the implication is that all proteins destined for fast transport pass through the GA. Such a common routing is of interest for future studies that must define the stage and the means by which specific proteins become committed to undergo fast axonal transport. It seems reasonable, for instance, that the GA may be involved in sorting out fast-transport proteins (that ultimately appear associated with axolemma or membrane structures at the nerve terminal) from classes of proteins that become associated with other membranous organelles of the cell (e.g., lysosomes and mitochondria). A similar obligatory passage through the GA, independent of whether carbohydrate addition is required, has been proposed for the intracellular transport of secretory proteins in the exocrine pancreas (see reference 38).

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[1] After this study was completed, a report appeared describing the inhibition of galactosyltransferase activity in rat embryo fibroblasts treated with monensin (R. P. Kitson and C. C. Widnell. 1981. *J. Cell Biol.* 91 [2, Pt. 2]: 405a[Abstr.].) Of particular interest is that monensin did not affect the activity of this enzyme when added directly to an in vitro assay system.

It is also of interest that monensin appears to inhibit all individual fast-transported species to a similar extent. This is in contrast to evidence suggesting that cobalt ions affect subpopulations for fast-transported proteins to differing extents at a step before passage through the GA (35). These populations are also distinguishable on the basis of molecular weight (35), transit-time in the soma (34), and by glycosylation (36). That subpopulations are not seen with respect to sensitivity to monensin suggests a common handling of fast-transported materials distal to the site of monensin action in the GA.

Present findings also strengthen the emerging view of fast axonal transport as a movement of assembled membrane (1, 9, 12, 17, 25, 32). That monensin blocks transport of glycerol-labeled material in a manner similar to that of leucine-labeled protein can be added to observed similarities in rates, Q_{10} values and subcellular distribution (e.g., reference 1) to indicate that fast-transport of lipid and protein are closely interrelated. The marked accumulation of smooth-membrane cisternae observed in pre-GA regions of monensin-treated cells is also consistent with a block of lipid and protein transport. Thus, monensin may impede delivery of newly synthesized protein and lipid to the axon by preventing assembled membrane structures from completing their obligatory passage through the GA.

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