Effect of monensin on plant Golgi: re-examination of the monensin-induced changes in cisternal architecture and functional activities of the Golgi apparatus of sycamore suspension-cultured cells

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SUMMARY

We have re-examined the effects of the ionophore monensin on the Golgi apparatus of sycamore maple suspension-cultured cells using a combination of high pressure freezing, immunocytochemical and biochemical techniques. Exposure of the cells to 10 μM monensin, which reduces protein secretion by ~90%, resulted first in the swelling of the *trans*-Golgi network, then of the *trans*-most *trans*-cisterna, the remaining *trans*-cisternae, and finally of the *cis* and medial cisternae. We postulate that these different rates of swelling reflect an underlying hierarchy of compartmental acidification with the *trans*-Golgi network being the most acidic compartment. Recovery occurred in the reverse sequence. Previous studies have suggested that the large swollen vesicles that accumulate in the cytoplasm of monensin-treated cells arise from the swelling and detachment of entire *trans*-cisternae. However, based on the many membrane blebbing configurations seen in association with the *trans*-Golgi network and the *trans*-Golgi cisternae of monensin-treated cells, and the fact that the surface area of the *trans*-Golgi cisternae is about five times greater than the surface area of the swollen vesicles, it appears that the swollen vesicles are produced by a budding mechanism. After 35-40 min of monensin treatment, cells with smaller, non-swollen, compact Golgi stacks began to appear and rapidly increased in number, contributing >60% of the cell population after 60 min and >80% after 100 min. In contrast, large numbers of swollen vesicles persisted in the cytoplasm of all

cells for over 100 min. Since azide treatment of monensin-treated cells can prematurely induce the un-swelling response and cellular ATP levels drop substantially after 45 min of monensin treatment, we propose that un-swelling of the Golgi stacks is due to a monensin-induced decline in ATP levels in the cells.

Immunocytochemical labeling of the high pressure frozen cells with anti-xyloglucan antibodies demonstrated that the concentration of xyloglucan, a hemicellulose, in the swollen vesicles increased with time. This increase in vesicle contents may explain why these swollen vesicles do not contract in parallel with the Golgi stacks. In vivo labeling experiments with [3H]fucose, [3H]UDP-glucose and [3H]leucine demonstrated that monensin-treatment not only inhibited protein secretion, but also cellulose synthesis. Protein synthesis, on the other hand, was reduced only slightly during the first 30 min of treatment, but quite strongly between 30 and 60 min, consistent with the observed drop in ATP levels after >40 min of exposure to monensin. Taken together, our findings indicate that the Golgi apparatus of plant cells responds to monensin in a much more differentiated manner than envisaged to date, thereby opening up interesting new possibilities for analyzing its functional organization.

Key words: Golgi apparatus, monensin, swelling, un-swelling, swollen vesicles, xyloglucan (XG)

INTRODUCTION

The Golgi apparatus of plant cells serves as the site of assembly of the oligosaccharide side chains of glycoproteins and proteoglycans, and the site of synthesis of the complex polysaccharides of the cell wall matrix. The glycoprotein and proteoglycan assembly pathways have much in common with those found in animal cells (Chrispeels, 1991; Faye et al., 1989; Kaushal et al., 1988), whereas those

involved in the production of complex polysaccharides are unique to plants (Zhang and Staehelin, 1992). Since both types of molecules are assembled side by side in Golgi stacks (Moore et al., 1991), but synthesis of the polysaccharides is initiated in different cisternae (Zhang and Staehelin, 1992), it is of interest to determine to what extent the site of synthesis affects the trafficking of different Golgi products.

Monensin, a monovalent cation ionophore that exchanges

Na^{+}, K^{+} and protons across membranes and thereby affects the acidification of acidic membrane compartments (Pressman and Fahim, 1982), has been used extensively to study the function of the Golgi apparatus in a variety of animal and plant systems. In animal cells, monensin has been shown (1) to cause swelling of Golgi cisternae and secretory vesicles, (2) to slow or arrest intra-Golgi transport of newly synthesized secretory proteins, (3) to interfere with the late Golgi functions such as proteolytic processing and the addition of terminal sugars to *N*-linked glycoproteins and (4) to lead to the accumulation of membrane and secretory protein precursors in medial or *trans*-Golgi cisternae, or condensing "vacuoles" (reviewed by Farquhar, 1985; Tartakoff, 1983).

The effects of monensin on the secretion of plant cells are less clearcut. Ultrastructural studies have shown that monensin leads to the accumulation of Golgi-derived swollen vesicles in the cytoplasm of treated cells (Mollenhauer et al., 1988; Morré et al., 1983; Shannon and Steer, 1984). According to Boss et al. (1984), the formation of these swollen vesicles is dependent on the active pumping of protons into *trans*-Golgi cisternae and on the monensin-mediated exchange of lumenal protons with more osmotically active cations. Furthermore, based on a careful kinetic analysis of the swollen vesicles in the cytoplasm, and in accordance with the cisternal progression model of Golgi function, Morré et al. (1983) have postulated that these vesicles arise from swollen *trans*-Golgi cisternae that became separated from the stacks. However, since swelling artifacts are difficult to control in chemically fixed samples processed for electron microscopy, the exact extent of swelling of different Golgi compartments has been difficult to evaluate (Mollenhauer et al., 1988). Monensin has also been reported to block the biosynthesis and/or the transport of fucose- and galactose-labeled macromolecules to the bean cotyledon walls, to block the transport of phytohemagglutinin from the Golgi apparatus to protein bodies (Chrispeels, 1983), to interfere with the post-translational processing of proteins in vacuoles and protein bodies (Stinissen et al. 1985), to block the secretion of -amylase from barley aleurone cells (Heupke and Robinson, 1985; Melroy and Jones, 1986), to inhibit the transfer of cellulose-synthesizing particle rosettes (Rudolph and Schnepf, 1988) and very long chain fatty acid-containing phospholipids (Bertho et al., 1991) to the plasma membrane and to cause mistargeting of storage proteins to the cell wall of pea cotyledon cells (Bowles et al., 1986; Craig and Goodchild, 1984). On the other hand, whereas Shannon and Steer (1984) observed a complete cessation of slime droplet formation by root cap cells of *Zea mays*, Sticher and Jones (1988) found in their biochemical studies no effect of monensin on the secretion of polysaccharide slime by root tips of corn. Similarly, Driouich et al. (1989) have reported that although monensin inhibits the incorporation of fucose and *N*-acetylglucosamine into the complex glycans of acid hydrolases of sycamore suspension culture cells, it does not prevent their secretion.

To set the stage for more detailed biochemical studies on the effect of monensin on the functional organization of the secretory pathway of plant cells, we have re-examined the effects of monensin on the structural organization of the different compartments of the secretory apparatus of sycamore maple suspension culture cells, using high pressure freezing/freeze-substitution techniques. In parallel, we have also monitored biochemically the effects of monensin on the synthesis and secretion of glycoproteins and polysaccharides, and probed by immunocytochemical techniques for the presence of xyloglucan in the swollen vesicles.

MATERIALS AND METHODS

Plant materials and drug treatment

Suspension-cultured sycamore (*Acer pseudoplatanus* L.) cells were kindly provided by Dr M. G. Hahn (Complex Carbohydrate Research Center, University of Georgia, Athens, GA), cultured in modified M6 medium (Torrey and Shigemura, 1957) on a shaker (125 revs/min) at 25°C in the dark, and harvested for treatment at log phase of growth.

Treatments in sycamore culture cells were conducted by adding a certain volume of monensin (Sigma Chemical Co., St. Louis, MO, USA) stock solution (1 mM) in methanol to 10 ml or more of cell suspension cultures. The final concentration of monensin was 10 μM.

Sample preparation for electron microscopy

After exposure to monensin for 5 to 100 min, the sycamore culture cells were collected by centrifugation (1000 g) and mixed with 22-25% (w/v) aqueous dextran (M_r: 38,800) in culture medium for high pressure freezing, as described by Zhang and Staehelin (1992). For studying the time course of recovery from monensin, the cells were transfered to a 30 μm nylon mesh, washed three times with fresh medium and then fixed as described at 5, 10, 15, 40 and 80 min after removing monensin from the growth medium. After high pressure freezing, the specimen cups were transfered to 1.8 ml cryogenic vials for freeze-substitution and subsequent embedding in Epon 812 for the morphological and in LR White for the immunolabeling experiments, as described by Zhang and Staehelin (1992). Sections on copper grids were counterstained with uranyl acetate and lead citrate and finally examined in a Philips CM 10 electron microscope.

Measurement of surface area

To compare the surface area of *trans*-Golgi cisternae and swollen vesicles that accumulated in the cytoplasm in the presence of monensin, we have determined the average diameter of these two membrane-bound compartments from measurements made on 20 selected electron micrographs, and then calculated the average surface area (S) of *trans*-Golgi cisternae, assuming a disc configuration (S=2 r^2), and the average surface area of medium and large swollen vesicles, assuming the shape of a sphere (S=4 r^2).

Immunolabeling experiments

As described in detail by Zhang and Staehelin (1992), the LR White sections were incubated first with a milk solution, then with the primary antiserum and finally with the Protein A-gold or the secondary antibody-gold particles. The antibodies used in this study were polyclonal anti-xyloglucan (anti-XG) antibodies and the monoclonal antibody CCRC-M1, the specificity of which has been discussed by Zhang and Staehelin (1992).

Radiolabeling experiments and monensin treatment

Sycamore cells were harvested, filtered, washed and resuspended in fresh culture medium lacking sucrose (3 g of cells per 10 ml

medium), as described by Driouich et al. (1989, 1992). The cells were incubated for 30 or 60 min with 10 µCi of [³H]leucine, [³H]fucose or [³H]UDP-glucose, and monensin added to a final concentration of 10 µM. The controls lacked monensin.

Measurement of cytoplasmic ATP levels

100 µl samples of control and monensin-treated cells in Eppendorf tubes were quickly frozen three times in a dry ice/acetone bath and warmed to 0°C to break open the cells. After the third thawing, 1 ml of distilled water was added to each sample and quickly mixed. 10 µl of this cell extract was transferred to the reaction tube of a TD-20e Luminometer (Turner Designs, Inc., Mt View, CA, USA) followed by 50 µl of a luciferase-luciferin solution (13.3 mg/ml; Sigma). After a 3 s delay to allow the mixture to equilibrate, the total number of light units produced over a 5 s interval (3000 to 10,000) were counted.

Precipitation of secreted proteins and extraction of hemicelluloses

At the end of the labeling experiments, the culture medium was collected by filtration and divided into two fractions; one fraction was used to precipitate secreted proteins, and the other to precipitate hemicelluloses. Secreted proteins were precipitated with trichloroacetic acid (TCA), as described by Driouich et al. (1992), and then resuspended in a buffer containing 20 mM Tris-HCl, pH 8.6, 1% SDS and 0.3% -mercaptoethanol to ensure complete solubilization. Hemicelluloses secreted into the culture medium were precipitated with 85% ethanol (final concentration) containing 0.5% (w/v) ammonium formate, according to Fry (1988), and dissolved in distilled water.

To extract hemicelluloses from the cell wall, the cells were washed with fresh medium and stirred in phenol/acetic acid/H_2O (2/1/1, by vol.) for 16 h at 25°C to remove cytoplasmic material (Fry, 1988). The insoluble residues (cell wall) were washed extensively with H_2O and the hemicelluloses extracted with 6 M NaOH/1% NaBH$_4$ (w/v), as described by Fry (1988). The cytoplasmic material was precipitated with acetone and ammonium formate (Fry, 1988). A sample of each fraction was mixed with scintillation liquid in plastic vials and the radioactivity measured in a Beckman LS 6800 scintillation counter.

RESULTS

Swelling response of the secretory pathway compartments to monensin

Fig. 1A illustrates the electron microscopic appearance of the cytoplasm of a control sycamore maple suspension-cultured cell preserved by high pressure freezing/freeze-substitution. The organization of the endoplasmic reticulum and the Golgi stacks is typical of plant suspension-cultured cells in general. In the context of this paper, the lack of large secretory vesicles should be noted. Most Golgi stacks in these cells consist of five to six cisternae, which can be subdivided into cis, medial and *trans*-types based on their morphology and their immunocytochemical labeling patterns (Figs 2-4; Zhang and Staehelin, 1992). "*cis*" cisternae occupy the end of the stack opposite the most densely staining *trans*-cisternae. They have the widest lumina, the most variable shape, and pick up the least stain, making them often difficult to discern against the background. Virtually all Golgi stacks of our cultured sycamore maple cells possess only one cis type of cisterna. "Medial" cisternae

are narrower and their mottled contents stain more heavily than those of the cis cisternae. Typical of "*trans*" cisternae is a collapsed, very thin lumen filled with uniformly dense products. In sycamore maple suspension culture cells not all Golgi stacks possess a clearly definable *trans*-Golgi network (TGN; Zhang and Staehelin, 1992). When present, it appears to consist of rounded, densely staining vesicles that are interconnected by branched, tubular elements, and clathrin coats are occasionally discernable on the rounded vesicles (Fig. 1B). In cases where no distinct TGN is seen (Fig. 1A), the terminal *trans*-cisterna often assumes an intermediate morphology by displaying some densely staining rounded and/or branching vesicular configurations, suggesting that in these Golgi stacks the trans-most cisterna(e) may assume some TGN functions. The TGN shown in Fig. 1B is larger and more distinct than the average TGN of most Golgi stacks of sycamore suspension culture cells.

Upon exposure of the cells to 10 µM monensin, changes in the morphology of high pressure frozen Golgi stacks become evident in less than 5 min (Fig. 2A). The first structure to be affected is the TGN, whose rounded vesicles and interconnecting tubes become swollen and lose their ability to bind stain (compare Figs 1B and 2A). The next compartment to swell is the terminal *trans*-cisterna (Fig. 2A). By 10 min, all of the *trans*-cisternae appear swollen, and some of the larger TGN-derived swollen vesicles begin to separate from the TGN (Fig. 2B). After 20 min of monensin treatment all of the Golgi cisternae appear swollen, but the *trans*-cisternae still exhibit the greatest amount of distortion (Fig. 3A,B). However, as seen in Fig. 3B, swelling of the cis and medial cisternae may continue, making them increasingly difficult to distinguish from the actual *trans*-cisternae. According to Morré et al. (1983), the number of swollen vesicles increases in a time-dependent manner for up to an hour. In our samples, those that accumulated within the first 20 min tended to be larger and their contents more translucent than those formed later (compare Figs 3A and 5A). Although it is impossible to deduce from static images the exact origin of the different vesicles, it appears likely that the more lightly stained vesicles arise by budding from the TGN and the more densely stained ones from the *trans*-Golgi cisternae (Figs 3A,B, 6). To test the hypothesis of Morré et al. (1983) that the swollen vesicles arise from the swelling and detachment of whole *trans*-Golgi cisternae, we have calculated the surface area of these two structures, based on their apparent diameter in our micrographs. These calculations indicated that an average swollen vesicle has a surface area of about 0.16 µm², whereas the average surface area of a *trans* cisternae is approximately 0.97 µm².

The next striking change in Golgi morphology becomes evident after about 40 min exposure to monensin, with the appearance of two types of Golgi stacks, one with greatly swollen cisternae (Fig. 4A), the other with smaller, but nearly normal-looking, non-swollen ones (Figs. 4B, 5A). Both are surrounded by large numbers of swollen vesicles. Only the TGN of the non-swollen, compact type of Golgi stacks retains some amount of swelling when present (Figs. 4B, 5A). Careful analysis of the cells exposed to monensin for 40 to 60 min shows that the two types of Golgi stacks are largely limited to different sets of cells. Thus, one type
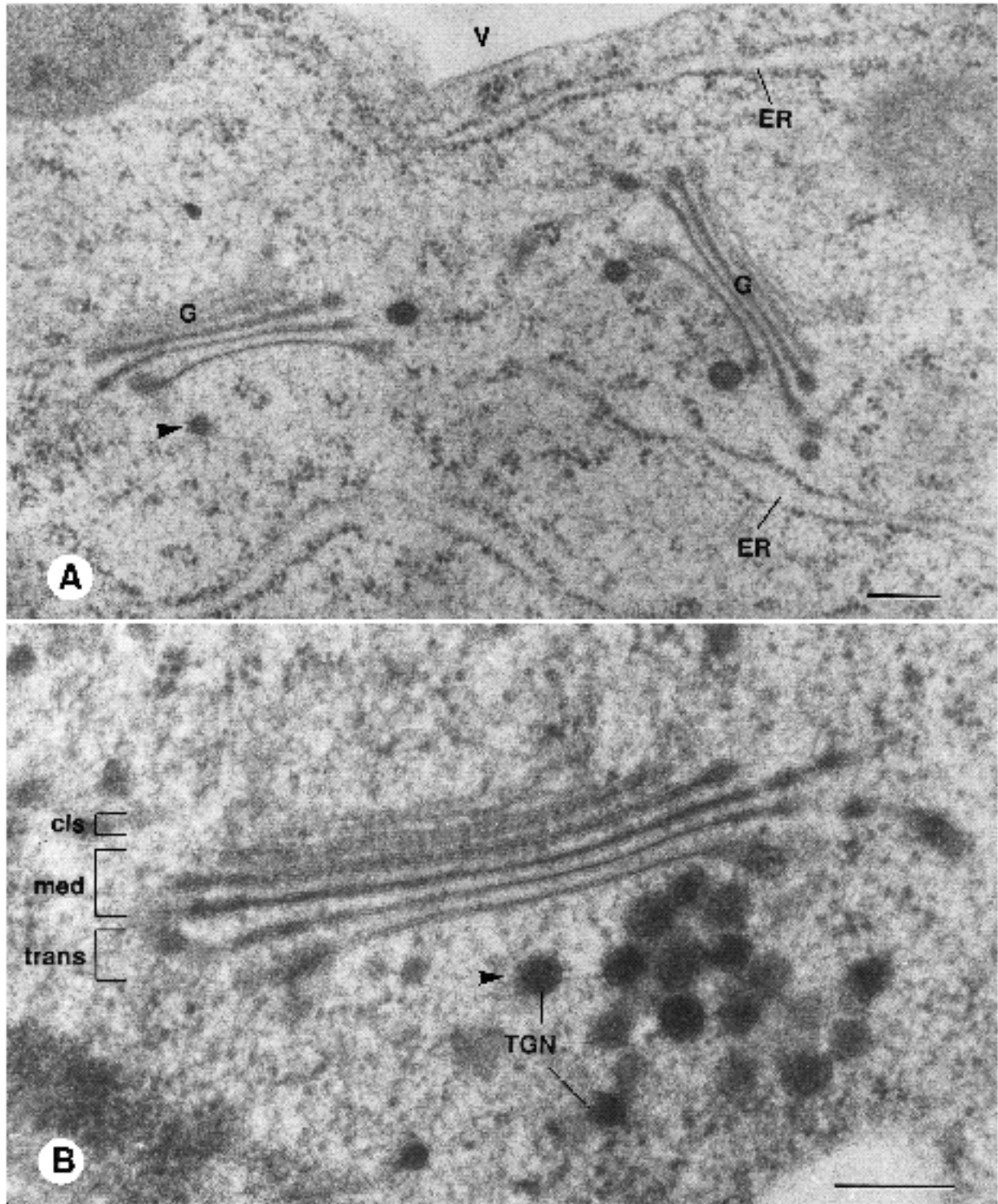
Fig. 1. Electron micrographs of suspension-cultured sycamore maple cells (no monensin treatment) preserved by high pressure freezing/freeze-substitution techniques. (A) Typical example of the cytoplasm of a control cell exhibiting the Golgi stacks (G), endoplasmic reticulum (ER), vacuole (V) and dense vesicles. Note the absence of swollen vesicles in the cytoplasm. (B) Higher magnification image of a Golgi stack and associated *trans*-Golgi network (TGN). The stack displays a distinct structural polarity that can be used to distinguish one *cis*, three medial and two *trans* cisternae. The unusually large TGN consists of a number of small dense vesicles. Arrowheads in A and B point to clathrin-coated vesicles. Bar, 0.2 μm.
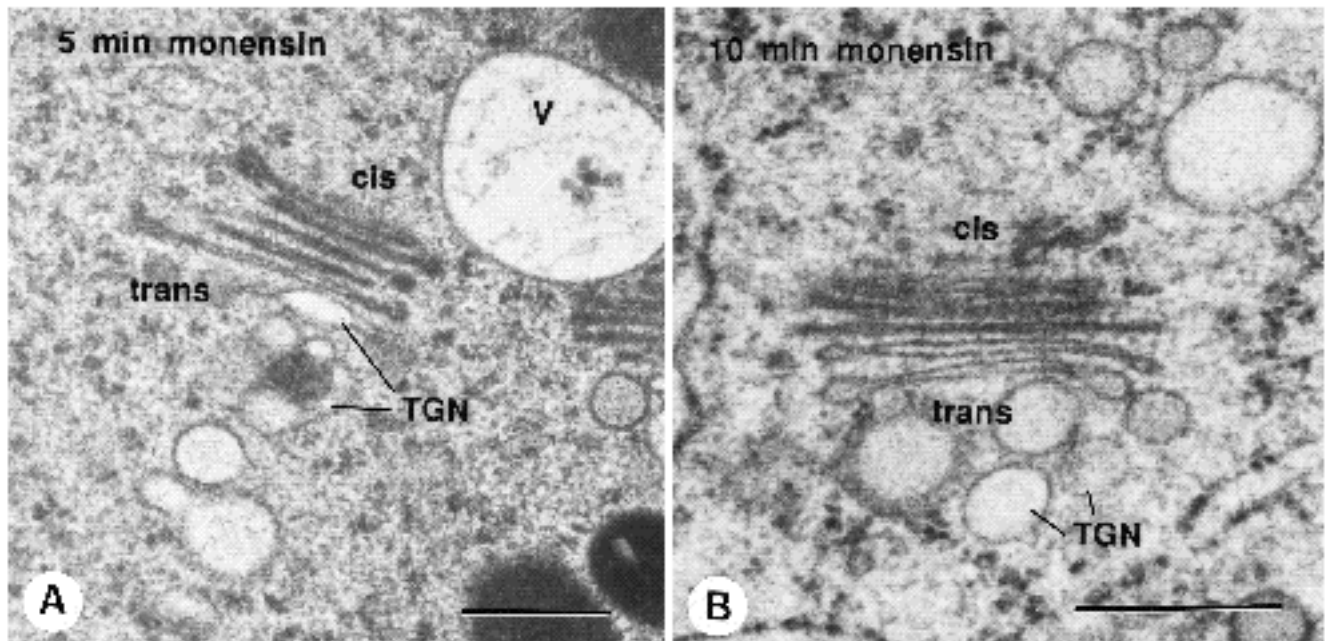
Fig. 2. Golgi stacks of sycamore culture cells treated with 10 μM monensin for 5 min (A) and 10 min (B). In A, only the vesicles which constitute the TGN are swollen; in addition, the lumen of contents of the terminal *trans*-Golgi cisterna appear to have lost their stainability. After 10 min (B), both the TGN and the *trans* cisternae appear swollen, enlarged and lack stained contents. TGN, *trans*-Golgi network; V, vacuole. Bar, 0.5 μm.

of cell exhibits only swollen Golgi stacks and the other only smaller, non-swollen ones. Very few cells possess both swollen and non-swollen side by side in their cytoplasm. Between 40 min and 60 min of monensin treatment, the percentage of cells containing non-swollen Golgi stacks increased in our samples from 20% to over 60%, and by 100 min to over 80%. However, as discussed in the biochemical section below, this structural "recovery" is not matched by a functional recovery of the secretory apparatus.

To test whether the above un-swelling of Golgi stacks after >40 min of monensin treatment is caused by the reduction of ATP levels in those treated cells, we have incubated sycamore culture cells with 10 μM monensin and 10 mM sodium azide, a competitive inhibitor of cytochrome oxidase, to reduce ATP levels in the cytoplasm (Persson et al., 1988). As expected, no swollen Golgi stacks and associated compartments were seen in such samples (data not shown). In a second set of experiments, we have determined directly the effect of monensin on cytoplasmic ATP levels using the luciferase-luciferin assay. The most striking change was found between the 45 min and 100 min monensin-treated samples, where a drop of close to 50% in cellular ATP levels was measured.

Cup-shaped Golgi stacks (Glas and Robinson, 1982) were most evident in the 5 to 10 min and the >60 min monensin samples, but never accounted for more than 25% of the stacks. Typical of monensin-treated cells is also a proliferation of multivesicular bodies (Fig. 5C), which suggests that monensin may also affect the endocytic pathway (Tanchak and Fowke, 1987).

Taken together, these micrographs of high pressure frozen sycamore maple suspension culture cells demon-

strate that the initial swelling response of the Golgi apparatus to monensin develops in a reproducible and sequential manner. Swelling first involves the TGN, then the *trans*-cisternae, and finally the medial and cis cisternae. Furthermore, based on the images and the surface area calculations, all of the swollen vesicles that accumulate in the cytoplasm appear to arise from blebbing structures associated with the TGN and the *trans*-cisternae, and not from the detachment of complete swollen *trans*-cisternae from the stacks as postulated by Morré et al. (1983). After about 40 min exposure to 10 μM monensin, cells containing non-swollen Golgi stacks but large numbers of swollen vesicles are observed with increasing frequency.

Structural changes associated with the recovery from monensin

Cells exposed to 10 μM monensin for 35 min recover completely within about 100 min of removal of the drug from the growth medium, as shown by their return to a normal cellular morphology, and the resumption of normal amounts of protein secretion (data not shown). Structural recovery (un-swelling) of *cis* and medial Golgi cisternae in our samples required 5 to 10 min. As demonstrated in Fig. 6A of a 10 min recovery sample, cis and medial cisternae become normal-looking, yet the central regions of the collapsed (trans) cisternae initially appear nearly empty when compared to those of control cells (Fig. 1), and virtually all products are confined to the swollen margins and the outermost portions of the flattened cisternae. During the next 30 min incubation in monensin-free growth medium, the Golgi stacks returned to very nearly the normal morphology, except for a slight swelling of the TGN and the presence of swollen vesicles around the stack (Fig. 6B). The
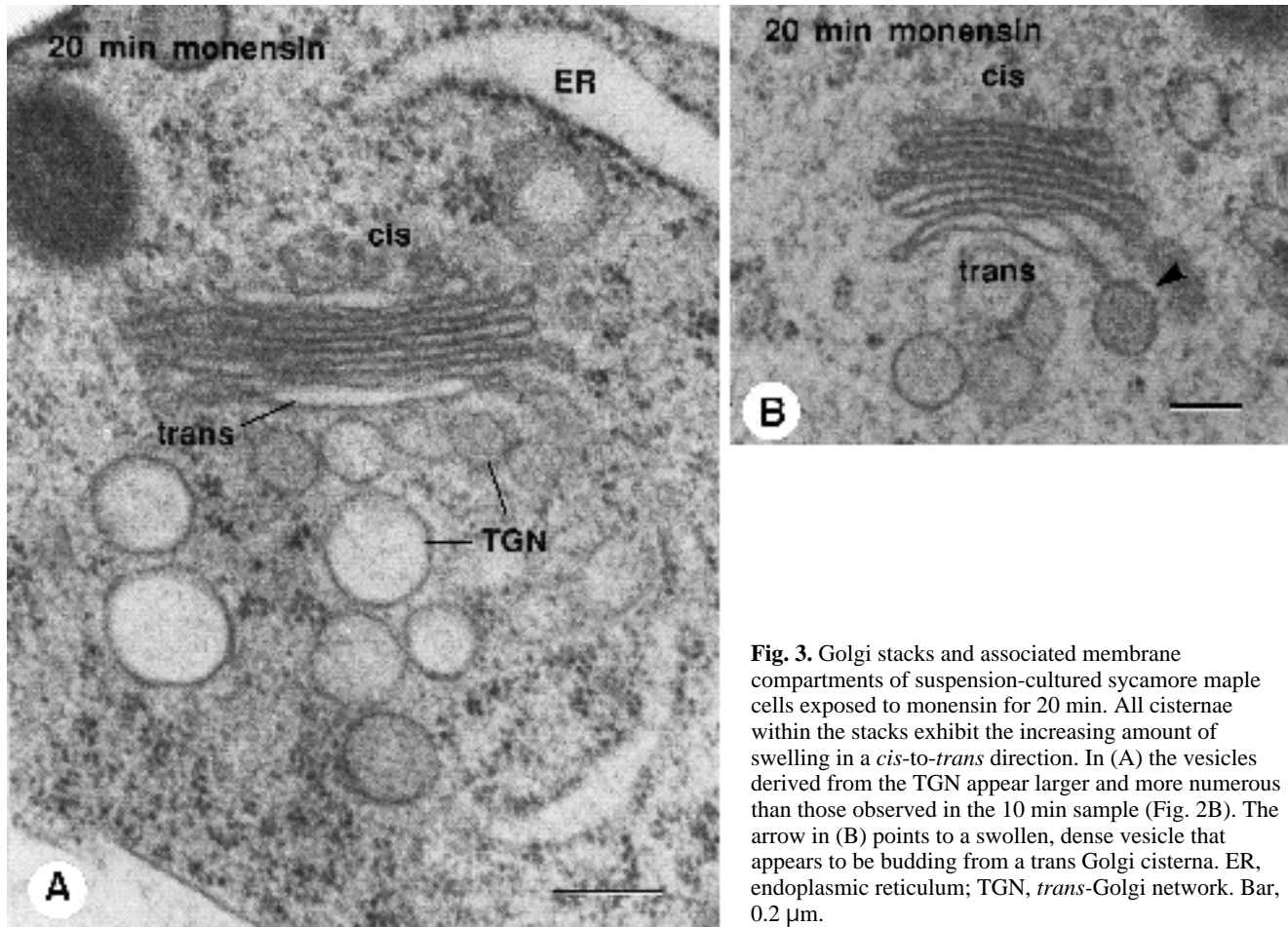
Fig. 3. Golgi stacks and associated membrane compartments of suspension-cultured sycamore maple cells exposed to monensin for 20 min. All cisternae within the stacks exhibit the increasing amount of swelling in a *cis*-to-*trans* direction. In (A) the vesicles derived from the TGN appear larger and more numerous than those observed in the 10 min sample (Fig. 2B). The arrow in (B) points to a swollen, dense vesicle that appears to be budding from a trans Golgi cisterna. ER, endoplasmic reticulum; TGN, *trans*-Golgi network. Bar, 0.2 μm.

time-dependent decrease in the number of swollen vesicles and an increase in their stainability is shown in Fig. 6A,B. By 80 min in the fresh medium without monensin, virtually all Golgi stacks exhibited a normal morphology and very few swollen vesicles remained in the cytoplasm (data not shown).

Unlike the Golgi stacks, the swollen vesicles in the cytoplasm do not "un-swell" within 1 to 2 h of removal of the ionophore. Instead their numbers slowly decrease over time. They probably disappear by fusing with the plasma membrane and discharging their contents into the cell wall, as indicated by Fig. 5B.

Immunocytochemical characterization of the monensin-induced swollen vesicles

As discussed above, the most striking effect of monensin on plant suspension culture cells is the time-dependent accumulation of swollen, Golgi-derived vesicles in the cytoplasm. A novel finding is that the swollen vesicles differ both in size and in their staining density (Fig. 5A). In most instances there is an inverse relationship between vesicle size and intensity of staining of the vesicular contents.

To analyze the contents of these vesicles and possible changes of the synthetic capabilities of the *trans*-Golgi cisternae and the TGN, we have immunolabeled thin sections of high pressure frozen/freeze-substituted cells embedded in LR White resin with antibodies raised against the cell wall matrix polysaccharide, XG. XG was chosen for this analysis because it is made exclusively in *trans*-Golgi cisternae and the TGN (Zhang and Staehelin, 1992). As shown in Fig. 7A-C, the labeling of the swollen vesicles with the anti-XG backbone antibodies (Lynch and Staehelin, 1992) increases unexpectedly in a time-dependent manner. Similar results were obtained with the monoclonal antibody CCRC-M1 (Puhlmann et al., 1991), that recognizes the terminal fucose of the trisaccharide sidechains of XG (data not shown). Although the structural preservation of the membranous organelles in the LR White-embedded samples does not match that of the Epon-embedded samples, it is still possible to see that on average the more densely staining, smaller vesicles exhibit a higher density of antibody binding than the more swollen and translucent vesicles. Taken together, these immunocytochemical results demonstrate that monensin stimulates the intracellular accumulation of XG within the large, Golgi- and TGN-derived swollen vesicles.

Effects of monensin on the intracellular accumulation and secretion of proteins and on the synthesis of cellulose

To confirm that the conditions used in the current investigation inhibit the secretion of proteins as reported in the literature, and to provide a baseline for comparison with other monensin studies (eg., Chrispeels, 1983; Driouich et al., 1989),
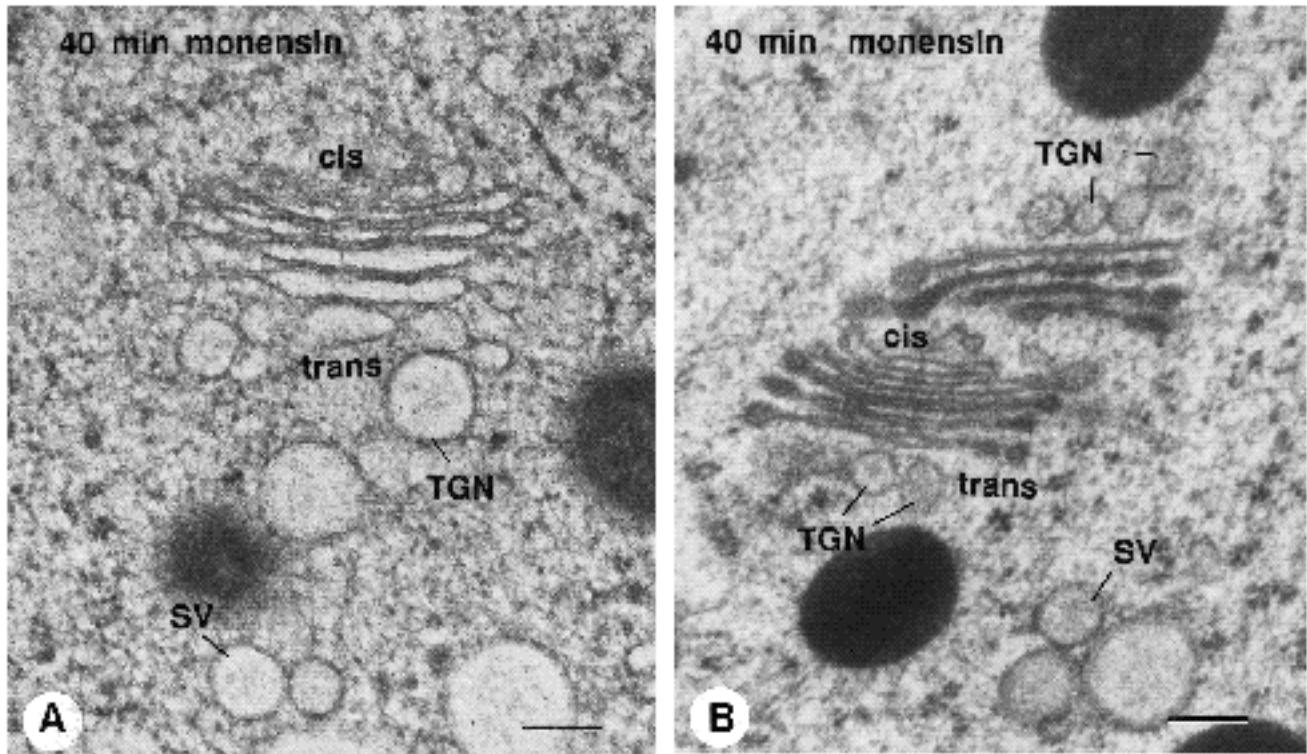
Fig. 4. Examples of two types of Golgi stacks observed in sycamore cells treated with monensin for 40 min. (A) depicts a greatly swollen Golgi stack and associated TGN, whereas (B) shows a nearly normal looking, non-swollen one. However, swollen vesicles are observed in the vicinity of both types of Golgi stacks and elsewhere in cytoplasm of these cells. SV, swollen vesicles; TGN, *trans*-Golgi network. Bar, 0.2 μm.

we have analyzed the effects of monensin on the incorporation of [3H]leucine into intracellular and secreted proteins. As shown in Table 1, 10 μM monensin inhibits protein secretion of sycamore suspension culture cells by 80% during the first 30 min of treatment, and by 90% over the period of an hour. Incorporation of [3H]leucine into intracellular proteins, on the other hand, is reduced by only 20% in the 30 min samples and by about 45% in the 60 min samples. These findings support the hypothesis that the primary effect of monensin is on the secretory pathway itself and that the large drop in protein secretion during the first 30 min of treatment is not caused by the inhibition of protein synthesis.

To determine if the appearance of non-swollen Golgi stacks after >40 min of monensin treatment (Fig. 6) reflects a functional recovery of these Golgi stacks, we have also compared the rate of protein secretion between 0 min and 40 min and between 40 min and 80 min of monensin treatment. In both samples protein secretion was inhibited by 90%, indicating that the reformation of non-swollen Golgi stacks itself is insufficient to permit resumption of protein secretion.

To test whether monensin also affects the synthesis of cellulose fibrils as suggested by the morphological studies of Rudolph and Schnepf (1988), we have labeled sycamore maple suspension culture cells with [3H]glucose and measured the radioactivity incorporated into the insoluble cellulose residues after removing starch, pectins and hemicellulosic polymers from the cell wall fraction (Fry, 1988). The results presented in Fig. 8 demonstrate that the incorporation of [3H]UDP-glucose into cellulose is significantly reduced in the monensin-treated cells compared to the control cells. The reduction was about 38% after 30 min and 60% after 60 min of treatment.

Effects of monensin on the intracellular accumulation and secretion of hemicelluloses

As discussed above, our immunolabeling experiments, with two types of anti xyloglucan antibodies (Fig. 7A-C; data not shown), suggest that monensin treatment leads to the intracellular accumulation of XG in large cytoplasmic vesicles. To confirm these observations and to determine to what extent monensin interferes with the secretion of XG, we have monitored the incorporation of [3H]UDP-xylose and [3H]fucose into the hemicellulosic polysaccharide fraction of the culture medium and the cell wall, as well as into the remaining cytoplasmic materials (intracellular polysaccharides and glycoproteins, and intracellular, unincorporated free tritiated sugars).

When sycamore cells were labeled with [3H]UDP-fucose, the amount of label incorporated into the cytoplasmic material fraction increased by 36% after 30 min and by 62% after 60 min treatment with monensin (Fig. 9). This observation is consistent with the notion that the secretion of fucose-containing glycoproteins and polysaccharides is inhibited by monensin, and with the finding that XG accumulates in swollen vesicles in the cytoplasm (Fig. 7A-C). Unexpectedly, Fig. 9 also demonstrates an increase in the amount of fucose-containing "hemicellulosic" polysaccharides that are secreted into the culture medium in the presence of monensin, but this increase in culture medium XG
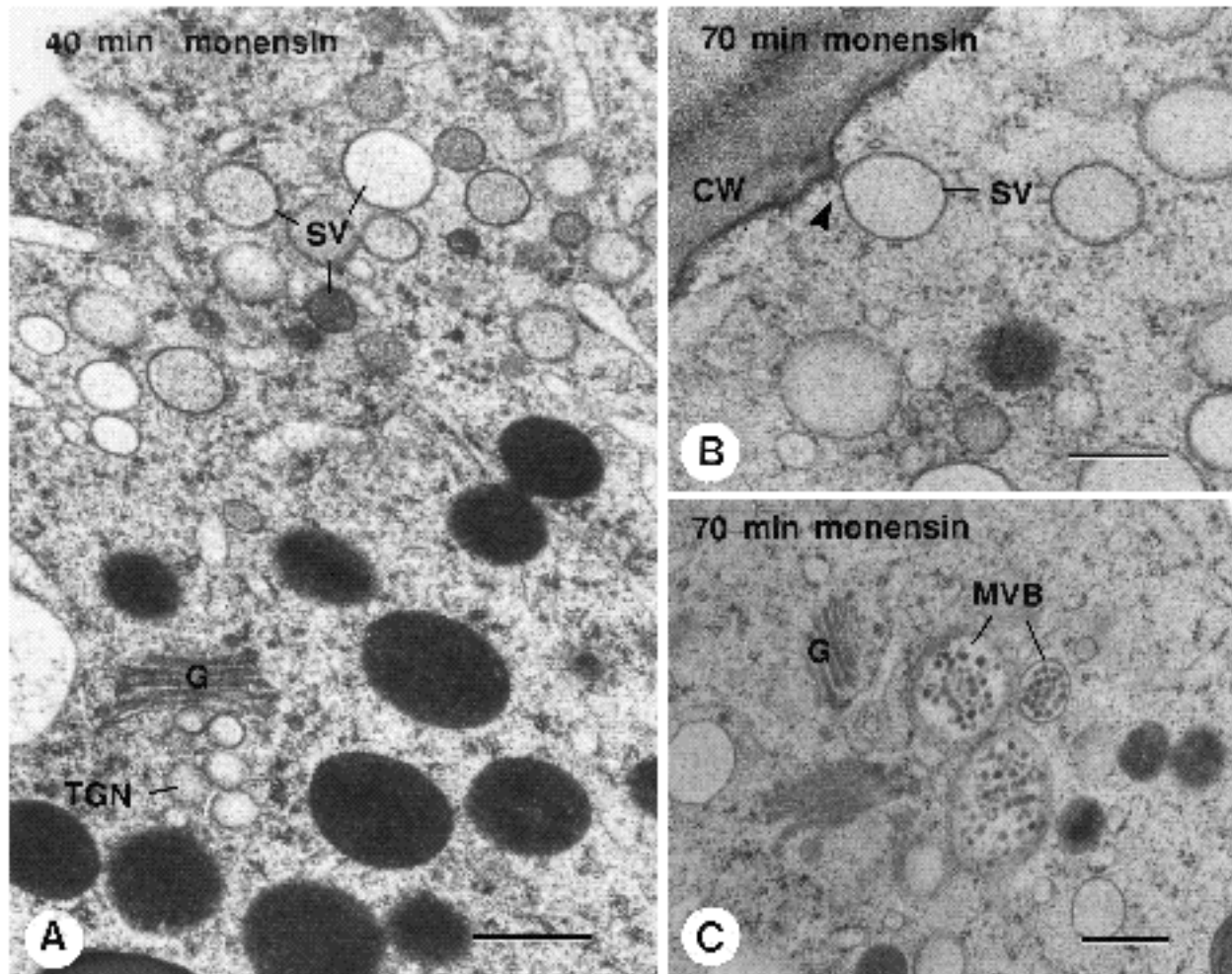
Fig. 5. Portions of three sycamore culture cells exposed to monensin for 40 min (A) and 70 min (B and C). (A) highlights the diversity in the stainability of the swollen vesicles. (B) illustrates a swollen vesicle that is attached to the plasma membrane in a configuration typical of a vesicle getting ready to fuse and discharge its contents. (C) shows the increase in the number of multivesicular bodies. CW, cell wall; G, Golgi; MVB, multivesicular body; SV, swollen vesicle. Bar, 0.5 μm.

appears to be partly compensated for by a slight decrease in cell wall-extractable XGs.

DISCUSSION

The goal of this research was to reinvestigate the morphological responses of plant cells (sycamore maple suspension culture cells) to the ionophore monensin using high pressure freezing/freeze-substitution techniques, and to correlate the structural changes in the secretory apparatus with alterations in secretory activity. The main advantage of the high pressure freezing/freeze-substitution technique over conventional chemical fixation and dehydration methods is that, because of the 1000 times faster rate of fixation and the low temperature processing, the ultrastructure of cellular organelles can be preserved with greater precision (eg. Craig and Staehelin, 1988; Dahl and Staehelin, 1989). The novel findings, partly summarized in Fig. 10, are that monensin causes (1) a sequential swelling response of different compartments of the Golgi apparatus; (2) the formation of swollen vesicles that arise from the TGN and *trans*-Golgi

cisternae; (3) the un-swelling of Golgi stacks but not of swollen vesicles after >40 min of treatment; (4) the gradual accumulation of XG in the swollen vesicles after their separation from the Golgi apparatus.

Origin of the monensin-induced swelling of Golgi-associated compartments

The monensin-induced swelling of different compartments of the Golgi apparatus is thought to result from a monensin-mediated exchange of protons with Na^+ and K^+ ions across the membranes of acidified compartments, thereby causing them to swell osmotically (Geisow and Burgoyne, 1982). Swelling of the compartments and maintenance of the swollen state require the presence and maintenance of a proton gradient (Boss et al., 1984). Since the acidification of Golgi compartments is brought about by H^+-ATPases both in animal (Glickman et al., 1983; Zhang and Schneider, 1983) and in plant cells (Ali and Akazawa, 1986; Boss et al., 1984; Chanson and Taiz, 1985), researchers have used the *amount* of cisternal swelling brought about by ionophores as a measure of cisternal acidification. Based on this criterion, Boss et al. (1984) and Griffing and Ray
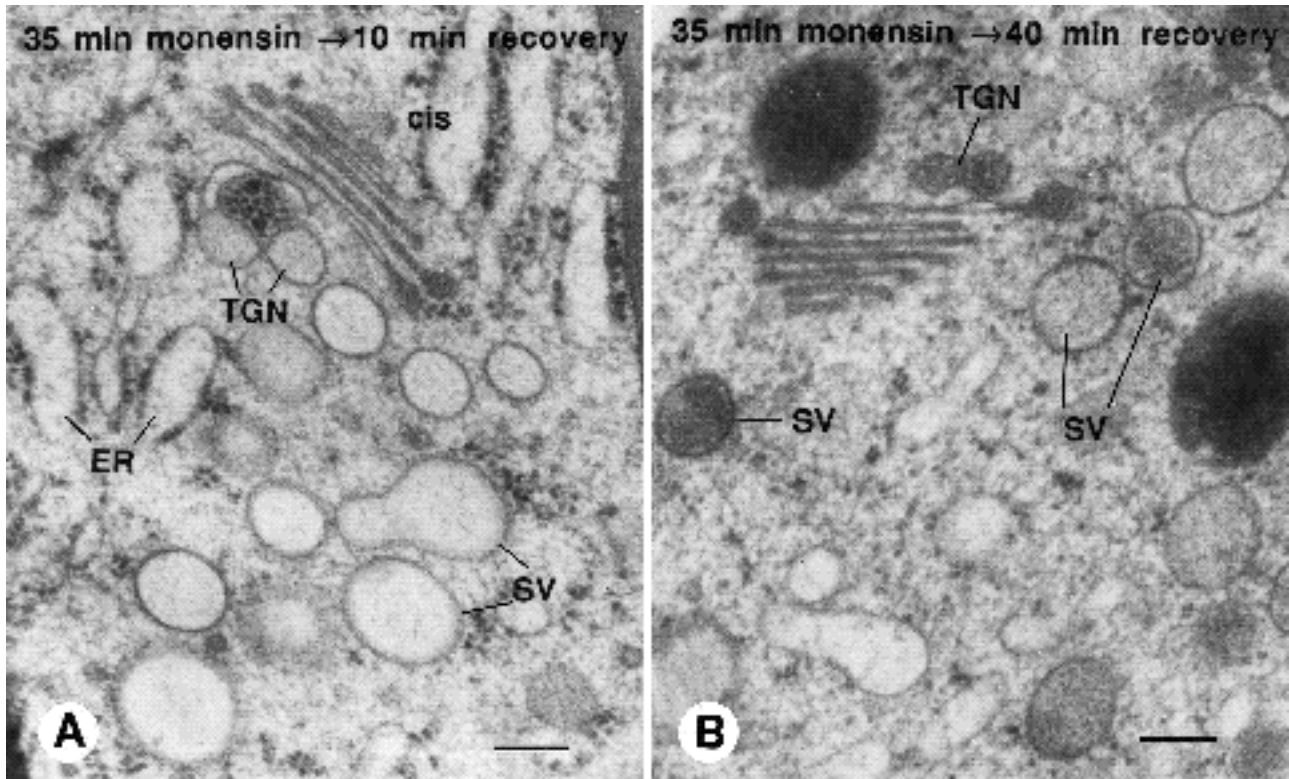
Fig. 6. Golgi stacks of sycamore cells treated for 35 min with monensin and then transferred to a monensin-free recovery medium for 10 min (A) and 40 min (B). Note that the cisternal luminae of the unswollen Golgi stack in A pick up much less stain than those of the Golgi stack in (B). In addition, the swollen vesicles around the Golgi stack of (A) are larger and more translucent than those seen in Fig. 6B. ER, endoplasmic reticulum; TGN, *trans*-Golgi network; SV, swollen vesicle. Bar, 0.2 μm.

(1985) have proposed that the *trans*-Golgi cisternae are the most acidic compartments of the plant Golgi apparatus.

Here we postulate that the *rate* of monensin-induced swelling is a more accurate measure of the degree of acidification of the secretory pathway compartments. To this end, our micrographic data suggest the following hierarchy of acidification of Golgi compartments: TGN>trans-most *trans*-Golgi cisterna>remaining *trans*-cisternae>cis and medial cisternae. These findings are consistent with more direct measurements of cisternal acidification in animal cells, which have pointed to the TGN and the *trans*-Golgi cisternae as being the most acidic Golgi compartments (Anderson and Pathak, 1985). Interestingly, immunolabeling experiments with V-ATPase antibodies have demonstrated the presence of H+-ATPases in all types of Golgi cisternae (Hurley and Taiz, 1990), but no quantitative data on the labeling patterns was reported. Why some elements of the endoplasmic reticulum (ER) swell in response to monensin (Figs 3A, 6) is unclear to us.

Origin of swollen vesicles

Based on the size and the kinetics of accumulation of swollen vesicles in chemically-fixed, monensin-treated cells, Boss et al. (1984) and Morré et al. (1983) have postulated that the vesicles arise by swelling of entire Golgi cisternae and subsequent release into the cytoplasm. Careful analysis of our micrographs of high pressure frozen/freeze-substituted, monensin-treated cells does not seem to lend support to this hypothesis. Thus, based on our

calculations, the surface area of an average *trans*-Golgi cisternae is about 5 times larger than the average size of a swollen vesicle. In addition, all of the initially formed swollen vesicles are clearly associated with what appear to be blebbing configurations of the TGN (Figs 2,3A) and *trans*-Golgi cisternae (Fig. 3B). We conclude, therefore, that the swollen vesicles that accumulate in monensin-treated cells arise mostly by a budding mechanism from the TGN and from *trans*-Golgi cisternae.

Cause of un-swelling of Golgi stacks exposed to 10 μM monensin for >40 min

One of the most unexpected findings of this study was the un-swelling of Golgi stacks exposed to monensin for >40 min. This un-swelling was probably missed in earlier studies because of the general morphological variability of Golgi stacks in chemically fixed cells, and also because the prominent swollen vesicles do not seem to participate in the un-swelling response. This transition from a swollen to a non-swollen configuration appears to occur quite rapidly as shown by our inability to observe "half swollen" Golgi stacks. Also, the fact that, despite intense searches, very few cells could be found that displayed both swollen and non-swollen Golgi stacks, suggests that the "signal" that triggers the unswelling response spreads quickly throughout a given cell.

As postulated by Boss et al. (1984), maintenance of the swollen state of acidic Golgi compartments in the presence of monensin requires constant pumping of protons into
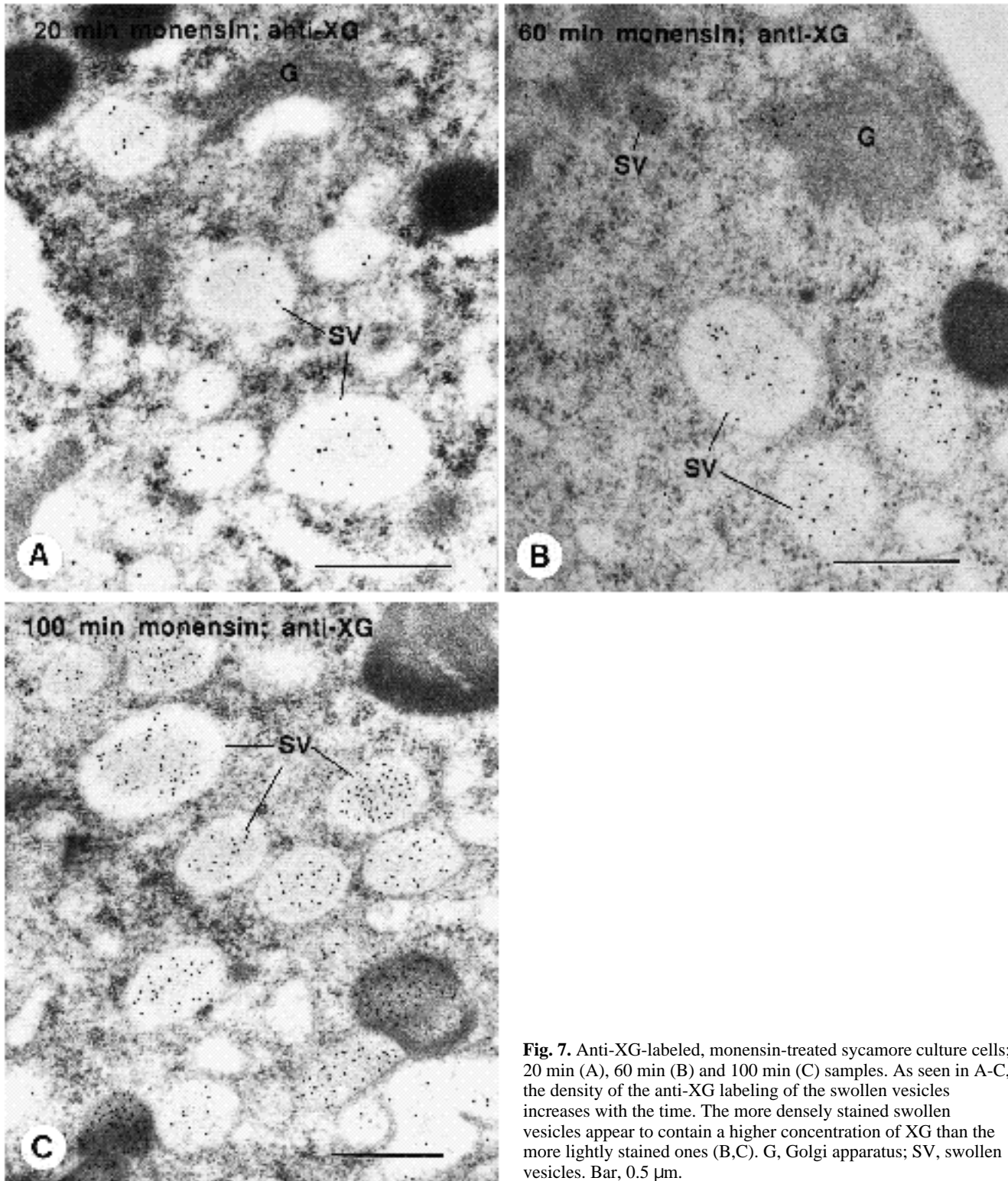
Fig. 7. Anti-XG-labeled, monensin-treated sycamore culture cells; 20 min (A), 60 min (B) and 100 min (C) samples. As seen in A-C, the density of the anti-XG labeling of the swollen vesicles increases with the time. The more densely stained swollen vesicles appear to contain a higher concentration of XG than the more lightly stained ones (B,C). G, Golgi apparatus; SV, swollen vesicles. Bar, 0.5 μm.

those compartments by H^+-ATPases. Thus, a possible explanation for the observed un-swelling of Golgi stacks after >40 min of monensin treatment is that this ionophore not only dissipates the pH of Golgi compartments, but also affects the pH across the inner mitochondrial membrane, thereby reducing the production of ATP and cellu-lar levels of ATP, as we have observed. With less ATP available to drive the H^+-ATPases, the monensin-induced swollen state of the cisternae would not be maintained. This decrease in cellular ATP levels may also explain why the incorporation of [^{3}H]leucine into intracellular proteins is reduced by only 20% during the first 30 min of mon-

Table 1. Effect of monensin on the synthesis and secretion of proteins

Sample type	Intracellular proteins (cts/min)	Secreted proteins (cts/min)
30 min: Control	236208	2478
Monensin	189475 (−20%)	425 (−80%)
60 min: Control	246714*	4015
Monensin	139042 (−43%)	421 (−90%)

3 g of cells were labelled with [³H]leucine in the presence or absence of monensin (10 µm), precipitated as described Materials and Methods, and the total radioactivity incorporated into each fraction measured.
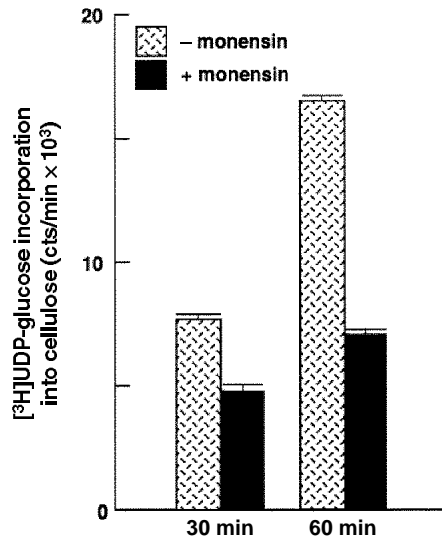

Fig. 8. Effect of monensin on the incorporation of [³H]UDP-glucose into cellulose. The cells were labeled with [³H]UDP-glucose for 30 min or 60 min in the presence or absence of monensin. The radioactivity was then measured in the insoluble cellulose fraction obtained after extraction of starch, pectins and hemicellulose polymers from the cell wall (Fry, 1988).

ensin treatment, but drops by 40% over the period of an hour.

An alternative explanation for the un-swelling of Golgi stacks is that monensin might lead to the loss of H⁺-ATPases from Golgi cisternae to swollen vesicles. An evaluation of this theory is presented in the next section, in the context of the failure of swollen vesicles to un-swell in parallel with the Golgi stacks.

Accumulation of XG in swollen vesicles

The failure of the swollen vesicles of monensin-treated cells to undergo an un-swelling response at the same time as the Golgi stacks is difficult to explain in the context of the ATP depletion theory presented in the foregoing section. As mentioned in the 'Introduction', proper acidification of the Golgi compartments appears to be of critical importance for a number of Golgi functions. These include the post-translational processing of proteins and glycoproteins (Barasch and Al-Awqati, 1992; Stinissen et al., 1985) and the sorting, packaging and trafficking of products destined for vacuoles and the cell surface (Bowles et al., 1986; Craig and Goodchild, 1984; Rothman et al., 1989). The inability of
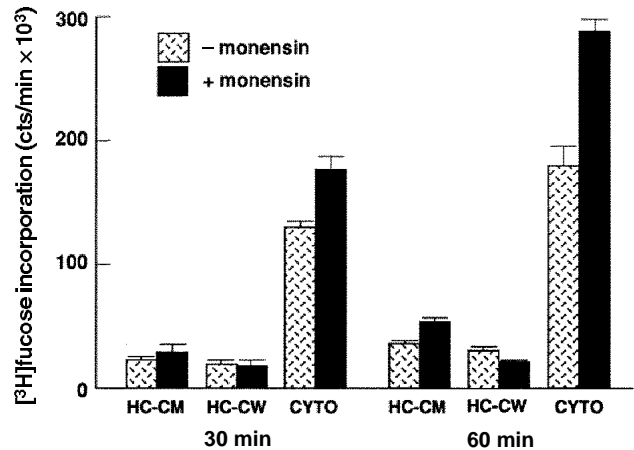

Fig. 9. Effect of monensin on the incorporation of [³H]fucose into hemicellulose. Sycamore cells were labeled with [³H]fucose in the presence or absence of 10 µM monensin for 30 min or 60 min. The hemicellulose fraction was extracted from the cell wall (HC-CW) or from the culture medium (HC-CM), as described in Materials and methods. Total radioactivity incorporated into these fractions as well as the cytoplasmic (CYTO) material were determined.

monensin-treated plant cells to process glycoproteins properly has already been reported (Chrispeels, 1983; Driouich et al., 1989). Similarly, the inhibition of secretion (Table I) and the accumulation of swollen vesicles in the cytoplasm are well-documented monensin effects (Figs. 2-4, 5A,B; Morré et al., 1983; Shannon and Steer, 1984). In this context, we propose that the monensin-induced changes in the pH of the TGN also interfere with the retrieval of *trans*-Golgi cisternal enzymes that become inadvertently packaged into secretory vesicles or delivered to the TGN, as shown in diagram Fig.10.

This latter hypothesis leads to a number of predictions that can be experimentally tested. For example, if *trans*-Golgi cisternal enzymes, such as those involved in the synthesis of XG, are "lost" to the swollen vesicles in monensin-treated cells, then synthesis of XG could potentially continue in these vesicles after their release into the cytoplasm. The increasing concentration of XG epitopes in swollen vesicles of 20 min, 60 min and 100 min monensin samples, as shown by our immunolabeling experiments (Fig. 7A-C), and the concomitant increase of fucose-containing compounds in the cytoplasmic vesicle fraction (Fig. 9), provide strong support for the hypothesis that the mechanism responsible for retrieving "lost" *trans*-Golgi cisternal enzymes is a pH sensitive process.

Based on this hypothesis, one can also postulate two non-exclusive mechanisms to explain the failure of swollen vesicles to un-swell when the adjacent Golgi stacks un-swell in response to a prolonged monensin treatment. The first suggests that the vesicles remain swollen because of the transfer of the H⁺-ATPases from and the Golgi cisternae to the swollen vesicles. The second builds on the observation that XG synthesis continues after the swollen vesicles have formed, and suggests that the accumulated XG molecules prevent the vesicles from un-swelling. We have obtained experimental support for this latter theory from examining the changes in cell morphology after returning monensin-
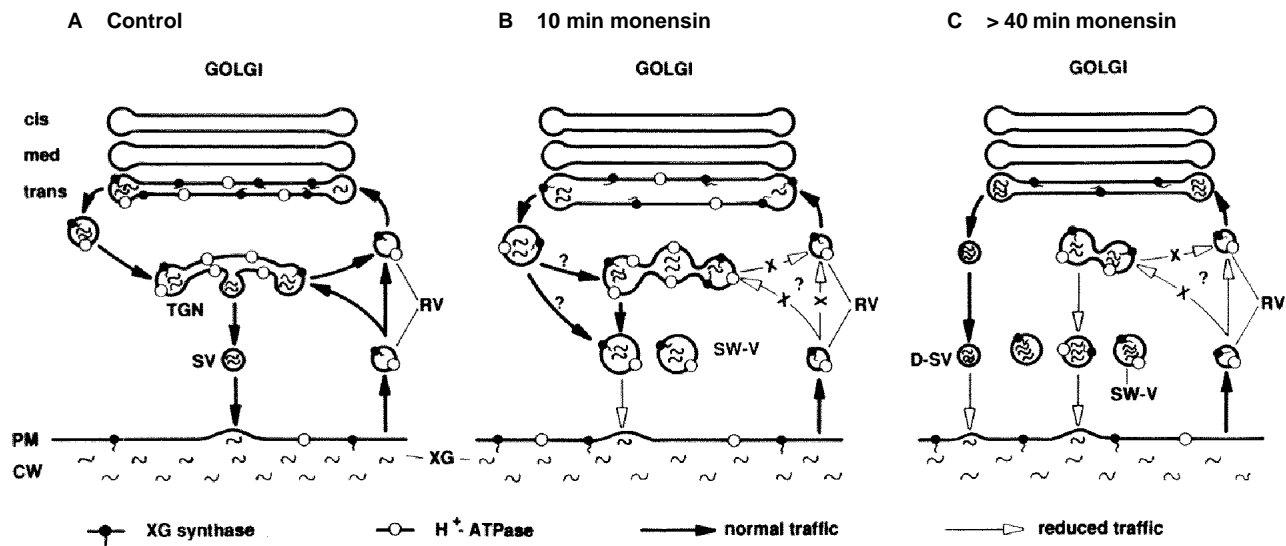
Fig. 10. Model of effects of monensin on the Golgi apparatus and secretory pathways of sycamore maple suspension-culture cells. In control cells (A), the secretory products pass through the TGN where they are sorted and packaged into secretory vesicles (SV) prior to their delivery to the cell surface. The TGN also serves as sorting/recycling station for trans cisternal enzymes that inadvertently are transferred to the TGN and have to be returned to the Golgi stack by means of recycling vesicles (RV). After 10 min monensin treatment (B), the TGN and *trans*-Golgi cisternae are greatly swollen, and swollen vesicles derived mostly from the TGN accumulate in the cytoplasm. The swollen vesicles lack the ability to efficiently deliver their contents to the cell surface. TGN sorting/recycling functions are also impaired resulting in a loss of *trans*-Golgi cisternal and TGN enzymes to the swollen vesicles. After >40 min monensin (C) the Golgi stacks undergo an un-swelling response, probably due to a decrease in cytoplasmic ATP levels. In contrast, the swollen vesicles do not un-swell, most likely because of the continued slow filling of the swollen vesicles with new XG molecules produced by displaced XG synthase molecules. Sorting/recycling functions remain impaired. PM, plasma membrane; CW, cell wall.

treated cells to a normal growth medium. In such recovering cells (Fig. 6A,B; and data not shown) the Golgi stacks return to a normal morphology within a few minutes, whereas the swollen vesicles persist for up to two hours. In this context it is interesting to note that in the absence of terminal fucose residues, the removal of some of the galactose residues from XG side-chains (Reid et al., 1988) causes the thus modified XG molecules to aggregate into irreversible gels, which could prevent un-swelling.

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