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Monensin and Brefeldin A Differentially Affect the Phosphorylation and Sulfation of Secretory Proteins*

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Chromogranin B and secretogranin II, two members of the granin family, are known to be post-translationally modified by the addition of *O*-linked carbohydrates to serine and/or threonine, phosphate to serine and threonine, and sulfate to carbohydrate and tyrosine residues. In the present study, chromogranin B and secretogranin II were used as model proteins to investigate in which subcompartment of the Golgi complex secretory proteins become phosphorylated. Monensin, a drug known to block the transport from the medial to the trans cisternae of the Golgi stack, inhibited the phosphorylation of the granins, indicating that this modification occurred distal to the medial Golgi. Monensin also blocked the addition of galactose to *O*-linked carbohydrates and the sulfation of the granins, confirming previous data that these modifications take place in the trans Golgi. To distinguish, within the trans Golgi, between the trans cisternae of the Golgi stack and the trans Golgi network, we made use of the previous observation that brefeldin A results in the redistribution to the endoplasmic reticulum of membrane-bound enzymes of the trans cisternae of the Golgi stack, but not of the trans Golgi network. Brefeldin A treatment abolished granin sulfation but resulted in the accumulation of phosphorylated and galactosylated granins. Differential effects of brefeldin A on membranes of the Golgi stack *versus* the trans Golgi network were also observed by immunofluorescence analysis of marker proteins specific for either compartment. Our results suggest that the phosphorylation of secretory proteins, like their galactosylation, largely occurs in the trans cisternae of the Golgi stack, whereas the sulfation of secretory proteins on both carbohydrate and tyrosine residues takes place selectively in the trans Golgi network.

The transport of secretory proteins from the rough endoplasmic reticulum (rER)[1] to, and through, the Golgi complex is accompanied by a series of post-translational modifications that include glycosylation, phosphorylation, and sulfation (see Refs. 1–3 and references therein). Many data are available on the subcellular sites within the secretory pathway where secretory proteins become glycosylated and sulfated (1–3). The best characterized of these modifications is *N*-glycosylation, whose individual reactions have been used to biochemically define the various subcompartments of the Golgi complex (see Refs. 1, 2, and 4 and references therein). Concerning *O*-glycosylation, it has been reported that the first step, the addition of *N*-acetylgalactosamine to serine and/or threonine residues, takes place in either a pre-Golgi compartment (5, 6) or early (cis) Golgi cisternae (7–10). The subsequent maturation of *O*-linked oligosaccharides occurs during the transport of proteins through the Golgi complex. The exact sites within the Golgi complex where these glycosylation reactions take place may vary between different cells type. However, on the basis of the available data one can assume that incorporation of galactose into *O*-linked oligosaccharides occurs in the trans Golgi cisternae (Refs. 1, 4, and 11 and references therein). With respect to sulfation, the available data indicate that the sulfation of both carbohydrate and tyrosine residues takes place in the trans Golgi network (TGN) (12–14), but it is unclear to what extent these reactions also occur in the trans cisternae of the Golgi stack.

Much less is known about the subcellular site where secretory proteins become phosphorylated. Several constitutive as well as regulated secretory proteins have been shown to be phosphorylated, including caseins (15), vitellogenins (16), proteodermatan sulfate (17), fibronectin (18), pro-adrenocorticotropin and its proteolytic product corticotropin (19), enkephalin-containing peptides (20), prolactin (21), and growth hormone (22). Studies on vitellogenins indicate that the phosphorylation of these proteins is a late event during their secretion (16). In addition, Golgi-derived membrane vesicles have been reported to contain casein kinase activities orientated toward the vesicle lumen (15). Moreover, Hirschberg and colleagues (23) have demonstrated the presence of an ATP translocation system in Golgi vesicles. These studies suggest that the phosphorylation of secretory proteins occurs in the Golgi complex. However the precise site of phosphorylation within the Golgi complex remains to be established.

In the present study, we used chromogranin B (CgB, originally called secretogranin I) and secretogranin II (SgII), two members of the granin family (24), as model proteins to investigate in which subcompartment of the Golgi complex secretory proteins become phosphorylated. Besides phosphorylation on serine and, to a lesser extent, threonine residues, CgB and SgII undergo *O*-glycosylation on serine and/or threonine and sulfation on carbohydrate and tyrosine residues (see Ref. 25 and references therein). This allowed us to compare in rat pheochromocytoma cells (PC12) the subcel-

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[1] The abbreviations used are: (r)ER, (rough) endoplasmic reticulum; CgB, chromogranin B; SgII, secretogranin II; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; TGN, trans Golgi network.

lular site of their phosphorylation with that of their glycosylation and sulfation. Although increasing evidence suggests that compartmentalization of resident Golgi enzymes may vary between different cell types, we assumed, on the basis of observations in other cells (Refs. 1, 10, 11 and references therein), that in PC12 cells galactosylation of the granins occurs in the trans Golgi cisternae. Concerning sulfation, recent data indicate that in PC12 cells, sulfated granins and constitutively secreted proteoglycans are packaged into post-TGN vesicles rapidly after a sulfate pulse (13), which is consistent with the notion that sulfation of these molecules occurs very late in the Golgi complex (12, 14).

To dissect the phosphorylation, glycosylation and sulfation of CgB and SgII during their passage through the Golgi complex, we treated PC12 cells, which produce these proteins in relatively large amounts, with monensin or brefeldin A, two drugs known to affect intracellular membrane traffic at different sites. Monensin blocks or slows down protein transport through the Golgi stack (Refs. 26 and 27 and references therein) and affects protein glycosylation. It has been shown that in the presence of the drug many glycoproteins may fail to acquire terminal carbohydrates (27–31). Although monensin may have multipe sites of action depending on the cell type, with certain glycoproteins such as glycoproteins of the Semliki Forest virus and Sindbis virus (32, 33), the block in transport appears to be localized within the Golgi complex. Investigating the effects of monensin on baby hamster kidney cells infected with Semliki Forest virus using immunocytochemistry and biochemistry, Griffiths *et al.* (34) concluded that in these cells monensin blocks movement from medial to trans Golgi cisternae. Brefeldin A blocks biosynthetic protein transport between the rER and the Golgi complex (35). In addition, with respect to biosynthetic protein transport, brefeldin A has been reported to rapidly affect the Golgi complex and to cause a redistribution, to the ER, of membrane proteins that normally reside in the cis, medial, and trans cisternae of the Golgi stack (35–37), but not of membrane proteins that normally reside in the TGN (38). We, therefore, interpreted the effects of monensin and brefeldin A on phosphorylation, galactosylation, and sulfation of the granins on the basis of the following concept reflecting the availability or lack of proteins substrates: a post-translational modification occurring up to the medial cisternae of the Golgi stack should not be affected by either monensin or brefeldin A; a modification occurring in the trans cisternae of the Golgi stack should be blocked by monensin but not by brefeldin A; a modification occurring in the TGN should be blocked by both monensin and brefeldin A.

MATERIALS AND METHODS

Cell Culture, Metabolic Labeling, and Drug Treatments—PC12 cells (1×10^6), grown as previously described in 3.5-cm dishes (39), were used for labeling. In pulse-chase experiments, PC12 cells were preincubated for 1 h in tyrosine-free Dulbecco's minimum essential medium supplemented with 10% fetal calf serum dialyzed against 10 mM HEPES-NaOH, pH 7.4, containing 150 mM NaCl. Cells were then labeled for 5 min with 100 μCi/ml [^{3}H]tyrosine (TRK 530, Amersham International, Buckinghamshire, Great Britain) and chased for 0, 2.5, 5, and 10 min in medium containing twice the normal concentration of unlabeled tyrosine. To investigate the effect of monensin and brefeldin A on the synthesis, galactosylation, sulfation, and phosphorylation of the granins, PC12 cells were preincubated for 1 h in tyrosine-free, glucose-free, sulfate-free, or phosphate-free medium (39), respectively, with or without either 10^{-6} M monensin (Sigma; added from a 10^{-4} M stock in 90% ethanol) or 2.5 μg/ml of brefeldin A (Epicentre Technologies, Madison, WI; added from a 5 mg/ml stock in 100% methanol). Cells were then incubated for 3 h in the respective medium containing either 50 μCi/ml [^{3}H]tyrosine, 100 μCi/ml [^{3}H]galactose (NET-698, Du Pont-New England Nuclear, Dreieich, Germany), 100 μCi/ml [^{35}S]sulfate (SJS.1, Amersham International), or 500 μCi/ml [^{32}P]phosphate (PBS.11, Amersham International), in the continued absence or presence of the above final concentrations of monensin or brefeldin A. To investigate the effects of short-term treatment with brefeldin A, cells were preincubated for 3 h in sulfate-free or phosphate-free medium. Dishes were then incubated for 10 min with or without 2.5 μg/ml of brefeldin A followed by labeling for 5 min with either 200 μC/ml [^{35}S]sulfate or 1 mCi/ml [^{32}P]phosphate in the continued absence or presence of the drug.

Electrophoresis—After labeling, cells were washed three times at 4 °C with ice-cold phosphate-buffered saline and then solubilized by either of the following two protocols: (i) 0.4 ml of Laemmli sample buffer was added to each dish and the cell lysate was transferred to an Eppendorf tube and boiled for 5 min (39); (ii) 0.5 ml of ice-cold 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.3% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of pepstatin was added to each dish, the cell lysate transferred to an Eppendorf tube, boiled for 3 min, and a heat-stable supernatant highly enriched in CgB and SgII obtained by centrifugation (40). Proteins in either the total cell lysate or the heat-stable fraction were precipitated overnight at −20 °C using 80% acetone. The pellets were then solubilized in lysis buffer and analyzed by two-dimensional PAGE as described (41) using a mini gel system (Mini two-dimensional Electrophoresis cell, Bio-Rad Laboratories, Milan, Italy).

Immunofluorescence—PC12 cells were plated onto poly-L-lysine-coated coverslips and grown for 2 days. After incubation for various times in normal medium with or without 2.5 μg/ml brefeldin A, cells were analyzed by indirect immunofluorescence as described in Rosa *et al.* (42) with minor modifications. Paraformaldehyde-fixed cells were permeabilized for 15 min in phosphate-buffered saline containing 0.5% saponin, quenched for 30 min with 0.1 M glycine-Tris, pH 7.5, and then incubated with either an antiserum against the Golgi complex (43) or an antiserum recognizing an integral membrane protein of the trans Golgi network (TGN38) (44). Micrographs were taken with a Zeiss photomicroscope III equipped with epifluorescence.

RESULTS AND DISCUSSION

In the present study, the post-translational modification of CgB and SgII during their passage along the secretory pathway was investigated by two means, (i) the incorporation of radioactive label indicative of a certain post-translational modification and (ii) the electrophoretic mobility of CgB and SgII upon two-dimensional PAGE. We first defined this electrophoretic mobility for rat CgB and SgII from PC12 cells by performing pulse-chase experiments using radioactive tyrosine (Fig. 1). In the case of CgB, after a 5-min pulse, a spot with an M_r of ~ 97,000 and a pI of ~ 5.6, corresponding to the newly synthesized CgB as indicated by immunoprecipitation with a specific antibody (Ref. 40; data not shown), was observed (Fig. 1*A*, *open arrow*) and is referred to as form 1 (Fig. 1*E*). During the chase, this labeled form of CgB (Fig. 1, *B–D, open arrows*) decreased concomitantly with the successive appearance of several more acidic forms of CgB. Considering also the results obtained with Coomassie Blue staining described below (see Fig. 2), at least five post-translationally modified forms of CgB could be distinguished (referred to as forms 2–4b, Fig. 1*E*), of which forms 3a–4b with M_r values of 105,000–113,000 and a pI of ~ 5.0 corresponded to mature CgB (Fig 1, *C* and *D, arrows*). In the case of SgII, after a 5-min pulse, two spots with an M_r values of~ 85,000 and pI of ~ 5.0, corresponding to the newly synthesized SgII as indicated by immunoprecipitation with a specific antibody (Ref. 40; data not shown), were detected (Fig 1*A*, *open triangle*) and are referred to as forms 1a and 1b (Fig. 1*F*). As was observed for CgB, these labeled forms of SgII decreased concomitantly with the successive appearance of several more acidic forms of SgII. At least four post-translationally modified forms of SgII could be distinguished (see also Fig. 2; referred to as forms 2a–3b, Fig. 1*F*), of which forms 3a and 3b, with M_r values of~ 86,000 and a pI of ~ 4.8, corresponded to mature SgII (Fig. 1, *C* and *D arrowheads*). Based on previous results

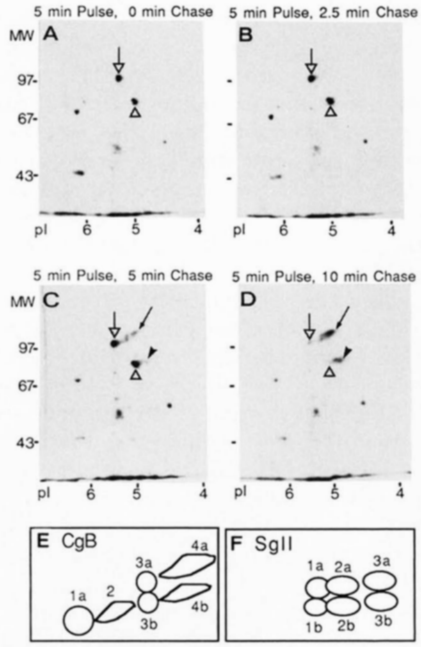

FIG. 1. **Newly synthesized, intermediate, and mature forms of CgB and SgII.** PC12 cells were pulse-labeled for 5 min with [^{3}H] tyrosine and then chased for 0, 2.5, 5, and 10 min (*A–D*). After each chase time, cell lysates (~30 μg of protein) were analyzed by two-dimensional PAGE followed by fluorography. *Open arrows*, newly synthesized CgB; *open triangles*, newly synthesized SgII; *arrows*, mature CgB; *arrowheads*, mature SgII. The diagrams in *E* and *F* show the various forms of CgB (*E*) and SgII (*F*) and summarize the results shown in *A–D*. For details, see text.

(Ref. 25 and references therein), we conclude that the successive changes in apparent molecular weight and isoelectric point of CgB and SgII after synthesis reflected the sequential post-translational addition of *O*-linked carbohydrates, phosphate, and sulfate that occurred during the passage of these proteins from the rER to, and through, the Golgi complex. The most acidic forms of CgB and SgII were the most extensively modified forms of the two proteins and represented the mature forms that had passed through the TGN and accumulated in secretory granules.

Whereas the galactosylation and sulfation of secretory proteins are thought to occur in the trans Golgi (1, 4, 11–14), the site of phosphorylation of secretory proteins within the secretory pathway is less clear. We used the drugs monensin and brefeldin A to investigate the site of granin phosphorylation and to determine where within the trans Golgi (trans cisternae of the Golgi stack *versus* TGN) the galactosylation and sulfation of the two granins takes place, on the basis of the following considerations. First, post-translational modifications occurring up to the medial cisternae of the Golgi stack should not be affected by either monensin or brefeldin A. Second, extrapolating from the results of Lippincott-Schwartz *et al.* (36, 37) and Chege and Pfeffer (38), modifications occurring in the trans cisternae of the Golgi stack might be expected to be blocked by monensin but not brefeldin A, whereas modifications occurring in the TGN might be expected to be blocked by both monensin and brefeldin A.

Secretory proteins are usually modified in an irreversible manner and are removed from the compartment in which a given modification reaction takes place. Hence, continuous delivery of protein substrate to the reaction compartment is required to detect the post-translational modification of secretory proteins by metabolic labeling. We therefore first examined the effects of monensin and brefeldin A on granin synthesis to exclude that any inhibitory action of these drugs on the post-translational modification of these proteins would be due to lack of protein substrate. This also provided information on the effects of monensin and brefeldin A on the various electrophoretic forms of CgB and SgII which were detected during the chase of pulse-labeled granins from the rER to secretory granules (see Fig. 1). In the experiments shown in Fig. 2, PC12 cells were labeled for 3 h with [^{3}H] tyrosine in the absence or presence of 10^{-6} M monensin or 2.5 μg/ml brefeldin A. Fluorography after two-dimensional PAGE indicated that neither monensin nor brefeldin A significantly inhibited the synthesis of CgB and SgII (Fig. 2, *D–F*). However, both drugs dramatically affected the electrophoretic mobility of the two granins, although in different ways. In the presence of either drug, the granins were not processed to the mature forms; however, after monensin treatment, all of the newly synthesized CgB accumulated as form 1 (monensin form: Fig. 2*E*, *open arrow*), whereas after brefeldin A treatment, only some of the newly synthesized CgB remained as form 1, and a large portion was processed to form 2 (brefeldin A forms: Fig. 2*F*, *arrow with asterisk*). Similar observations were made for SgII (Fig 2, *E* and *F*: monensin form, *open triangle*; brefeldin A forms, *arrowhead with asterisk*), although in this case the differences between monensin and brefeldin A were less evident since the differences in electrophoretic mobility between forms 1 and 2 are less marked (see Fig. 1*F*). The accumulation of forms 1 and 2 of CgB and SgII in the presence of monensin and brefeldin A could also be detected after analysis of the total cellular granins by Coomassie Blue staining of the two-dimensional gels (Fig. 2, *A–C*). These results indicated that monensin and brefeldin A differentially inhibited the post-translational modification of CgB and SgII.

To study the effects of monensin and brefeldin A specifically on the phosphorylation, galactosylation, and sulfation of the granins, PC12 cells were labeled for 3 h with either

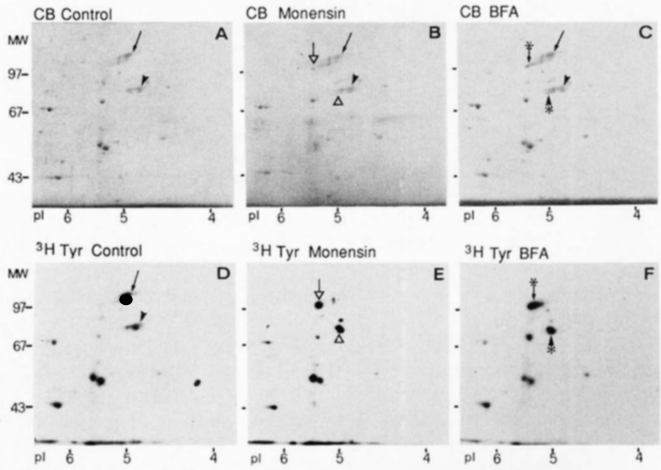

FIG. 2. **Monensin and brefeldin A differentially affect the maturation of CgB and SgII.** PC12 cells were preincubated for 1 h in tyrosine-free medium without (*Control*; *A* and *D*) or with either 10^{-6} M monensin (*Monensin*; *B* and *E*) or 2.5 μg/ml brefeldin A (*BFA*; *C* and *F*), and then labeled for 3 h with [^{3}H]tyrosine in the continued absence or presence of the drug. Cell lysates (~30 μg of protein) were analyzed by two-dimensional PAGE followed by Coomassie Blue staining (*CB*, *A–C*) or fluorography (*^{3}H Tyr*, *D–F*). *Arrows*, mature CgB corresponding to forms 3a–4b shown in Fig. 1*E*; *arrowheads*, mature SgII corresponding to forms 3a–3b shown in Fig. 1*F*; *open arrows*, monensin forms of CgB corresponding to form 1a shown in Fig. 1*E*; *open triangle*, monensin form of SgII corresponding to forms 1a-b shown in Fig. 1*F*; *arrows with asterisk*, brefeldin A forms of CgB corresponding to form 2 shown in Fig. 1*E*; *arrowheads with asterisk*, brefeldin A forms of SgII corresponding to forms 2a–2b shown in Fig. 1*F*.

[^{32}P]phosphate, [^{3}H]galactose, or [^{35}S]sulfate in the absence or presence of monensin or brefeldin A (Fig. 3). In the control condition (Fig. 3, *A, D,* and *G*), [^{32}P]phosphate, [^{3}H]galactose, and [^{35}S]sulfate labeling was predominantly observed for forms 3a–4b of CgB (*arrows*) and forms 3a–b of SgII (*arrowheads*), consistent with the conclusion that these were the mature forms of the granins. Compared with control, monensin virtually abolished the incorporation of radioactive sulfate (Fig. 3, *G* and *H*) and strongly reduced that of radioactive galactose (Fig. 3, *D* and *E*) and phosphate (Fig. 3, *A* and *B*) into CgB and SgII, with only a trace of [^{32}P]phosphate-labeled forms 2 and 3b being detectable in the case of CgB. In contrast, brefeldin A treatment, while also abolishing the incorporation of radioactive sulfate into the granins (Fig. 3*I*), allowed the accumulation of [^{32}P]phosphate- and [^{3}H]galactose-labeled form 2 of CgB and of [^{32}P]phosphate-labeled forms 2a–b of SgII (Fig. 3, *C* and *F*). ([^{3}H]Galactose-labeled forms 2a–b of SgII, possibly also accumulated in the cells after treatment with brefeldin A, could not be detected, since their position was obscured by other [^{3}H]galactose labeled glycoproteins after treatment with the drug.) The analysis of the total cellular [^{32}P]phosphate-labeled proteins also revealed that brefeldin A treatment selectively affected secretory proteins (in PC12 cells: CgB and SgII) as opposed to cytoplasmic proteins, since we did not detect any major effect of the drug on the phosphorylation of the other cellular, presumably cytoplasmic, proteins (Fig. 3, compare *A* and *C*).

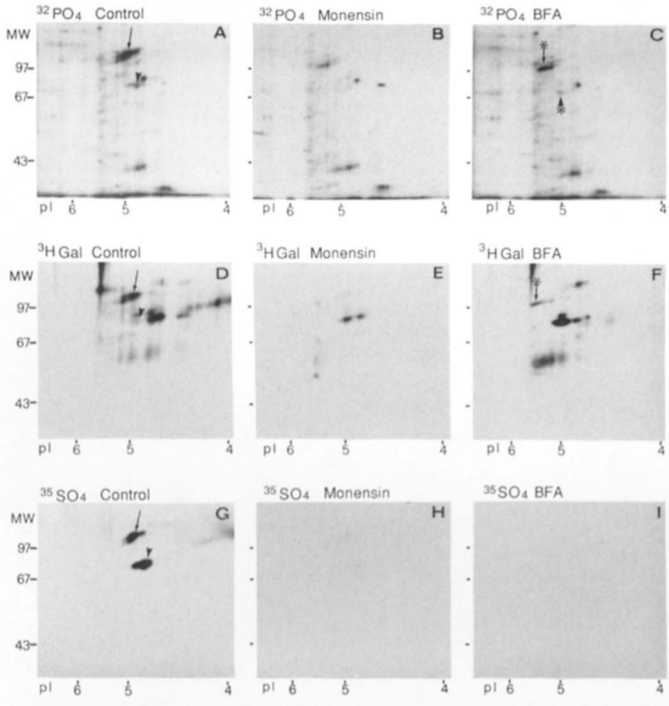

FIG. 3. **Brefeldin A, but not monensin, allows the accumulation of [^{32}P]phosphate- and [^{3}H]galactose-labeled CgB and SgII.** PC12 cells were preincubated for 1 h in phosphate-, glucose-, or sulfate-free medium without (*Control*; *A, D,* and *G*) or with either 10^{-6} M monensin (*Monensin*; *B, E,* and *H*) or 2.5 μg/ml brefeldin A (*BFA*; *C, F,* and *I*) and then labeled for 3 h with [^{32}P]phosphate (*^{32}PO$_4$, A–C*), [^{3}H]galactose (*^{3}H Gal, D–F*), or [^{35}S]sulfate (*^{35}SO$_4$, G–I*), respectively, in the continued absence or presence of the drug. Cell lysates (~30 μg of proteins) were analyzed by two-dimensional PAGE followed by autoradiography (*^{32}PO$_4$, A–C*) or fluorography (*^{3}H Gal* and *^{35}SO$_4$, D–I*). *Arrows*, mature CgB corresponding to forms 3a–4b shown in Fig. 1*E*; *arrowheads*, mature SgII corresponding to forms 3a–3b shown in Fig. 1*F*; *arrows with asterisk*, brefeldin A forms of CgB corresponding to form 2 shown in Fig. 1*E*; *arrowhead with asterisk*, brefeldin A form of SgII corresponding to forms 2a–2b shown in Fig. 1*F*. These labeled forms of CgB and SgII were identified by the position of the corresponding Coomassie Blue-stained forms (compare Fig. 2, *A* and *C*). Note the virtual abolishment of [^{35}S] sulfate incorporation into CgB and SgII in the presence of both monensin and brefeldin A and the large reduction of [^{32}P]phosphate and [^{3}H]galactose incorporation into CgB and SgII in the presence of monensin.

Assuming that the effects of monensin and brefeldin A on the incorporation of [^{32}P]phosphate, [^{3}H]galactose, and [^{35}S] sulfate into the granins resulted from the inhibitory action of these drugs on biosynthetic secretory protein transport, these results imply the following. First, the phosphorylation, galactosylation, and sulfation of the granins occur at sites in the secretory pathway that are distal to the site where transport is blocked by monensin. Second, at least some of the phosphorylation and galactosylation of the granins occurs in a compartment that still receives newly synthesized secretory protein in the presence of brefeldin A, whereas the sulfation of the granins occurs in a compartment that does not. In line with the data of Chege and Pfeffer (38), the former compartment may be the trans cisternae of the Golgi stack and the latter compartment may be the TGN.

Although the inhibitory effect of brefeldin A on sulfation is most likely explained by the lack of delivery of unsulfated granins to the TGN, other explanations, *e.g.* a direct effect of brefeldin A on the TGN, are also possible, in particular when one considers recent reports on the fusion of the TGN membranes with endosomal membranes (45, 46) and the fact that in the above experiments, the brefeldin A treatment had been performed for 4 h. We therefore investigated the effect of brefeldin A on the organization of the TGN in PC12 cells as revealed by the morphological appearance of TGN38, an integral membrane protein of the TGN (44). Immunofluorescence analysis of PC12 cells showed a clustered perinuclear localization of TGN38 in control conditions (Fig. 4*A*). This intracellular distribution of TGN38 was still largely maintained after 10 min in the presence of brefeldin A (Fig. 4*B*). In contrast, after 2 h of brefeldin A treatment, TGN38 was partly dispersed throughout the cytoplasm, showing a fine punctate pattern of staining, and partly found in a clustered perinuclear localization (Fig. 4*C*). The dispersion of TGN38-containing membranes throughout the cytoplasm appeared to be complete after 4 h of brefeldin A treatment (Fig. 4*D*). The absence of a dramatic change in the overall structure of the TGN after the first 10 min of brefeldin A treatment was not due to a general lack of effect of the drug, since the intracellular distribution of marker proteins of the Golgi stack, revealed by immunofluorescence using the antiserum described by Louvard *et al.* (43), was rapidly changed from a clustered perinuclear localization in control conditions (Fig. 4*E*) to a dispersed cytoplasmic pattern (Fig. 4, *F–H*). This rapid effect of brefeldin A on Golgi membranes proximal to the TGN is in line with results of several previous studies (35–38, 47), and the results obtained with TGN38 are consistent with those recently reported by Reaves and Banting (48).

Since during the first 10 min of treatment with brefeldin A, the overall structure of the TGN in PC12 cells did not appear to be dramatically affected, we examined the effect of the drug on the sulfation of the granins in PC12 cells pretreated with brefeldin A for 10 min and then labeled with radioactive sulfate for 5 min in the continued presence of the drug (Fig. 5). Also in this condition, brefeldin A abolished the radioactive sulfate incorporation into CgB and SgII as well as into a constitutively secreted sulfate proteoglycan (13) (Fig. 5, *A* and *B*). In contrast, brefeldin A treatment did not significantly reduce the incorporation of radioactive phosphate into CgB (Fig. 5, *C* and *D*). Also after this short

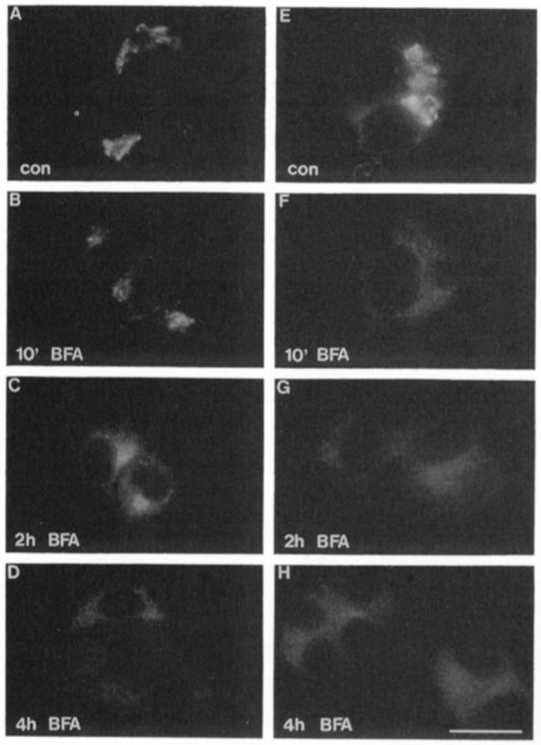

FIG. 4. **Effect of brefeldin A on the intracellular distribution of TGN38 in PC12 cells.** PC12 cells were incubated without (*con*; *A* and *E*) or with 2.5 μg/ml brefeldin A (*BFA*) for 10 min (*B* and *F*), 2 h (*C* and *G*), and 4 h (*D* and *H*). Cells were then fixed and immunolabeled using either an antiserum raised against a membrane protein of the TGN (TGN38, dilution 1:400) (*A–D*) or an antiserum raised against the Golgi complex (dilution 1:250). Primary antibodies were revealed using rhodamine-conjugated goat IgG directed against rabbit IgG. Bar = 10 μm.

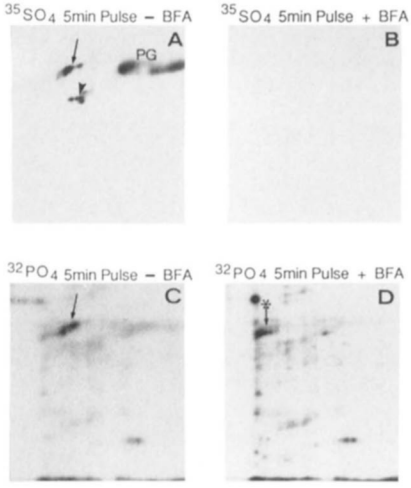

FIG. 5. **Brefeldin A prevents the sulfation, but not the phosphorylation, of the granins.** PC12 cells were preincubated for 10 min with (+*BFA*) or without (−*BFA*) 2.5 μg/ml brefeldin A and then labeled for 5 min with either [^{35}S]sulfate ($^{35}SO_4$, *A* and *B*) or [^{32}P] phosphate ($^{32}PO_4$, *C* and *D*) in the continued absence or presence of the drug. Heat-stable fractions (~20 μg of protein) prepared from the cell lysates were analyzed by two-dimensional PAGE followed by fluorography. *Arrows*, normal TGN form of CgB which is equal to the mature CgB (Fig. 1*E*, forms 3a–4b); *arrowhead*, normal TGN form of SgII which is equal to the mature SgII (Fig. 1*F*, forms 3a–3b); *arrow with asterisk*, brefeldin A form of CgB corresponding to form 2 shown in Fig. 1*E*. *PG*, a constitutively secreted sulfated proteoglycan.

brefeldin A treatment, phosphate-labeled CgB accumulated as form 2 (Fig. 5*D*), as opposed to forms 3 and 4 which were predominant in the control condition (Fig. 5*C*). (After such a short labeling with radioactive phosphate, SgII could not be clearly detected.)

In conclusion, we interpret our results as follows. The data obtained with monensin suggest that the phosphorylation of secretory proteins, as shown here for CgB and SgII which are phosphorylated predominantly on serine and to a minor degree on threonine (Ref. 25 and references therein), occurs in the trans Golgi, as is the case for their galactosylation on *O*-linked carbohydrate and their sulfation on *O*-linked carbohydrate and tyrosine residues. The lack of inhibition of granin phosphorylation and galactosylation in the presence of brefeldin A suggests that normally these modification reactions occur, within the trans Golgi, predominantly in the trans cisternae of the Golgi stack, whose membrane-bound enzymes (in the present case the galactosyltransferase and protein kinase) in the presence of brefeldin A, in analogy to previous work (Refs. 35–38 and 49 and references therein), recycle back to the ER and, therefore, are able to continuously modify the newly synthesized granins. With respect to the various forms of CgB, this implies that form 2 normally is generated in the trans cisternae of the Golgi stack.

In contrast, the inhibition of sulfation of the granins on both *O*-linked carbohydrate and tyrosine residues in the presence of brefeldin A suggests that these modification reactions occur selectively in the TGN, whose pool of unsulfated granins (as substrates for sulfation) is not replenished in the presence of the drug. With respect to the various forms of CgB, this implies that forms 3 and 4 normally are generated in the TGN. The conclusion that protein sulfation is a TGN-specific post-translational modification is consistent with previous kinetic studies (12) and with the observation that sulfated proteins are packaged into post-TGN vesicles very rapidly after a sulfate pulse (13). We cannot exclude that the virtually complete inhibition of [^{35}S]sulfate incorporation into secretory proteins after a brefeldin A treatment for as short as 10 min is caused not only by the lack of delivery of unsulfated secretory proteins to the TGN but in addition by other effects, direct or indirect, of the drug. For example, inhibition of [^{35}S] sulfate incorporation into secretory proteins after brefeldin A treatment may also result from the rapid loss of unsulfated secretory proteins, phosphoadenosine phosphosulfate (the co-substrate for sulfation), and/or sulfotransferases from the TGN, for example because of the fusion of TGN membranes with endosomal membranes (45, 46). However, we do not find it likely that the inhibitory effect of brefeldin A on sulfation was caused by an inhibitory effect of the drug on tyrosylprotein sulfotransferase activity, *e.g.* via affecting the pH of the TGN, because this enzyme exhibits a broad pH optimum (50, 51). The fact that tyrosylprotein sulfotransferase exhibits significant activity at neutral pH is also the main reason why we find it unlikely that the dispersed cytoplasmic distribution of TGN38 after 4 h of brefeldin A treatment, reflected the redistribution of TGN membrane components to the ER. Rather, this change in the intracellular distribution of a TGN marker should be considered in light of recent reports (Refs. 45, 46, and 49 and references therein) showing that the effects of brefeldin A are not confined to the compartments of the secretory pathway proximal to the TGN but also concern the structural organization of the TGN and endosomal compartments, resulting in their fusion. The ability to label proteins selectively in the TGN by using radioactive sulfate may be advantageous for investigating further this phenomenon.

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