Novel Blockade by Brefeldin A of Intracellular Transport of Secretory Proteins in Cultured Rat Hepatocytes

(Received for publication, March 11, 1986)

Yoshio Misumi, Yuko Misumi, Koichiro Miki, Akira Takatsuki, Gakuzo Tamura, and Yukio Ichihara

From the Department of Biochemistry, Fukuoka University School of Medicine Nanakuma, Jonan-ku, Fukuoka 814-01, the Department of Anatomy, Saga Medical School, Nabeshima, Saga 840-01, and the Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

We examined the effect of brefeldin A, an antiviral antibiotic, on protein synthesis, intracellular processing, and secretion in primary culture of rat hepatocytes. The secretion was strongly blocked by the drug at 1 µg/ml and higher concentrations, while the protein synthesis was maintained fairly well. Pulse-chase experiments with [35S]methionine demonstrated that brefeldin A completely blocked the proteolytic conversion of proalbumin to serum albumin up to 60 min of chase, although its conversion was observed as early as 20 min in the control cells. The drug also inhibited the terminal glycosylation of oligosaccharide chains of α1-protease inhibitor and haptoglobin. These two modifications have been shown to occur at the trans region of the Golgi complex. The drug, however, had no effect on the proteolytic processing of the haptoglobin pro-form which takes place within the endoplasmic reticulum. Such an effect by brefeldin A is very similar to that induced by the carboxylic ionophore monensin. However, in contrast to evidence that monensin causes a delayed secretion of the unprocessed forms of these proteins, brefeldin A allowed the completely processed forms to be secreted after a prolonged accumulation of the unprocessed forms. Morphological observations demonstrated that the endoplasmic reticulum was markedly dilated by treatment with the drug at 10 µg/ml which continuously blocked the secretion. On the other hand, brefeldin A caused no inhibitory effect on the endocytic pathway as judged by cellular uptake and degradation of 125I-asialofetuin. These results indicate that brefeldin A is a unique agent which primarily impedes protein transport from the endoplasmic reticulum to the Golgi complex by a mechanism different from those considered for other secretion-blocking agents so far reported.

Newly synthesized secretory proteins are transported from the rough endoplasmic reticulum to the cell surface via a sequence of compartments. After exit from the endoplasmic reticulum, through its transitional elements, these proteins obtain access to smooth-surfaced membranes, among which the best characterized component is the Golgi complex, itself a structurally, histochemically, and functionally composite organelle (1–3). Exit from the stack of the Golgi cisternae is followed by transport via secretory vesicles. Although the framework of such secretory process is now well established (4), it is becoming increasingly clear that very little information on the mechanism involved is available.

The use of drugs affecting the secretory process at distinct sites in the cell may provide valuable for more detailed studies of specific steps in secretion and may lead to an understanding of the molecular basis of the mechanism involved in intracellular transport. For example, the carboxylic ionophore monensin has been used as one of the most potential agents which perturb protein transport (5, 6). In monensin-treated cells, newly synthesized proteins accumulate in intracellular vacuoles that appear to be derived from the Golgi complex (5, 6), and some of the post-translational modifications such as terminal glycosylation and proteolytic cleavage are blocked (7–10). The available evidence on the primary action of monensin (6, 11), as clearly demonstrated for its effect on the receptor-mediated endocytosis (12, 13), suggested that there exists an acidic subcompartment in the Golgi complex. Indeed, recent observations have revealed that the Golgi complex has its own ATP-dependent proton pump (14–16) to maintain an acidic internal milieu (17), which may be functionally important in the secretory process as well as in the endocytic pathway.

Brefeldin A is a unique fungal metabolite of a 13-membered macrocyclic lactone ring (18) and shows a wide range of interesting biological activities including an inhibitory effect on virus multiplication (19, 20). Recently Takatsuki and Tamura (21) have demonstrated that brefeldin A causes intracellular accumulation of high mannose type G protein and inhibition of its expression on cell surface in vesicular stomatitis virus-infected baby hamster kidney cells. In the present study, we examined the effect of brefeldin A on biosynthesis, intracellular processing, and secretion of several plasma proteins in primary culture of rat hepatocytes. The drug inhibited the secretion as strongly as monensin; however, there was a marked difference between the two drugs in their modes of action as revealed by biochemical and morphological analyses.
secretion.

Fig. 1. Effect of brefeldin A on total protein synthesis and secretion. Rat hepatocytes (2 × 10⁶ cells/dish) were preincubated at 37 °C for 1 h in the absence or presence of brefeldin A at the indicated concentrations. The cells were labeled with 20 μCi of [3H]leucine for 10 min and chased for 1 h. Total protein synthesis (C) was determined by measuring the trichloroacetic acid-insoluble radioactivity incorporated into the cells during a 10-min pulse and expressed as percentages of that of the control cells. Protein secretion (○) was determined by measuring the acid-insoluble radioactivity secreted into the medium during the 1-h chase and expressed as percent inhibition compared with that of the untreated cells.

FIG. 2. Effect of brefeldin A on the time course of albumin secretion. Cells (2 × 10⁶ cells/dish) were preincubated at 37 °C for 1 h in the absence (○) or presence of brefeldin A at 0.1 (●), 1 (△), 5 (▲), and 10 μg/ml (×). The cells were pulse-labeled for 10 min with 100 μCi of [35S]methionine and then chased. The drug, when indicated, was present throughout the pulse-chase. At the indicated times of chase, aliquots of the medium were taken and used for immunoprecipitation of albumin. Values are expressed as percentages of the radioactivity secreted of the total radioactivity incorporated into the immunoprecipitable albumin during a 10-min pulse.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

Effects of Brefeldin A on Protein Synthesis and Secretion—Fig. 1 shows dose effects of brefeldin A on total protein synthesis and secretion in cultured rat hepatocytes which were pulse-labeled with [3H]leucine for 10 min. Up to 1 μg/ml concentration of the drug had little effect on protein synthesis, although 20% inhibition was observed at 10 μg/ml. When the secretion rate during a 60-min chase was compared with that of control cells, brefeldin A was found to cause an almost complete inhibition of the secretion at 1 μg/ml and higher concentrations.

Fig. 2 shows dose effects of brefeldin A on the albumin secretion as a function of chase time after pulse-label with [35S]methionine. In the control cells, the newly synthesized albumin appeared in the medium even at 10 min (10), increased linearly up to 1 h, and thereafter reached the maximum level corresponding to about 80% of the total radioactivity incorporated into albumin. When the cells were treated with brefeldin A, the albumin secretion was inhibited in a dose-dependent manner. In the presence of 1 μg/ml concentration of the drug, the secretion was almost completely blocked until 1 h; after that time, the newly synthesized albumin was rapidly secreted into the medium, reaching about 90% of the normally secreted level at 3 h of chase. Such a time course of secretion, the initial complete inhibition and time-dependent release from the blockade, is quite similar with that observed in the cells treated with 1 μM monensin under the same conditions (10, 25, 35). At higher concentrations of brefeldin A, the complete blockade of secretion continued for 2 h (5 μg/ml) or a longer period (10 μg/ml). Similar secretion profiles in the presence or absence of brefeldin A were obtained for α₁-protease inhibitor and haptoglobin (data not shown).

Effect of Brefeldin A on Proteolytic Conversion of Proalbumin—Albumin is initially synthesized as proalbumin, the propeptide of which is cleaved off during the intracellular transport (36, 37). Fig. 3 demonstrates the effect of brefeldin A on the proteolytic processing of proalbumin, in which 35S-labeled samples prepared as in Fig. 2 were analyzed by electrofocusing in polyacrylamide gels followed by fluorography. In the control cells, the conversion of proalbumin to serum albumin was detected as early as 30 min of chase. When the cells were treated with brefeldin A, its conversion was delayed

Fig. 3. Effect of brefeldin A on the proteolytic conversion of proalbumin. Cells (2 × 10⁶ cells/dish) were preincubated at 37 °C for 1 h in the absence (A) or presence of brefeldin A at 1 (B) and 5 μg/ml (C). The cells were labeled with 100 μCi of [35S]methionine for 10 min and chased. At the indicated times, cells lysates and medium were prepared and used for immunoprecipitation of albumin. The immunoprecipitates were analyzed by gel electrofocusing (pH 5–8) followed by fluorography as described under "Materials and Methods." PA and SA indicate the major forms of proalbumin and serum-type albumin, respectively. The pH gradient (8 to 5) was formed from the top to bottom of the gels.

1 Portions of this paper (including "Materials and Methods" and Figs. 6–9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-767, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The conversion of proalbumin was already detectable at 20 min of chase under the same conditions by more detailed analysis (9).
A Unique Secretion-blocking Agent Brefeldin A

FIG. 4 Effect of brefeldin A on glycosylation of α₁-protease inhibitor. Cells were preincubated at 37 °C for 1 h in the absence (A) or presence of brefeldin A at 1 μg/ml (B). The cells were then pulse-labeled with 100 μCi of [35S]methionine for 10 min at 37 °C and chased. At the indicated times, cell lysates and medium were prepared and used for immunoprecipitation of α₁-protease inhibitor. The immunoprecipitates before and after Endo H digestion were analyzed by fluorography. M, medium; TM, tunicamycin-treated. M, (×10^-3) of three forms of α₁-protease inhibitor are indicated at the left side of the gels.

depending upon the drug concentrations used; the intracellular converted form was detectable only after 2 h and 3 h of chase in the presence of 1 μg/ml and 5 μg/ml, respectively, of the drug (Fig. 3, B and C). Compared with the time course of secretion (Fig. 2), the inhibitory effect by the drug on the proalbumin conversion is found to be closely related with the delayed secretion. Previously, we reported that monensin (10, 35) and weakly basic amines (39) also caused a strong inhibition of the proteolytic conversion, resulting in release of proalbumin into the medium. In the presence of brefeldin A, however, the form finally secreted into the medium was found to be the processed albumin even after an extremely prolonged accumulation of proalbumin in the cells, although the latter form was detectable as a minor component in the medium (Fig. 3, B and C).

Effect of Brefeldin A on Glycosylation of α₁-Protease Inhibitor—Rat serum α₁-protease inhibitor is a glycoprotein containing 13.2% carbohydrate (24). When its biosynthesis was examined in cultured rat hepatocytes under the same conditions as above, the protein was initially synthesized as a molecule with $M_{r}$ = 51,000 and then converted to a form with $M_{r}$ = 56,000, which was secreted into the medium (Fig. 4A). Upon digestion with Endo H, the initially synthesized $M_{r}$ = 51,000 form was shifted to a form with $M_{r}$ = 46,000 which had the same mobility in SDS-polyacrylamide gel electrophoresis as that prepared from the tunicamycin-treated cells, while the $M_{r}$ = 56,000 form in the cell as well as in the medium did not change the mobility. Since α₁-protease inhibitor is a single polypeptide chain, the change in molecular mass reflects the processing of oligosaccharide chains in the molecule during its intracellular transport; the $M_{r}$ = 51,000 form was converted to the $M_{r}$ = 56,000 form by processing of its oligosaccharide chains from the high mannose type to the complex one, which was already detectable at 30 min of chase in the control cells.

In the presence of brefeldin A (1 μg/ml), the conversion to the $M_{r}$ = 56,000 form was completely blocked until 2 h of chase; after that, the mature form appeared in the cell and secreted into the medium (Fig. 4B). The results obtained with Endo H treatment confirmed that the oligosaccharide chains of the intracellularly retained form were the high mannose type. A higher concentration of the drug (5 μg/ml) caused a longer blockade of the oligosaccharide processing (data not shown). However, under the conditions tested, the form finally secreted after a long delay was found to be the mature form with $M_{r}$ = 56,000 (Fig. 4B). The finding that in the presence of brefeldin A the terminal glycosylation eventually took place before the secretion is in contrast with the effect by monensin under which the glycoprotein was finally secreted as the unprocessed form with $M_{r}$ = 51,000 (10, 35).

Effect of Brefeldin A on Intracellular Processing of Haptoglobin—As previously reported (25), haptoglobin was initially synthesized as prohaptoglobin with $M_{r}$ = 45,000, which was immediately cleaved into the subunits, α and β. The β-subunit with $M_{r}$ = 33,000 containing high mannose type oligosaccharide chains was further processed to a $M_{r}$ = 36,000 form which was already detectable at 30 min of chase in the medium (Fig. 5A). Upon treatment of the cells with brefeldin A (1 μg/ml), the precursor with $M_{r}$ = 45,000 observed just after pulse-label disappeared with the same time course as in the control cells, while the $M_{r}$ = 36,000 form of the β-subunit did not appear until 2 h of chase (Fig. 5B). The intracellularly accumulating β-subunit with $M_{r}$ = 33,000 was found to have the high mannose type oligosaccharide chains, since it was sensitive to Endo H. The β-subunit, however, was finally secreted into the medium after being converted to the $M_{r}$ = 36,000 form with the complex type oligosaccharide chains, which was secreted into the medium (Fig. 5A). Upon treatment of the cells with brefeldin A (1 μg/ml), the precursor with $M_{r}$ = 45,000 observed just after pulse-label disappeared with the same time course as in the control cells, while the $M_{r}$ = 36,000 form of the β-subunit did not appear until 2 h of chase (Fig. 5B). The intracellularly accumulating β-subunit with $M_{r}$ = 33,000 was found to have the high mannose type oligosaccharide chains, since it was sensitive to Endo H. The β-subunit, however, was finally secreted into the medium after being converted to the $M_{r}$ = 36,000 form with the complex type sugar chains. These results indicate that the drug has no effect on the proteolytic conversion of prohaptoglobin into the subunits, but exerts the same inhibitory effect on the processing in glycosylation of the β-subunit as observed for α₁-protease inhibitor.

Reversibility of the Effects of Brefeldin A—Fig. 6 shows the kinetics of the reversibility of the brefeldin A inhibition of secretion. In the presence of brefeldin A at 5 μg/ml, the secretion of [35S]albumin and [35S]α₁-protease inhibitor was completely blocked up to 2 h of chase. After removal of the drug from the medium at 1 h of chase, albumin was rapidly secreted with almost the same rate and reached the same maximum level in the medium as in the control cells (Fig. 6A). Essentially the same recovery of the secretion was observed for α₁-protease inhibitor (Fig. 6B), although the secretion rate of the latter was slower than that of albumin. When the samples were analyzed by electrophoresis/fluorography, it was found that the mature form of each protein was rapidly increased in the cells after removal of the drug (data not shown). These results indicate that the inhibitory effect of brefeldin A on the intracellular transport and processing of the secretory proteins was fully reversible immediately after removal of the drug.

Effect of Brefeldin A on Cellular Uptake and Degradation of [125I]-A-Asialofetuin—The representative secretion-blocking agent monensin is also known to block the receptor-mediated

---

The abbreviations used are: Endo H, endo-β-N-acetylglucosaminidase H; CCCP, carbonyl cyanide m-chlorophenylhydrazone; SDS, sodium dodecyl sulfate.

---

3 The abbreviations used are: Endo H, endo-β-N-acetylglucosaminidase H; CCCP, carbonyl cyanide m-chlorophenylhydrazone; SDS, sodium dodecyl sulfate.

4 The α-subunit was not identified here, because it contains no methionine.
endocytosis and concomitant events in endosomes and lysosomes (6, 12, 13, 38). On the other hand, weak bases originally known as lysosomotropic agents exert the inhibitory effect on the secretory process (39, 40). Hence, we examined a possible effect of brefeldin A on the endocytic pathway by measuring the cellular uptake and degradation of 125I-asialofetuin. As shown in Fig. 7, in the control cells, 125I-asialofetuin which had bound to the cells during a 90-min incubation at 4 °C was increasingly degraded as a function of incubation time at 37 °C, reaching about 70% degradation at 2 h. Almost the same time course and extent of the degradation was observed in the cells treated with brefeldin A at 1 μg/ml that effectively blocked the secretory process as described above. Even at a higher concentration (10 μg/ml), the drug exerted at most 20% inhibition of the degradation level normally observed (Fig. 7). Thus, the results suggest that brefeldin A does not specifically affect the receptor-mediated endocytosis and degradation process of the ligand, in contrast to the available evidence obtained with monensin and weak bases (6, 12, 38).

*Morphology of Brefeldin A-treated Hepatocytes*—It may be possible to consider that the strong inhibition of secretion by brefeldin A would be accompanied by any morphological alteration of organelles involved in the secretory process. In fact, monensin (5, 6) and weak bases (40) induce marked morphological changes of the Golgi complex and related elements. Fig. 8 shows electron micrographs of brefeldin A-treated cells. There was no significant change in the cells which had been treated with 1 μg/ml of the drug at 37 °C for 2 h (Fig. 8A); abnormal arrangement or distension of the Golgi cisternae and endoplasmic reticulum were not detectable in the treated cells. Under this condition, the secretion was blocked during the first hour of chase, but recovered to the control level by 3 h (Fig. 2). When the cells were exposed for 4 h to a 10 μg/ml concentration of the drug, which continuously blocked the secretion (Fig. 2), there appeared to be a marked alteration in the endoplasmic reticulum; endoplasmic reticulum with or without ribosomes were remarkably dilated and contained amorphous materials, possibly secretory proteins, within the lumens (Fig. 8B). Thus, the results suggest that the primary site impaired by brefeldin A is the endoplasmic reticulum. Dilatation of the endoplasmic reticulum might result from the complete blockade of protein transport for longer periods which required a high dose of the drug (10 μg/ml), because a transient inhibition by a lower dose caused no significant alteration in morphology.

*Effect of Brefeldin A on Cellular ATP Level*—Cellular metabolism is known to be essential for intracellular transport from the rough endoplasmic reticulum to the Golgi complex and for exocytosis in some regulated secretory cells (41). To compare with the previous evidence that the secretion block observed in the presence of CCCP is not accompanied by striking ultrastructural alterations in plasma cells (42), we examined a possible effect of brefeldin A on the cellular ATP level. As demonstrated in Fig. 9, however, in comparison with the control cells, the brefeldin A-treated cells showed no significant change in the cellular ATP level under the conditions tested, although 20% reduction was detected only after the cells were incubated for 3 h with a much higher dose (10 μg/ml) of the drug. These results are in contrast to a marked reduction of ATP in the CCCP-treated cells.

The present study clearly demonstrated that brefeldin A is a potent inhibitor of intracellular transport of secretory proteins in rat hepatocytes. The drug specifically blocked the secretion, but caused no significant effect on protein synthesis and protein degradation process following the receptor-mediated endocytosis. Takatsuki and Tamura (21) have recently reported that brefeldin A completely blocks translocation of the newly synthesized G protein to cell surfaces in vesicular stomatitis virus-infected baby hamster kidney cells, resulting in intracellular accumulation of G protein with high mannose type oligosaccharide chains. We obtained the same results in the present system of rat hepatocytes; in the presence of the drug (1 μg/ml), no 35S-labeled G protein was detectable in the medium during the 3-h incubation, and oligosaccharide chains of the intracellular G protein were sensitive to Endo H digestion. Thus, the inhibitory effect of brefeldin A on intracellular transport of exportable proteins seems to be slightly different between the membrane-bound G protein and soluble secretory proteins; transport of the membrane protein was continuously blocked, while that of secretory proteins was time dependently released from the blockade. The reason for this difference is not clear at present. In spite of such differences, these results strongly suggest that brefeldin A primarily blocks the protein transport before the trans Golgi in which terminal glycosylation of oligosaccharide chains in glycoproteins (43) and proteolytic processing of proalbumin (9, 10, 37) take place. Morphological observations

---

further indicate that the drug possibly blocks exit from the endoplasmic reticulum, since its dilution was coupled with the continuous blockade of secretion. Brefeldin A is known to be a lipophilic compound (18), which may easily interact with membranes. Through the interaction with the membrane, brefeldin A is supposed to cause a dysfunction of some particular component(s) involved in the transport mechanism from the endoplasmic reticulum to the Golgi complex, although details of the mechanisms remain to be solved. The mode of the brefeldin A action is clearly different from those of other secretion-blocking agents such as monensin (5–10), weak bases (39, 40), and the microtubule-affecting agents colchicine (10) and taxol (44). A further investigation on the mechanism of the brefeldin A action will shed light on the transport mechanism of secretory proteins, as well as membrane proteins.

Acknowledgments—We thank Drs. S. Masuko (Saga Medical School), S. Ogata, N. Hamasaki, and K. Oda (Fukuoka University) for useful suggestions and help in the present study.

REFERENCES
methanol/10% trichloroacetic acid/74% acetic acid for 1 h, and treated with ENTRANCE followed by drying and fluorography (39). SDS-polyacrylamide gel electrophoresis was carried out in slab gels with 10% and 12.5% acrylamide for Mr-protease inhibitor and hepatoglobin, respectively, according to Laemmli (31). Cells were processed for fluorography as above. Apparent molecular weights were determined by co-electrophoresis of marker proteins; rat transferrin (77,000), rat serum albumin (66,000), ovalbumin (45,000), and trypsin (23,000).

**Determination of Cellular ATP Level:** Hepatocytes (8 x 10^5 cells) which had been incubated in the presence or absence of brefeldin A or CCCP at the indicated concentrations were washed twice with Hanks' solution and suspended in 1 ml of NaCl/Pi. An equal volume of 6 M perchloric acid was added to the cell suspension and mixed well, followed by centrifugation at 32,000 x g for 5 min. The resulting supernatants were neutralized with 0.5 M KOH and used for determination of ATP, which was performed according to Minakami et al. (32).

**Uptake and degradation of [125]asialo-fetuin:** Cells which had been preincubated at 37°C for 1 h in the presence or absence of brefeldin A were washed with ice-cold Hanks' solution containing 1% bovine serum albumin (Hanks-BSA), and incubated at 4°C for 1 h with [125]asialo-fetuin (2 x 10^7 cpm/µg) in 1.2 ml of the Hanks-BSA solution. After removal of the medium containing [125]asialo-fetuin, the cells were washed 4 times with ice-cold Hanks-BSA solution and further incubated at 37°C in 1.5 ml of the same solution without [125]asialo-fetuin. Brefeldin A, when indicated, was present in the medium throughout the incubation at 4°C as well as at 37°C. At the indicated times, cells and medium were separately removed from the dishes, and cells were suspended in 1.5 ml of NaCl/Pi. The cell suspensions and medium were treated with trichloroacetic acid (finally 10%), and acid-insoluble and acid-soluble materials in each sample were separated by centrifugation at 5,000 x g for 10 min, followed by radioactivity determination.

**Electron Microscopy:** Cells which had been cultured on collagen-coated cover slips in dishes in the presence or absence of brefeldin A were washed with NaCl/Pi and fixed in 2% paraformaldehyde/0.1 M sodium cacodylate buffer (pH 7.4) containing 0.1 M sucrose at 4°C for 40 min. The cells were postfixed in 1% osmium tetroxide/0.1 M phosphate buffer (pH 7.2) for 1 h, samples were then dehydrated by passage through graded ethanols and embedded in Durvac's resin (33). Thin sections were cut with a Leica MT-2 ultramicrotome, stained with lead citrate and uranyl acetate (34), and viewed with a JEOL 100CX electron microscope.

**Fig. 6.** **Reversibility of brefeldin A effect on secretion.** Cells were preincubated at 37°C for 1 h in the absence (x) or presence of brefeldin A at 5 µg/ml (·). The cells were then pulse-labeled with 100 µCi of [35]methionine, and chased. In one experiment brefeldin A was continuously present in the medium throughout the pulse-chase period (○). In another experiment the drug was removed from the medium at 1 h chase (△). At the indicated times, cell lysates and medium were prepared and used for immunoprecipitation of albumin (A) and Mr-protease inhibitor (B). Values are expressed as percentages of the radioactivity secreted of the total radioactivity incorporated into the immunoprecipitated albumin (A) or Mr-protease inhibitor (B). The results are the means of three obtained from two separate experiments.

**Fig. 7.** **Degradation of [125]asialo-fetuin endocytosed by hepatocytes.** After preincubation at 37°C for 1 h in the presence or absence of brefeldin A, the cells were incubated at 4°C for 1 h with [125]asialo-fetuin (2.4 x 10^7 cpm/µg) in 1.2 ml of Hanks-BSA solution, washed and further incubated at 37°C in the fresh Hanks-BSA solution without [125]asialo-fetuin. Brefeldin A, when indicated, was present during incubation at 4°C and at 37°C. At the indicated times, cells and medium were taken and used for radioactivity determination as described in Materials and Methods. Degradation was measured as total acid-soluble radioactivity in the cells and medium, is expressed as percentages of total radioactivity associated with cells after 1 h incubation at 4°C control cells (4.7 x 10^6 cpm/dish) (■); cells treated with brefeldin A at 1 µg/ml (4.4 x 10^6 cpm/dish) (●); at 5 µg/ml (4.5 x 10^6 cpm/dish) (○), and at 10 µg/ml (3.9 x 10^6 cpm/dish) (△). Values represent the means of the results obtained from two separate experiments.

**Fig. 8.** **Electron micrographs of brefeldin A-treated hepatocytes.** Cells were incubated at 37°C in the presence of brefeldin A at 1 µg/ml for 2 h (A) or 10 µg/ml for 4 h (B). The cells were then fixed, embedded and thin-sectioned as described in the Materials and Methods section. G. Golgi complex; RER, rough endoplasmic reticulum; M, mitochondria; P, peroxisome. Arrows indicate ribosomes associated with the endoplasmic reticulum. Bar, 1 µm.

**Fig. 9.** **Effect of brefeldin A on cellular ATP level.** Cells (8 x 10^5 cells) were incubated at 37°C for 1 h (open bar) or 3 h (closed bar) in the presence or absence of brefeldin A or CCCP at the indicated concentrations. Cells exposed to 1.0% methanol, which was used as a solvent for brefeldin A, were taken as another control. Cellular ATP levels were determined as described in the Materials and Methods section. The results represent the means ± S.E. of three separate experiments.