Brefeldin A-induced Increase of Sphingomyelin Synthesis

ASSAY FOR THE ACTION OF THE ANTIBIOTIC IN MAMMALIGNAL CELLS*

(Received for publication, October 14, 1991)

Ansgar Brüning, Achim Karrenbauer, Eva Schnabel, and Felix T. Wielandt
From the Institut für Biochemie I, Universität Heidelberg, im Neuenheimer Feld 328, W-6900 Heidelberg, Germany

Brefeldin A leads to an increase of sphingomyelin in Chinese hamster ovary cells. The antibiotic is known to cause a dramatic morphological change of the endomembrane system in various mammalian cells resulting in a redistribution of Golgi resident proteins to the endoplasmic reticulum (Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H. P., Yuan, L. C., and Klausner, R. D. (1990) Cell 60, 821-836). A strict correlation was found between the brefeldin A-induced increase of sphingomyelin and the biochemical criteria that apply for this morphological change. From our data we conclude that the increase in sphingomyelin caused by brefeldin A reflects translocation of the enzyme sphingomyelin synthase from the Golgi apparatus to the endoplasmic reticulum. Using a radioactively labeled truncated ceramide this increase in sphingomyelin synthesis is easily detectable, and thus this method can serve as a convenient biochemical assay for the action of brefeldin A in mammalian cells.

The antibiotic brefeldin A (BFA) has attracted the interest of biochemists and cell biologists because it acts on the equilibrium state of intracellular organelles (1-3) and thus interferes with vesicular protein transport (4). These effects on the distribution of intracellular proteins as well as on transport have been demonstrated by fluorescence and electron microscopy using labeled antibodies against marker proteins (1-3) or by measuring the transport of proteins which have been pulse-labeled in vivo (2, 3, 5, 6). Such experiments, though very effective, are either not quantitative at all or technically complicated and time consuming.

The molecular mechanism of action of the antibiotic is still unclear. An immediate effect is to cause dissociation of coat proteins (7) from the Golgi (4, 8). Subsequently, the organelle appears to disintegrate by vesiculation, and after a few minutes Golgi enzymes are translocated to the endoplasmic reticulum (ER) (2, 3). These effects are fully reversible, provided that ATP is not depleted in the cells (1-3). The dissociation of coat proteins from the Golgi caused by BFA can be inhibited by preincubation with GTPyS or AlF₃, indicating that trimeric G-proteins are somehow involved in this process (4, 9). Nocodazole, a microtubuli-disassembling drug, inhibits translocation of Golgi enzymes to the ER but does not inhibit BFA-induced dissociation of coat proteins from the Golgi (10). These findings have supported a model of recycling of Golgi constituents to the ER via an intermediate (10, 11) or salvage (12) compartment.

We have developed a ceramide analogue that is truncated in both hydrophobic chains (13, 14). This water-soluble and amphiphilic compound C₈C₈-Cer readily permeates biological membranes and is converted in vivo to yield the sphingolipid analogues "truncated sphingomyelin" (C₈C₈-SM) and "truncated glucosylceramide" (C₈C₈-GlcCer). Biosynthesis of sphingomyelin in CHO cells occurs by transfer of a phosphatidylcholine moiety from phosphatidylcholine to ceramide. The enzyme sphingomyelin synthase is located in the cis (15) or cis and medial (16) Golgi, whereas both substrates, phosphatidylcholine and ceramide, are synthesized in the ER (17, 18), a membrane system of about 10 times the size of the Golgi apparatus, with correspondingly more phosphatidylcholine and ceramide present than in the Golgi. If the availability of one of these substrates is rate-limiting in physiological sphingomyelin synthase in the Golgi, then translocation of sphingomyelin synthase from the Golgi to the ER caused by BFA should lead to an increase of sphingomyelin. This has led us to probe sphingomyelin synthase in the presence of BFA. We show here that sphingomyelin synthase from endogenous ceramide is indeed increased after administration of BFA to CHO cells. An even more drastic increase is observed with the exogenously added analogue C₆C₆-Cer. Stimulation of C₆C₆-SM biosynthesis observed after administration of BFA was found to be in accordance with all biochemical criteria so far defined for the action of BFA on the endomembrane system. Using the model substrate C₆C₆-Cer, sphingomyelin synthase activity can quickly and quantitatively be followed. This assay may be useful for an easy and effective screening of cells for their sensitivity to BFA as well as for drugs antagonistic to BFA.

EXPERIMENTAL PROCEDURES

Materials—BFA and its derivatives were kindly provided by R. E. Kneusel and Dr. U. Matern (Freiburg). Forskolin and nocodazole were purchased from Sigma, München. All cell culture reagents were from Biochrom KG, Berlin. Truncated ceramide (C₆C₆-Cer) and [³H] C₆C₆-Cer were prepared as described (13).

Cell Culture—CHO cells (ATCC CCL 61) were grown in suspension cultures in α-MEM containing 7.5% fetal calf serum and 100 units of penicillin plus 100 units of streptomycin per ml. Cells were harvested at a density of 6-8 x 10⁶ cells/ml and resuspended at 5 x 10⁶ cells/ml in α-MEM without fetal calf serum and without antibiotics but supplemented with 20 mM Hepes buffer (pH 7.2).

PIK-2 cells (ATCC CCL 56, kindly provided by Dr. F. Bautz, Heidelberg) were cultured in 25-cm² flasks (Falcon, GB) in the medium as described above for the growth of CHO cells. Immediately before an experiment, cells were washed with phosphate-buffered saline (140 mM NaCl, 2.6 mM KCl, 6 mM Na₂HPO₄, 1.5 mM KH₂PO₄).

*This work was supported by Grant SFB 352 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 06221-564156; Fax: 06221-564366.

interferes with vesicular protein transport, which may affect the distribution of intracellular organelles and thus be relevant for the pathogenesis of certain diseases. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 352). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" under 18 U.S.C. 1734 solely to indicate this fact.
trypsinized (0.125% trypsin, 0.05% EDTA in phosphate-buffered saline), washed with α-MEM, and resuspended at a concentration of 5 × 10^6 cells/ml.

Labeling of CHO Cells with [3H]Choline—1 × 10^6 CHO cells each were plated out in plastic Petri dishes (3.5 cm) in the medium described under "Cell Culture" and grown to near confluency were plated out in plastic Petri dishes.

After preincubation with BFA (5 μg/ml) at 37 °C for 15 min, 5 μCi of [3H]choline (75 Ci/mmole, Amersham Corp.) was added, and the cells were kept at 37 °C for 1, 3, 6, and 16 h, respectively, in a humidified atmosphere of 5% CO2. Thereafter, the cells were harvested using trypsin as described above for PtK cells. Membrane lipid extraction was carried out according to Ref. 13 with the following modifications. The cell pellets were resuspended in 3 volumes of water, then n-methyl and chloroform were added to obtain ratios of 0.8/2/1 for H2O/CH3OH/CHCl3. After sonication for 5 min (Sonorex, Bandelin, Berlin), more CHCl3 was added to give ratios of 0.8/2/2 for H2O/CH3OH/CHCl3. After another 5 min of sonication, water was added to a final ratio of 1.8/2/2 for H2O/CH3OH/CHCl3, and the two phases were separated. The lipids under study (phosphatidylcholine and sphingomyelin) were found exclusively in the CHCl3 phase and subsequently separated by TLC (HPTLC Silica Gel 60). After sonication for 30 min at 37 °C, the cells were chilled on ice, centrifuged (30,000 × g, 15 min), and the cell pellet was extracted with 50 μl of methanol/water (1/1). After centrifugation at 13,000 × g for 5 min, 5-μl aliquots of the cell extracts and of the corresponding media were subjected to TLC on Silica Gel 60 (Merck) with butanone/water/acetone/formic acid (8/10/6/0.5) as the solvent system. The radioactivities of C8Ce-SM and C8Ce-GlcCer were determined by two-dimensional radioscanning as described above.

RESULTS AND DISCUSSION

Sphingomyelin Synthesis Is Increased in the Presence of BFA—Two different pathways of sphingomyelin synthesis are discussed in the literature: (i) transfer of phosphorylcholine from phosphatidylcholine to ceramide (both reaction partners are generated in the ER) or rather a direct influence of BFA on the activity of sphingomyelin synthase from the Golgi to the mixed ER-Golgi organelle, in which a large supply of phosphatidylcholine is available for increased sphingomyelin synthesis. This concept was corroborated by the experiments described in the following sections.

BFA-induced Increase in Sphingomyelin Is Specific to BFA—In order to assess the specificity of the observed effect, structural analogues of BFA were tested. BFA proved to be highly specific because at 5 μg/ml neither 7-oxo-BFA, 4,7-

| Incubation time | [3H]Sphingomyelin formed | % of control |
|-----------------|--------------------------|-------------|
| 1               | 128                      |             |
| 3               | 135                      |             |
| 6               | 172                      |             |
| 16              | 194                      |             |

FIG. 1. BFA increases the synthesis of C8Ce-SM but not of C8Ce-GlcCer. CHO cells were incubated in the absence or presence of 5 μg BFA/ml for 15 min at 37 °C followed by a 30-min incubation with [3H]C8Ce-Cer (for details see "Experimental Procedures"). Cell extracts and media were chromatographed on silica gel plates, and their contents of [3H]C8Ce-SM and [3H]C8Ce-GlcCer determined by two-dimensional radioscanning. The counts measured in 1 h in the individual spots are given in the ordinate. Numbers in the bars represent the C8Ce-SM and C8Ce-GlcCer content in the presence of BFA divided by the content of control cells and media.

To be drastically increased by BFA (Fig. 1). A maximal cellular content of [3H]C8Ce-SM representing almost 5-fold the amount found in control cells is already observed 30 min after the addition of BFA. In contrast, the level of [3H]C8Ce-GlcCer is essentially uninfluenced by the drug. Does this increase reflect the described BFA-induced fusion of the Golgi with the ER or rather a direct influence of BFA on the activity of the enzyme sphingomyelin synthase? In order to exclude the latter possibility, we investigated [3H]C8Ce-SM synthesis in vitro using isolated intact CHO Golgi membranes as described (15). Membranes were incubated at 37 °C for 15 min with various concentrations of BFA (0–200 μg/ml), and subsequently [3H]C8Ce-SM synthesis was determined. No influence of the antibiotic on sphingomyelin synthase was detected. This finding suggests that the increase of sphingomyelin observed after administration of BFA in vivo indeed reflects fusion of ER with Golgi membranes, i.e. translocation of sphingomyelin synthase from the Golgi to the mixed ER-Golgi organelle, in which a large supply of phosphatidylcholine is available for increased sphingomyelin synthesis. This was shown by experiments described in the following sections.
dioxo-BFA, nor BFA-acid caused an increase of C₈C₈-SM synthesis (not shown). These substances are all naturally occurring derivatives of BFA. The inability of the synthetic derivatives 4-epi-BFA and O-methylated BFA species to block non-clathrin-coated vesicle formation has been shown in a cell-free system (4), suggesting a highly specific binding of BFA to its target.

**BFA-induced Increase in Sphingomyelin Is Prevented by Nocodazole and by Fluoride**—Dissociation of the Golgi can be prevented by preincubating the cells with the microtubule-destabilizing reagent nocodazole before addition of BFA (10). A similar inhibition of the antibiotic's effect by GTPγS or AIF₇ has been described (9). Therefore, nocodazole and fluoride were tested for their ability to inhibit BFA-induced sphingomyelin increase. CHO cells were incubated with nocodazole or fluoride, followed by BFA, and then sphingomyelin synthase activity was probed by the addition of [³H]C₈C₈-Cer. As shown in Fig. 2, both drugs cause a strong reduction of the BFA effect when compared with the control. In addition, fluoride completely blocks the formation of C₈C₈-GlcCer (not shown). This indicates that the high concentration of fluoride used has led to energy depletion, because C₈C₈-GlcCer synthesis depends on UDP-Glc, the formation of which is dependent on the energy charge of a cell. Thus, the inhibition of BFA-induced C₈C₈-SM synthesis by fluoride may be explained by two effects: interference with trimeric G-proteins (21) and/or energy depletion. Both conditions have been described to inhibit the antibiotic's effect on the endomembrane system (2, 9).

**BFA-induced Increase in Sphingomyelin Is Antagonized by Forskolin and Is Reversible**—Forskolin, a drug known for its activation of adenylate cyclase has been shown to antagonize the BFA-induced Golgi dissociation/ER fusion process in a cAMP-independent way (22). This drug inhibits translocation of Golgi markers into the ER when applied before adding BFA and leads to a relocation to Golgi structures of Golgi markers when administered after the addition of BFA. Likewise, the effects of BFA can be reversed by simply washing out the drug (1–3). In order to probe the described BFA-induced increase in sphingomyelin synthase under the above mentioned conditions, cells were either pretreated with forskolin and then challenged with BFA, or forskolin was added after the challenge. The results are depicted in Fig. 3; forskolin at 200 or 20 µg/ml clearly reduced the BFA effect when added before BFA. In addition, the BFA-induced increase in sphingomyelin was partially reversed by adding forskolin after BFA. We have never observed a total block of the C₈C₈-SM increase in accordance with the idea that forskolin acts as a competitive antagonist to BFA (22). Likewise, a single washing of the cells with a BFA-free medium caused a strong reduction of [³H]C₈C₈-SM even after a recovery time as short as 15 min.

**BFA-resistant PtK Cells Do Not Respond to BFA with an Increase in Sphingomyelin**—Recently, PtK-1 cells (rat kangaroo kidney cells from the marsupial Potorous tridactylus) have been described to be resistant against BFA. Even at high concentrations, the antibiotic (up to 50 µg/ml) did not induce translocation of Golgi markers into the ER (23). If the sphingomyelin increase described above is a consequence of the BFA-induced ER/Golgi fusion, this increase must not occur in PtK cells. Therefore PtK-2 cells were incubated with various concentrations of BFA, and their [³H]C₈C₈-SM synthesis was determined. As clearly shown in Fig. 4, no increase in sphingomyelin synthesis is detectable even at a BFA concentration of 200 µg/ml, indicating BFA resistance of both established PtK cell lines. It may be of interest to test if this resistance is restricted to PtK cells or if other marsupials are resistant against BFA as well.

Taken together, these data provide strong evidence that the increase in sphingomyelin synthesis induced by BFA reflects dissociation and fusion of the Golgi with the ER. The most straightforward explanation for the increased synthesis of sphingomyelin is the translocation of sphingomyelin synthase into a mixed ER-Golgi organelle, where a larger pool of substrate is available than in the Golgi alone. However, the amount of endogenous free ceramide in this mixed compartment might still be limiting because addition of an excess of exogenous membrane-permeable ceramide analogue further increased the levels of the sphingomyelin analogue.
practical assay to screen cell lines or organisms for their sensitivity to BFA or to screen drugs for an antagonistic action. This will lead to a better understanding of the still unknown mechanism of the dramatic action of this antibiotic. In addition, the assay should help to characterize the cellular organelle using this assay. Presently we attempt to isolate and characterize the mixed ER-Golgi organelle using this assay.

Acknowledgments—We thank Gaby Weiss for her excellent and reliable maintenance of the cell cultures, Irene Speckhard and Barbara Schröter for their help with the manuscript, and Dr. Heiner Schimer for critically reading the manuscript. PtK cells were a gift of Dr. Friedlinde Bautz. Brefeldin A and its derivatives used here were kindly provided by Richard E. Kneusel and Dr. Ulrich Matern, Freiberg.

REFERENCES
1. Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988) J. Biol. Chem. 263, 18540–18552
2. Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S., and Klausner, R. D. (1989) Cell 56, 801–813
3. Doms, R. W., Russ, G., and Yewdell, J. W. (1989) J. Cell Biol. 109, 61–72
4. Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J. G., Lippincott-Schwartz, J., Klausner, R. D., and Rothman, J. E. (1991) Cell 64, 1183–1195
5. Takatsuki, A., and Tamura, G. (1985) Agric. Biol. Chem. 49, 899–902
6. Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., and Ikehara, Y. (1986) J. Biol. Chem. 261, 11398–11403
7. Serafini, T., Stenbeck, G., Brecht, A., Lottspeich, F., Orci, L., Rothman, J. E., and Wieland, F. T. (1991) Nature 349, 215–220
8. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) J. Cell Biol. 111, 2295–2306
9. Donaldson, J. G., Lippincott-Schwartz, J., and Klausner, R. D. (1991) J. Cell Biol. 112, 579–588
10. Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A, Berger, E. G., Hauri, H. P., Yuan, L. C., and Klausner, R. D. (1990) Cell 60, 821–836
11. Armstrong, J., and Warren, G. (1990) Nature 344, 383–385
12. Pelham, H. R. B. (1989) Annu. Rev. Cell Biol. 5, 1–23
13. Karrenbauer, A., Jeckel, D., Just, W., Birk, R., Schmidt, R. R., Rothman, J. E., and Wieland, F. T. (1990) Cell 63, 259–267
14. Helms, J. B., Karrenbauer, A., Wirtz, K. W. A., Rothman, J. E., and Wieland, F. T. (1990) J. Biol. Chem. 265, 20027–20032
15. Jeckel, D., Karrenbauer, A., Birk, R., Schmidt, R. R., and Wieland, F. T. (1990) FEBS Lett. 261, 155–156
16. Puttermann, A. H., Steiger, B., Hubbard, A. L., and Pagano, R. E. (1990) J. Biol. Chem. 265, 8650–8657
17. Pagano, R. E. (1988) Trends Biochem. Sci. 13, 202–205
18. van Meer, G. (1988) Annu. Rev. Cell Biol. 5, 247–275
19. Bligh, E. G., and Dyer, W. J. (1957) Can. J. Biochem. Physiol. 37, 911–917
20. Stoffel, W., and Meisner, J. (1980) Hoppe Seylers Z. Physiol. Chem. 361, 755–771
21. Kahn, R. A. (1991) J. Biol. Chem. 266, 15596–15597
22. Lippincott-Schwartz, J., Glickman, J., Donaldson, J. G., Robbins, J., Kreis, T. E., Seamon, K. B., Sheetz, M. P., and Klausner, R. D. (1991) J. Cell Biol. 112, 567–577
23. Kiistakos, N. T., Roth, M. G., and Bloom, G. S. (1991) J. Cell Biol. 113, 1009–1023
24. Urbani, L., and Simoni, R. D. (1990) J. Biol. Chem. 265, 1919–1925
25. Vance, J. E., Asman, E. J., and Szarka, R. (1991) J. Biol. Chem. 266, 8241–8247

Fig. 4. PtK cells do not respond to BFA with a C8-C8 SM increase. 2.5 x 10^6 PtK-2 cells in 50 μl of α-MEM were incubated with various BFA concentrations at 37 °C for 30 min and processed as described for CHO cells to determine their [3H]C8-C8-SM contents. As a control 2.5 x 10^6 CHO cells in 50 μl of α-MEM were incubated in the presence and absence of 5 μg of BFA/ml and analyzed in the same way.

Under physiological conditions, the truncated sphingolipids are efficiently secreted from the cells. In the experiments with BFA presented here, the ratio of the cellular content of sphingomyelin to that of the corresponding media was increased by a factor of 2 (usually measured 30 min after the addition of BFA) (cf. Fig. 1). This cellular accumulation indicates that C8-C8-SM is retained. This is in line with previous observations that BFA inhibits the transport of proteins from the ER via the Golgi to the plasma membrane. On the other hand, transport of some membrane lipids has been reported to be uninfluenced by BFA (24, 25). The increase of [3H]C8-C8-SM induced by BFA can easily and quickly be determined by TLC. This system provides a practical assay to screen cell lines or organisms for their sensitivity to BFA or to screen drugs for an antagonistic action. This will lead to a better understanding of the still unknown mechanism of the dramatic action of this antibiotic. In addition, the assay should help to characterize the cellular target of BFA, in semi-intact cells or (as a classical biochemical complementation assay) during protein purification. Presently we attempt to isolate and characterize the mixed ER-Golgi organelle using this assay.