Forskolin Stimulates Detoxification of Brefeldin A*

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Walter Nickel‡, J. Bernd Heims, Richard E. Kneusel, and Felix T. Wieland

From the Institut für Biochemie I, Ruprecht-Karls Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany and Institut für Biologie II, Universität Freiburg, Schänzelestrasse 1, 79104 Freiburg, Germany

Forskolin has been shown to prevent the effects of brefeldin A (BFA) exerts on many mammalian cells with respect to the disassembly of the Golgi apparatus as well as an increase of sphingomyelin synthesis (Lippincott, S. 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). It has been speculated that forskolin interferes with the action of BFA by competition for the binding of BFA to its target protein, which is most likely the Golgi-localized nucleotide exchange factor specific for ADP-ribosylation factor 1. Here we show that in vitro forskolin does not prevent inhibition of Golgi-catalyzed nucleotide exchange by BFA. Therefore it appears unlikely that forskolin and BFA bind to the same target protein. Using [3H]BFA we have measured detoxification of BFA by Chinese hamster ovary (CHO) cells. BFA is secreted from CHO cells as cysteine and glutathione conjugates (Brüning, A., Ishikawa, T., Kneusel, R. E., Matern, U., Lottspeich, F., and Wieland, F. T. (1992) J. Biol. Chem. 267, 7726–7732). We present evidence that forskolin treatment of CHO cells results in increased levels of Cys-BFA, the major BFA conjugate secreted by CHO cells, in the medium. Elevated levels of Cys-BFA are also found intracellularly. The effect of forskolin is shown to be independent of its ability to raise the intracellular concentration of cyclic AMP. Therefore, we suggest that the effect of forskolin on BFA-induced disassembly of the Golgi apparatus might be due to an enhanced detoxification of the drug.

Treatment of mammalian cells with BFA, an inhibitor of protein secretion (7), causes the complete disassembly of the Golgi complex resulting in an endoplasmic reticulum-Golgi mixed compartment (8). Under these conditions coat proteins that associate with Golgi membranes in order to form coat protein I (COPI)-coated transport vesicles are redistributed into the cytoplasm (9). The effects of BFA are fully reversible when the drug is removed from the medium of cultured cells indicative for the detoxification of BFA (6). Forskolin has originally been described as an activator of adenyl cyclase (10). Forskolin stimulates cAMP synthesis by a direct interaction with the catalytic subunit of the enzyme (11). In addition, forskolin has also been shown to bind to a number of membrane-spanning plasma membrane proteins including the glucose transporter (12), the nicotinic acetylcholine receptor (13), the γ-aminobutyric receptor (14), voltage-dependent K+ channels (15), and possibly the P-glycoprotein multidrug transporter (16, 17). Although these interactions are not well characterized it seems that forskolin inhibits the activities of these membrane transporters (18). Finally, it has been reported that forskolin both inhibits and reverses the effects of BFA on Golgi morphology and on sphingomyelin synthesis, respectively (1, 2). Forskolin action was shown to be independent of its ability to stimulate adenyl cyclase (1). Since the action of forskolin was found to be dose-dependent and could be overcome by increased levels of BFA, it was proposed that forskolin and BFA act on the same target.

This target of BFA has been described to be a Golgi-localized nucleotide exchange enzyme specific for ADP-ribosylation factor 1 (3–5) or a factor regulating this enzyme. These data could imply that forskolin is able to bind to the BFA target, thereby inhibiting BFA action. However, the BFA target has not yet been purified. Even direct cross-linking experiments with [3H]BFA did not reveal the identity of this factor (6). Isolation of forskolin-binding proteins from Golgi-enriched subcellular fractions by use of a forskolin affinity matrix also did not result in the identification of a potential BFA target (data not shown).

We present data that indicate an indirect role of forskolin in maintaining the integrity of the Golgi apparatus in the presence of BFA. Employing an in vitro assay to reconstitute nucleotide exchange onto ARF we show that forskolin cannot rescue this activity by competition with BFA. Therefore, we conclude that the target of BFA might be different from that of forskolin.

On the basis of a previously published assay (6), we present evidence that treatment of cells with forskolin results in a significantly increased formation and secretion of Cys-BFA, the major conjugate secreted by CHO cells. Our results suggest that forskolin prevents the effects of BFA on the secretory pathway by stimulating detoxification of BFA rather than by interacting with proteins (e.g. the ARF-specific nucleotide exchange factor) that are involved in vesicular transport.

EXPERIMENTAL PROCEDURES

Materials—Tritiated BFA (2.075 Ci/mmol) and its conjugates were synthesized and purified as described earlier (19). Forskolin was a generous gift of Dr. H. Metzger (Hoechst AG). [α-32P]GTP was from Amersham Corp. All cell culture reagents were from Biochrom (Berlin, Germany). All chemicals used in this study were of analytical grade.

Guaniie Nucleotide Exchange Assay—This assay was performed exactly as described in Helms et al. (20) Purification of recombinant myristoylated ADP-ribosylation factor 1 and CHO Golgi membranes was as described by Balch et al. (21).

Préincubation with forskolin was as described in the legend to Fig. 1.

Cell Culture—CHO cells (ATCC CCL 61) were grown in suspension cultures in α-minimum Eagle's medium containing 7.5% fetal calf serum, 100 units of penicillin, and 100 units of streptomycin per ml. BFA Secretion Experiments—For BFA secretion experiments, cells were collected at a density of 5 × 10^6 cells/ml and then resuspended in medium lacking fetal calf serum at a density of 2 × 10^6 cells/ml. For kinetic experiments (see Fig. 2, A and B) 100 μl of cells were used per experimental condition. 10 μCi of [3H]BFA were added (final concentration, 13 μg of BFA/ml of medium), and aliquots of 10 μl were removed at the times indicated. Samples were centrifuged and pellets

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† To whom correspondence should be sent. Tel.: 49-6221-544 688; Fax: 49-6221-544 366; E-mail: c0@x.urz.uni-heidelberg.de.

The abbreviations used are: BFA, brefeldin A; ARF, ADP-ribosylation factor; CHO, Chinese hamster ovary.
Secretion of BFA Conjugates Is Decreased When Cells Are Depleted of ATP—To investigate alternative mechanisms by which forskolin could prevent BFA action we considered the possibility that forskolin acts by stimulating the process of detoxification of BFA. For this purpose we employed a previously described assay, which monitors the detoxification of BFA by CHO cells (6). BFA was shown to be secreted as cysteine and glutathione conjugates, respectively. When BFA is added to CHO cells, intracellularly formed conjugates are rapidly secreted. This mechanism has been suggested to be the basis of the observed reversibility of BFA action (6) upon removal of the drug.

A typical BFA secretion experiment is shown in Fig. 2A. CHO cells were incubated with [3H]BFA at 37°C. At the times indicated aliquots were removed from the cell suspension, and the medium was analyzed by TLC. Cys-BFA and GSH-BFA are the major conjugates formed (identified by comparison with synthesized standards as described in Brüning et al. (6)). Secretion of BFA conjugates starts approximately 5–10 min after addition of [3H]BFA. After 90 min considerable amounts of both Cys-BFA and GSH-BFA are secreted into the medium. The amount of Cys-BFA in the medium is comparable with the amount of BFA found intracellularly (data not shown). There are at least three additional minor BFA derivatives secreted into the medium that have not yet been identified.

Secretion of Cys-BFA is strongly reduced when cells are depleted of ATP (Fig. 2C; similar data were obtained for GSH-BFA, data not shown). In this experiment the amounts of Cys-BFA secreted into the medium after a 90-min incubation were analyzed. Cys-BFA is still secreted but at a largely reduced rate. This could indicate that the formation of BFA conjugates is dependent on ATP. Alternatively the ATP dependence of BFA conjugate secretion might indicate that BFA derivatives are actively secreted into the medium involving an ATP-driven pump localized to the plasma membrane. Since intracellular Cys-BFA is not accumulating under these conditions (Fig. 2C) and the ratio of intracellular versus extracellular Cys-BFA remains unchanged, these data seem to indicate that conjugate formation rather than secretion is affected. However, it cannot be excluded that conjugate secretion is ATP-dependent as the decrease of intracellular ATP might not be sufficient to inhibit a putative ABC transporter involved in BFA conjugate secretion.

Detoxification of Brefeldin A Is Increased in Forskolin-treated CHO Cells—The experiments shown in Fig. 2, B and C, demonstrate that forskolin-treated cells are able to remove BFA more efficiently than untreated cells. CHO cells were incubated for 1 h at 37°C in the presence of 100 μM forskolin before the addition of [3H]BFA. At the times indicated the media were analyzed by TLC and the amounts of Cys-BFA and GSH-BFA quantified (not shown for GSH-BFA). After 90 min of incubation the amount of Cys-BFA in the medium was enhanced by a factor of ~2 when cells were pretreated with forskolin. The intracellular level of Cys-BFA was also increased in the presence of forskolin (Fig. 2C). This indicates that forskolin treatment of CHO cells results in enhanced conjugation of BFA to cysteine. The formation of GSH-BFA was not affected by the addition of forskolin (data not shown).

When cells were depleted of ATP, forskolin was no longer able to stimulate the formation of Cys-BFA (intracellular pool, Fig. 2C). Accordingly, the amounts of Cys-BFA found in the medium also remained unchanged (Fig. 2C).

A membrane-permeating analogue of cyclic AMP, dibutylryl-cAMP, did not mimic the effects observed with forskolin (Fig. 3). Therefore, the stimulatory effect of forskolin on BFA conjugate formation is cAMP-independent. These data are consist-

RESULTS AND DISCUSSION

Forskolin Does Not Rescue BFA-inhibited Guanine Nucleotide Exchange onto ARF in Vivo—The effect of forskolin on Golgi-catalyzed guanine nucleotide exchange onto ARF was investigated using the assay published by Helms and Rothman (4). Purified recombinant ARF and isolated CHO Golgi membranes were incubated in the presence of [α-32P]GTP, and ARF were added. Protein-bound [α-32P]GTP was counted. Shown values are corrected with respect to spontaneous nucleotide exchange occurring in the absence of Golgi membranes as well as in the absence of ARF. Nucleotide exchange under control conditions was set to 100% enzyme activity. Results shown are typical for three independent experiments.

Extracted with 10 μl of methanol/water (1:1). Cell extracts and the corresponding media were analyzed by TLC. In some experiments the total amounts of BFA, Cys-BFA, and GSH-BFA in cell extracts and the corresponding media were determined after 90 min of incubation (see Figs. 2C and 3). ATP depletion was performed by adding 50 mM deoxyglucose, 0.05% sodium azide for 15 min at 37°C right before addition of [3H]BFA. Where indicated cells were preincubated with forskolin (final concentration, 100 μM) for 1 h at 37°C before radiolabeled BFA was added.

Thin Layer Chromatography—BFA and its conjugates were separated on Silica Gel 60 plates purchased from Merck. Chloroform/methanol/water/formic acid (10:10:3:0.6) was used as solvent. Radiolabeled BFA derivatives were analyzed either by fluorography according to Ref. 22 or by the use of a two-dimensional TLC radio scanner (Berthold, Wildbad, Germany). Identification of Cys-BFA and GSH-BFA was by comparison to synthesized standards as well as by fast atom bombardment mass spectrometry (6).

Detoxification of Brefeldin A Is Increased in Forskolin-treated CHO Cells—The experiments shown in Fig. 2, B and C, demonstrate that forskolin-treated cells are able to remove BFA more efficiently than untreated cells. CHO cells were incubated for 1 h at 37°C in the presence of 100 μM forskolin before the addition of [3H]BFA. At the times indicated the media were analyzed by TLC and the amounts of Cys-BFA and GSH-BFA quantified (not shown for GSH-BFA). After 90 min of incubation the amount of Cys-BFA in the medium was enhanced by a factor of ~2 when cells were pretreated with forskolin. The intracellular level of Cys-BFA was also increased in the presence of forskolin (Fig. 2C). This indicates that forskolin treatment of CHO cells results in enhanced conjugation of BFA to cysteine. The formation of GSH-BFA was not affected by the addition of forskolin (data not shown).

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**Fig. 1. Forskolin does not restore Golgi-catalyzed guanine nucleotide exchange onto ARF inhibited by BFA.** The standard assay was performed in a final volume of 100 μl. Forskolin (FK) and BFA were added from methanol stock solutions adjusted to the final concentrations indicated. Methanol did not contribute more than 1% (v/v) to the final mixture. Reactions containing forskolin were preincubated for 15 min at 37°C before BFA, [α-32P]GTP, and ARF were added. Protein-bound [α-32P]GTP was counted. Shown values are corrected with respect to spontaneous nucleotide exchange occurring in the absence of Golgi membranes as well as in the absence of ARF. Nucleotide exchange under control conditions was set to 100% enzyme activity. Results shown are typical for three independent experiments.
ent with the finding that forskolin can prevent the disassembly of the Golgi complex in the presence of BFA via a cAMP-independent mechanism (1) and suggest that forskolin-enhanced detoxification prevents disassembly of the Golgi complex in the presence of BFA.

After 90 min of incubation about 15% of radiolabeled BFA was found associated with cells (data not shown). The amount of BFA in the cells was similar to the amount of Cys-BFA in the medium. In forskolin-treated cells this amount of extracellular Cys-BFA was doubled although the total intracellular amount of BFA remained unchanged. How can forskolin reverse the effects of BFA in vivo if the intracellular level of BFA is not affected by forskolin? One explanation could be that only a small pool of intracellular BFA affects membranetraffic. When [3H]BFA was added to an H2O/octanol mixture (octanol is similar in hydrophobicity to biological membranes), only 0.4% of [3H]BFA could be recovered from the H2O phase. The presence of forskolin did not alter this distribution (data not shown) indicating that forskolin is not able to facilitate the release of BFA from membranes. From this phase distribution we consider it likely that the bulk of BFA is randomly dissolved in the cellular endomembrane system. BFA nonspecifically sticking to membranes may not contribute to the effect BFA exerts on membrane traffic and is not expected to be conjugated to cys-

**Fig. 2. Effects of ATP and forskolin on formation and secretion of Cys-BFA.** A, kinetics of BFA conjugate secretion were performed as described under "Experimental Procedures." 100 μl of CHO cells were either untreated or incubated in the presence of either forskolin (final concentration, 100 μM) or dibutyryl-cAMP (final concentration, 10 μM) for 1 h at 37 °C. Radiolabeled BFA was added as described in the legend to Fig. 2A. After a 90-min incubation, medium and cells were analyzed as described under "Experimental Procedures." Standard aberrations are shown (n = 3).

**Fig. 3. Forskolin affects detoxification of BFA by a cAMP-independent mechanism.** The secretion assay was performed exactly as described in the legend to Fig. 2A. 100 μl of CHO cells were either untreated or incubated in the presence of either forskolin (final concentration, 100 μM) or ATP-depleting reagents or incubated under control conditions before [3H]BFA was added. Where indicated ATP depletion was performed by adding 50 mM deoxyglucose, 0.05% sodium azide for 15 min at 37 °C. Finally radiolabeled BFA was added as described for A. After a 90-min incubation medium and cells were analyzed as described under "Experimental Procedures." Standard aberrations are shown (n = 3).
Detoxification of BFA

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