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Brefeldin A inhibits protein synthesis in cultured cells

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The fungal metabolite brefeldin A (BFA) is known to disrupt the Golgi apparatus resulting in redistribution of Golgi proteins to the endoplasmic reticulum and inhibition of protein secretion. BFA was found to inhibit protein synthesis in rat glioma C6 cells by up to 70% between 0.1 and 1 μg/ml. Inhibition was both time-dependent and reversible. BFA inhibited protein synthesis to varying degrees in a number of other cell lines but not in BFA-resistant marsupial kidney cells. The same concentrations of BFA which inhibited protein synthesis, also blocked the inhibitory effects of *Pseudomonas* exotoxin and ricin on BFA-sensitive cells. BFA, however, was unable to block the inhibition of protein synthesis by the toxins in the resistant marsupial kidney cells.

Brefeldin A; Protein synthesis; *Pseudomonas* exotoxin; Ricin

1. INTRODUCTION

Brefeldin A, a metabolite of the fungus *Eupenicillium brefedianum* with a 13-membered macrocyclic lactone ring [1], has antiviral activity [2,3] and inhibits cell surface expression of virus G proteins in virus-infected cells [4]. BFA also blocks protein secretion [5], causes the intracellular accumulation of incompletely processed glycoproteins [4–7], inhibits complex glycosphingolipid synthesis [8,9], and enhances sphingomyelin synthesis [10]. It is now known that BFA mediates its effects through the disassembly of the Golgi apparatus and the redistribution of resident Golgi proteins into the endoplasmic reticulum (reviewed in [11]). Some of the earliest effects of BFA are the dissociation of coat proteins from Golgi membranes [12–14]. BFA does not affect endocytosis (see [11]) and has been reported to have little if any effect on macromolecular synthesis including protein synthesis [5]. Thus, BFA is widely used as a highly specific blocker of Golgi functions.

While investigating the effects of BFA on the intoxification of cultured cells by several bacterial toxins, we observed that the drug itself was a potent inhibitor of protein synthesis. We describe this inhibition and show that it occurs at concentrations of BFA known to disassemble the Golgi apparatus.

2. MATERIALS AND METHODS

2.1. *Materials*

BFA was obtained from Sigma as well as from Boehringer/Mannheim and Epicentre Technologies (Madison, WI); it was stored as a stock solution of 1 or 5 mg/ml in ethanol at −20°C. *Pseudomonas* exotoxin A was from List Biological Laboratories (Campbell, CA); ricin from Sigma; and [^{3}H]leucine (150–180 Ci/mmol), [^{35}S]methionine (1180 Ci/mol) and ^{14}C-labeled amino acids were from DuPont-New England Nuclear.

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2.2. *Cells and cell culture*

Rat glioma C6 cells were grown as previously described [15]. S49 and CHW cells were grown in DMEM supplemented with 10% horse and foetal bovine serum, respectively. Other cell lines were obtained from the American Type Culture Collection and grown according to its recommendations except for SK-N-MC and Caco-2 cells where NuSerum IV (Collaborative Biomedical Products, Bedford, MA) was substituted for foetal bovine serum. Cells for experiments usually were grown to confluency in 24 × 16-mm cluster plates.

2.3. *Protein synthesis*

Routinely, the medium was changed to 0.5 ml/well of serum-free DMEM or EMEM (SK-N-MC, Caco-2) buffered with 25 mM HEPES and containing 0.01% bovine serum albumin. For PtK_1 and PtK_2 cells, EMEM containing 10% foetal bovine serum was used. BFA was added to the medium, the cells were incubated for 2.5 h at 37°C and then pulsed with 1 μCi of [^{3}H]leucine for 1 h. For the experiments involving time courses, the cells were pulsed in leucine-free medium for 15 min. Cells were exposed to the toxins 30 min after adding BFA and incubated for 2 or 4 h before being pulsed with [^{3}H]leucine. After the pulse, the medium was rapidly removed and the cells were washed 3 times with 1 ml of ice-cold 10% trichloroacetic acid. The cell layers were dissolved in 0.2 M NaOH overnight and analyzed for incorporation of label by liquid scintillation counting. Each data point represents the mean ± S.D. of triplicate wells from a representative experiment.

3. RESULTS

3.1. *Inhibition of protein synthesis by BFA*

BFA from three different sources inhibited the incorporation of [^{3}H]leucine into proteins by rat glioma C6 cells (Fig. 1A). The inhibitory dose range was 0.1 to 1

μg/ml. These same concentrations of BFA also inhibited the incorporation of [^{35}S]methionine and a mixture of ^{14}C-labeled amino acids (Fig. 1B), thus making it unlikely that BFA was interfering with amino acid transport. BFA also was an effective inhibitor when the cells were exposed to it in different media such as DMEM containing either 5% NuSerum (the normal culture medium for C6 cells) or 10% foetal bovine serum (data not shown). To indirectly address the effects of BFA on protein synthesis by free and membrane-bound polysomes, the incorporation of [^{3}H]leucine into cytosolic and membrane-bound proteins was determined (Table I). Label was incorporated almost equally into the two pools, and BFA inhibited labelling of the two pools to a similar extent.

The inhibition by BFA occurred relatively rapidly (Fig. 2A) and was completely reversible (Fig. 2B). As the cells were pulsed with [^{3}H]leucine for only 15 min in these latter experiments, it seemed unlikely that BFA might be mediating its effects by increasing protein degradation. To directly address this possibility, however, control and BFA-treated C6 cells were pulsed with [^{3}H]leucine for different times over a 1-h period (Fig. 3A). The cells incorporated the label into proteins at a linear rate, and at all times assayed (even 5 min), the BFA-treated cells incorporated > 60% less label. In addition, control and BFA-treated cells were pulsed with [^{3}H]leucine in leucine-free medium for 30 min, washed, and chased in regular medium ± BFA for up to 6 h (Fig. 3B). Fifty percent of the label disappeared from control cells by 6 h, and the presence of BFA slightly increased the half-life to 7 h.

BFA-mediated inhibition of protein synthesis was not unique to C6 cells as it was observed in a variety of cultured cell lines of human, hamster, and mouse origin (Table II). The only exceptions appeared to be the PtK_1 and PtK_2 cell lines from marsupial (potaroo or rat kangaroo) kidney which are reported to be resistant to BFA [16,17]. The inhibitory potency of BFA was intermediate between *Pseudomonas* exotoxin and cycloheximide (Fig. 4A). By contrast to the latter agents, BFA only partially inhibited protein synthesis even at high concentrations.

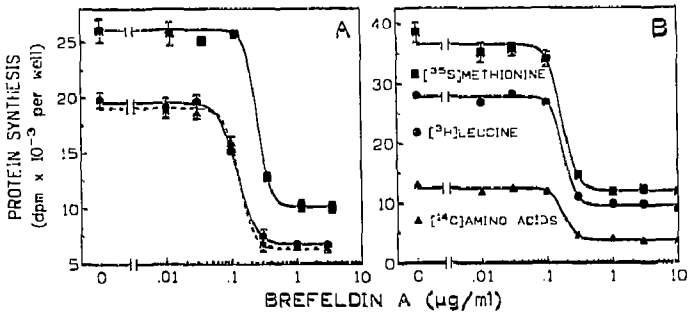

Fig. 1. (A) Rat glioma C6 cells were exposed to increasing concentrations of BFA from three different sources: SC (●), BM (▲), and ET (■). After 2.5 h, the cells were pulsed with [^{3}H]leucine and assayed for incorporation into protein as described in section 2. (B) The cells were exposed to BFA from the same source (SC) and pulsed with 1.2 μCi [^{3}H]leucine (●), 0.21 μCi [^{14}C]-amino acids (▲) or 1.6 μCi [^{35}S]methionine (■).

Table I

Inhibition of membrane-bound and cytosolic protein synthesis by BFA in rat glioma C6 cells

Cell fraction	[^{3}H]Leucine incorporated (dpm/flask)		Inhibition by BFA %
	Control	BFA-treated	
Lysate	278,200 ± 2990	117,300 ± 3680	57.8
Membrane	152,600 ± 3030	66,000 ± 780	56.7
Cytosol	129,600 ± 4400	50,400 ± 880	61.1

Cells grown in 25 cm^2 flasks were incubated in the absence and presence of 1 μg/ml BFA for 2.5 h, pulsed with 2 μCi/ml of [^{3}H]leucine for 1 h, and washed 3 times with ice-cold PBS. The cells then were lysed in 6 ml of ice-cold 1 mM Tris/2 mM EDTA (pH 7.4), and 5 ml of each lysate were centrifuged at 150,000 × g for 1 h. Each of the fractions was assayed for incorporation into trichloroacetic acid-insoluble protein as described in section 2.

3.2. *Effects of BFA and toxins*

BFA has been reported to block the cytotoxic effects of several bacterial and plants toxins including *Pseudomonas* exotoxin and ricin [17–19]. Because the effects of these toxins are usually assayed by their ability to inhibit protein synthesis and we found that BFA by itself inhibited, we decided to confirm these studies. At low concentrations of BFA (up to 30 ng/ml), the exotoxin was a potent inhibitor of protein synthesis by C6 cells

Table II

Inhibition of protein synthesis by BFA in different cell lines

Cell line		Protein synthesis in the presence of BFA (% of control)
Rat glioma	C6	36
Chinese hamster ovary	CHO-K1	72
Chinese hamster fibroblast	CHW	59
Baby hamster kidney	BHK-21 (tk$^-$ts13)	55
Mouse fibroblast	L (clone 929)	71
Mouse lymphoma	S49	53
Human colonic adenocarcinoma	Caco-2	67
Human epidermoid carcinoma	A431	53
Human neurotumor	SK-N-MC	81
Potaroo kidney	PtK_1	100
Potaroo kidney	PtK_2	100

Cells were incubated in the absence and presence of 1 μg/ml BFA for 2.5 h, pulsed with 1 μCi (2 μCi for PtK_1 and PtK_2 cells) of [^{3}H]leucine for 1 h and assayed for incorporation into protein as described in section 2.

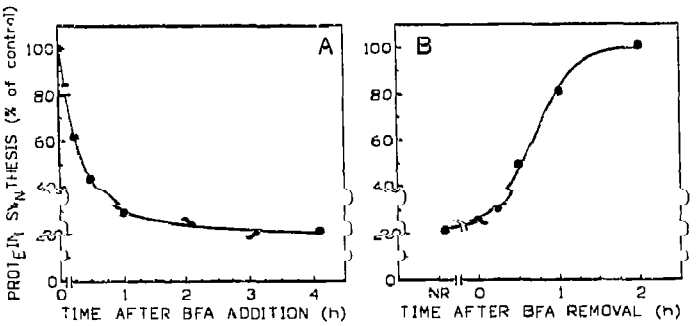

Fig. 2. C6 cells were exposed to 1 μg/ml BFA for the indicated times (A), or for 2 h, then washed and incubated in BFA-free medium for the indicated times (B). Then the cells were pulsed for 15 min in leucine-free medium containing [^{3}H]leucine with (A) and without (B) BFA. Control cells incorporated 82,000 ± 6,130 dpm/well.

(Fig. 4B); at the same higher concentrations of BFA which blocked protein synthesis, the inhibitory effects of the toxin were alleviated. This was more apparent when the inhibition due to the toxin was normalized to that due to BFA (Fig. 4B, ×-----×). Similar results were observed with A431, SK-N-MC, CHO and L cells (data not shown). As we found PtK$_1$ cells to be insensitive to *Pseudomonas* exotoxin, we tried ricin on these cells (Fig. 5A). Ricin inhibited protein synthesis by 98% and concentrations of BFA as high as 10 μg/ml were unable to prevent its cytoxicity. This confirmed that PtK$_1$ cells are resistant to BFA [16]. By contrast, the inhibitory effect of ricin on SK-N-MC cells was blocked by BFA (Fig. 5B). Similar results were observed in CHO cells (data not shown).

4. DISCUSSION

We found that BFA was an inhibitor of protein synthesis in a wide variety of cultured cells lines. This was somewhat unexpected as BFA is widely used and many recent reports cite the work of Misumi et al., 1986 [5] as evidence that BFA does not inhibit protein synthesis. The latter authors reported that 1 μg/ml BFA completely blocks protein secretion in rat hepatocytes without any effect on protein synthesis whereas a 26% inhibition is observed at 10 μg/ml BFA [5]. Ulmer and Palade found that 1 μg/ml BFA slightly stimulated protein synthesis in murine erythroleukemic cells [20]. Others, investigating the effect of BFA on the cytoxicity of bacterial and plant toxins which act by inhibiting protein synthesis, made no mention of any inhibition by BFA itself [17–19]. It may be that some cell lines are less sensitive or insensitive to this effect of BFA; however, some of the same cell lines (Caco-2, A431, BHK, CHO, L) were used in these studies as we used in ours. We can not rule out the possibility that differences in treatment conditions might account for the differing results. In addition, most of the published data are presented as % of control so that a modest inhibition by BFA itself may not have been considered important when compared to the large effects caused by the toxins. In this regard, Hudson and Grillo [19] reported in the legend to Fig. 2 that human lymphoma Jurkat cells incorporate 21,700 and 14,476 cpm of [^{14}C]leucine per sample in the absence and presence of 0.5 μg/ml BFA, which represents a 33% inhibition. Finally in 1966, Betina and Montagneir reported that BFA at 5–10 μg/ml inhibits protein synthesis in BHK-21/13 cells by 60% [21].

The mechanism by which BFA inhibits protein synthesis remains unclear. We observed that BFA inhibited the incorporation of different amino acids into proteins to the same extent; BFA was equally effective at blocking synthesis of membrane-bound and cytosolic proteins; BFA even at high concentrations did not totally inhibit protein synthesis in contrast to *Pseudomonas* exotoxin and cycloheximide; and the extent of BFA inhibition varied among the different cell lines. It is well-established that BFA blocks protein secretion by causing the disassembly of the Golgi apparatus [reviewed in 11]. Golgi-resident proteins as well as newly synthesized proteins are found in the ER. BFA appears to shift the balance of the anterograde and retrograde transport pathways between ER and Golgi by inhibiting the former and enhancing the latter. The earliest observed effects of BFA are the release of coat proteins such as β-COP [12] and ARF [13] from Golgi membranes and γ-adaptin from the trans-Golgi network [14]

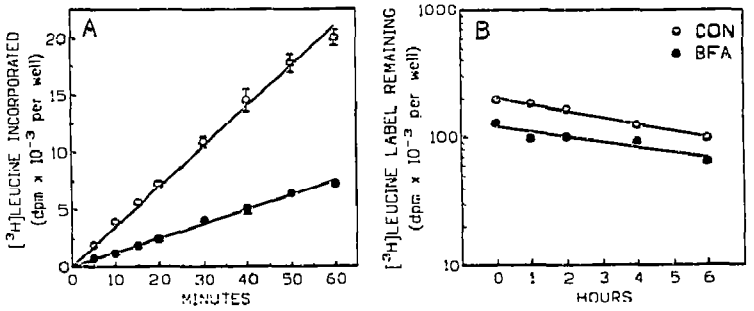

Fig. 3. (A) C6 cells were incubated in the absence (○) and presence (●) of 1 μg/ml BFA for 2.5 h, and pulsed with [^{3}H]leucine for the indicated times. (B) C6 cells were incubated in leucine-free medium in the absence (○) and presence (●) of 1 μg/ml BFA for 30 min, pulsed with [^{3}H]leucine for 30 min and washed three times with ice-cold PBS. The cells then were chased in regular culture medium ± BFA for the indicated times.

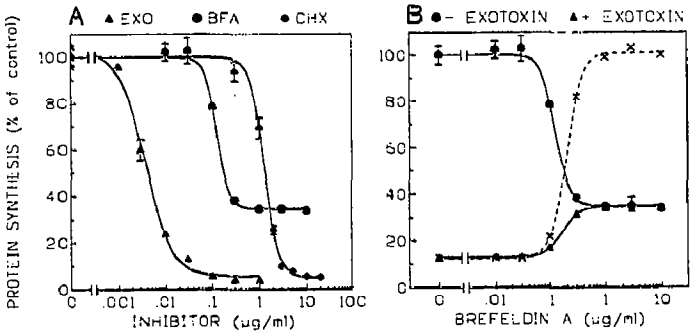

Fig. 4. (A) C6 cells were exposed for 4 h to *Pseudomonas* exotoxin (▲), BFA (○) or cycloheximide (●) and then pulsed with [^{3}H]leucine. (B) C6 cells were exposed to BFA for 30 min; then 1 µg/ml exotoxin was added to some of the wells (▲) and after 2 h, the cells were pulsed with [^{3}H]leucine. (x---x) Incorporation in the presence of exotoxin normalized to that in its absence (see text).

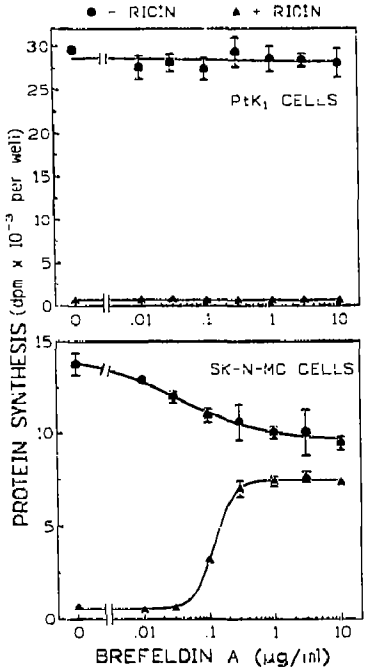

Fig. 5. PtK$_1$ (A) and SK-N-MC (B) cells were exposed to BFA for 30 min; then 1 µg/ml ricin was added to some of the wells (▲) and after 4 h, the cells were pulsed with [^{3}H]leucine.

into the cytosol. This inhibition of coat protein assembly appears in turn to lead to the extension of uncoated tubules and the development of a tubular-vesicular network.

It is possible that the accumulation of secreted proteins and Golgi-resident proteins in the ER affects protein synthesis in the ER. This would not explain why BFA inhibited protein synthesis in both membranes and cytosol to a similar extent. More likely, disruption of intracellular trafficking/processing by BFA may lead to the inappropriate processing or location of some factor(s) required for protein synthesis. This may explain why BFA did not totally inhibit protein synthesis, and why the extent of BFA inhibition varied among the different cell lines. Although we cannot rule out the possibility that BFA is having a direct effect on protein synthesis, it appears unlikely. Thus, BFA inhibited protein synthesis in the same concentration range which causes its characteristic morphological effects on the Golgi apparatus. In addition, we found that these same concentrations blocked the action of ricin and *Pseudomonas* exotoxin. Others have shown a close correlation between Golgi disassembly and protection against these toxins [17–19]. Finally, BFA did not inhibit protein synthesis in PtK$_1$ and PtK$_2$ cells known to be resistant to BFA-induced Golgi disassembly [16,17].

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