Volume 288, number 1,2, 143–146 FEBS 10096 August 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3.50
ADONIS 001457939100807T

Guanosine nuceleotides modulate the inhibitory effect of Brefeldin A on protein secretion

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Received 17 June 1991

Brefeldin A (BFA) causes rapid redistribution of Golgi proteins into the endoplasmic reticulum (ER), leaving no definable Golgi-apparatus, and blocks transport of proteins from the ER to distal secretory compartments of the cell. Using pulse-chase experiments the present study shows that BFA (1 μg/ml) inhibits basal and CCK-stimulated protein secretion in isolated pancreatic acinar cells by 65 ± 6% and 84 ± 5%, respectively. In isolated permeabilized cells higher concentrations of BFA (30 μg/ml) were necessary to obtain inhibition of protein secretion. In parallel experiments protein secretion was stimulated by GTP (1 mM). BFA had no inhibitory effect on protein secretion in the presence of GTP, indicating that BFA might act on a GTP-binding protein. Investigating the effect of BFA on small molecular weight GTP-binding proteins we observed that [α-^{32}P]GTP binding to a 21 kDa protein in a subcellular fraction enriched in ER was increased in the presence of BFA. We conclude that this 21 kDa and possibly also other GTP-binding proteins may be the molecular target of Brefeldin A in pancreatic acinar cells.

Brefeldin A; Protein synthesis; Protein secretion; Pulse-chase experiment; Small molecular weight GTP-binding protein; Guanosine nucleotide

1. INTRODUCTION

Brefeldin A (BFA) induces rapid disassembly of the Golgi-apparatus, redistribution of resident and itinerant Gilgi proteins as well as of Golgi lipids into the endoplasmic reticulum (ER) and blocks protein transport within the secretory pathway beyond the Golgi-apparatus [1,2]. The effects of BFA are rapidly and completely reversed by removing the drug. The molecular target(s) of BFA remain to be determined.

Inhibition of intracellular protein transport by BFA has been shown in cell systems delivering proteins constitutively to the cell surface [3,4]. The effect of BFA in pancreatic acinar cells where secretory proteins enter a regulated secretion pathway has not yet been characterized.

Evidence suggests that ras-related small molecular weight GTP-binding proteins regulate vesicular transport between different intracellular compartments, e.g. between the ER and the Golgi complex [5]. We therefore investigated whether BFA affects small molecular weight GTP-binding proteins in pancreatic acinar cells. Furthermore, using pulse-chase experiments in permeabilized acinar cells, we have investigated the effects of GTP and of GTPγS on protein transport and secretion in the presence or absence of BFA. We found that BFA-induced inhibition of protein transport was abolished in the presence of guanosine nucleotides and that [α-^{32}P]GTP binding to a 21 kDa protein in a subcellular fraction of ER was increased by BFA.

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2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase Typ III, 132 U/mg from *Clostridium histolyticum* was from Worthington (Freehold, NJ, USA). Brefeldin A was a kind gift of Sandoz (Basel, Switzerland). It was stored at −20°C as a stock solution of 5 mg/ml in methanol. Cholecystokinin (CCK), adenosine-triphosphate (ATP), guanosine-triphosphate (GTP), bovine serum albumin (BSA), creatine phosphate, and digitonin were from Sigma (München, FRG), acridine orange and sodium-azide from Merck (Darmstadt, Germany), oligomycin from Serva (Heidelberg, Germany), trypsin-inhibitor, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) and creatine kinase from Boehringer (Mannheim, Germany), [^{3}H]leucine (146 Ci/mmol) from Amersham Buchler (Braunschweig, Germany), Eagles minimum essential medium (MEM) from Gibco (Eggenstein, Germany) and [α-^{32}P]GTP (3000 Ci/mmol) was obtained from NEN (Dreieich, Germany).

2.2. *Methods*

2.2.1. *Cell preparation.* Acinar cells were isolated from the pancreas of male Wistar rats (200–250 g) by collagenase digestion as previously described [6]. Isolated cells were permeabilized as indicated with digitonin (5 μg/ml).

2.2.2. *Pulse-chase experiments.* Isolated cells were preincubated with or without Brefeldin A at the indicated concentration in Eagles MEM, pulse-labelled for 10 min with [^{3}H]leucine (20 μCi/ml) in Eagles MEM lacking unlabelled leucine and subsequently chased for 120 min at 37°C in complete Eagles MEM supplemented with HEPES 20 mM, glucose 5 mM, BSA 2 g/l and trypsin inhibitor 0.1 g/l [7]. Permeabilized cells were chased in a 120 mM KCl-buffer (KCl 120 mM, KH_2PO_4 1.2 mM, $MgCl_2$ 2 mM, $CaCl_2$ 0.1 mM, HEPES 20 mM, glucose 10 mM, BSA 2 g/l, trypsin inhibitor 0.1 g/l, pH 7.0 with Tris)

which was supplemented with MEM amino acids, ATP 1 mM and an ATP-regenerating system [8]. BFA was present in the medium throughout the pulse-chase period at concentrations as indicated. Total protein synthesis was determined by measuring the trichloroacetic insoluble radioactivity incorporated into the cells during a 10 min pulse.

2.2.3. *[α-^{32}]GTP binding assay.* Isolated pancreatic acinar cells were incubated for 20 min at 37°C with or without CCK and BFA, respectively. Subsequently, cells were homogenized, the homogenate centrifuged at 1000×*g* for 12 min at 4°C. The resulting supernatant was centrifuged at 11 000×*g* for 15 min at 4°C and the fluffy layer (enriched 2 to 3-fold in ER) on top of the 11 000×*g* pellet was carefully removed [9]. Proteins were resolved by SDS-PAGE gels) [10] followed by an electrophoretical transfer to nitrocellulose paper (0.2 μm) [11,12]. The nitrocellulose sheets were incubated for 60 min at 24°C with [α-^{32}P]GTP as described [13]. After 5–7 washes the blots were air-dried and bound radioactivity was detected by autoradiography. The autoradiography were scanned by a computerized laser-densitometer (Ultrascan, Pharmacia-LKB, Freiburg, Germany).

2.2.4. *Measurement of proton-gradient formation with Acridine orange.* Rat pancreatic microsomal vesicles were prepared from isolated acinar cells as described [9,14]. Measurement of H^+-uptake was performed as previously described using Acridine orange [14].

2.2.5. *Statistics.* All results are expressed as mean ± SE. Statistical analysis was performed using Student's paired *t*-test.

3. RESULTS

Fig. 1 shows the dose–response curve of BFA on total protein synthesis and CCK-stimulated protein secretion in isolated intact and permeabilized pancreatic acinar cells, respectively. The cells were pulse-labelled with [^{3}H]leucine for 10 min and total protein secretion was measured after 120 min. No difference in total protein synthesis was observed in control and CCK-stimulated cells. BFA had only a small effect on protein synthesis, a 5% inhibition was observed at 10 μg/ml. In isolated intact acinar cells the protein secretion rate during a 120 min chase was half-maximally inhibited at 0.05 μg/ml BFA, maximal inhibition occured at 1 μg/ml. However, in isolated permeabilized cells higher BFA concentrations were necessary to obtain inhibition of protein secretion (1.5 μg/ml for half-maximal and 10 μg/ml for maximal inhibition). BFA inhibited CCK-stimulated protein secretion by 84 ± 5% and 55 ± 6% in intact and permeabilized cells, respectively.

Fig. 2 shows the effect of BFA (1 μg/ml) on total protein secretion in intact cells as a function of chase time after pulse-labelling with [^{3}H]leucine for 10 min. After a chase period of 120 min basal and CCK-stimulated protein release was inhibited by 67 ± 2% and 86 ± 2%, respectively. No significant difference was observed in the protein secretion rate of cells treated with BFA (1 μg/ml) or cells treated with CCK (1 nM) and BFA (1 μg/ml) (Fig. 2).

To investigate the effects of GTP and GTPγS on intracellular protein transport permeabilized pancreatic

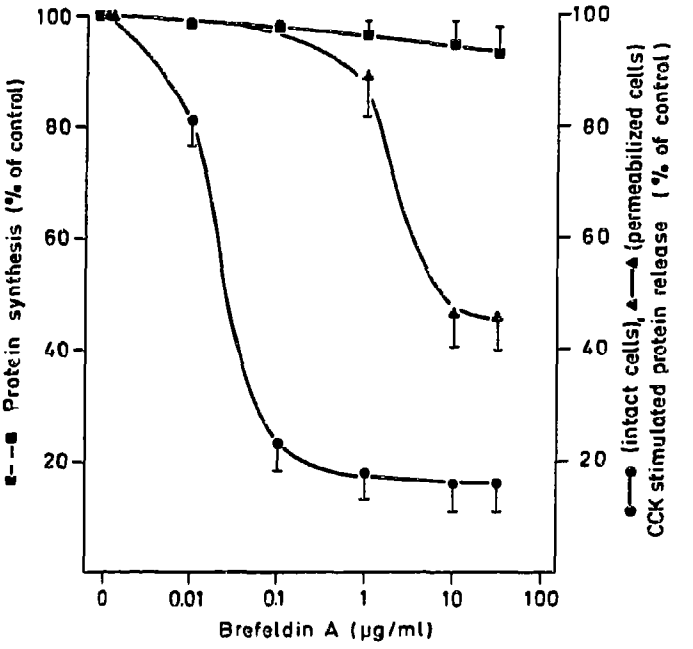


Fig. 1. Effect of BFA on total protein synthesis (■—■) and CCK (1 nM) stimulated protein secretion in isolated intact (●—●) and permeabilized (▲—▲) pancreatic acinar cells. Isolated cells were preincubated with or without BFA for 30 min, pulse-labelled for 10 min with [^{3}H]leucine (20 μCi/ml) and chased for 120 min at 37°C. BFA was present in the medium throughout the pulse-chase period at concentrations as indicated. Total protein synthesis is defined as radioactivity incorporated into proteins during a 10 min pulse. ($n = 3$).

acinar cells were used. In the presence of ATP and an ATP-regenerating system CCK (1 nM) and GTP (1 mM) stimulated protein secretion during a 120 min chase period by 72 ± 9% ($P < 0.001$, $n = 7$) and 112 ± 28% ($P < 0.0001$, $n = 7$), respectively. GTPγS (100 μM) had no significant effect on basal protein secretion (P = n.s., $n = 6$) (Fig. 3). However, incubation of CCK-stimulated cells with GTPγS resulted in a significant inhibition of protein secretion by 68 ± 6% ($P <$

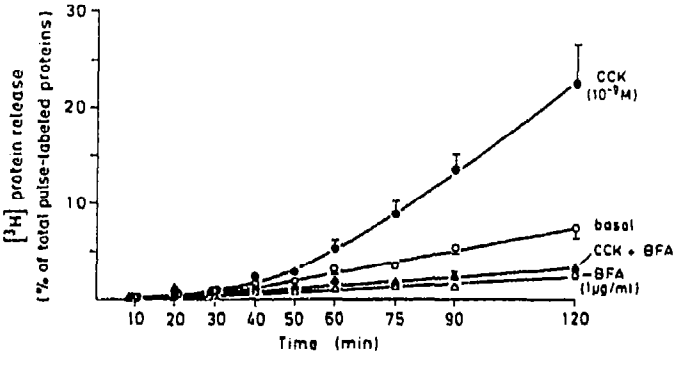


Fig. 2. Effect of BFA (1 μg/ml) on total protein secretion in isolated intact cells as a function of chase time after pulse-labelling with [^{3}H]leucine for 10 min. At the indicated times of chase aliquots were taken, the trichloroacetic acid-insoluble radioactivity released into the medium was measured and expressed as percent of total incorporated radioactivity ($n = 3$).

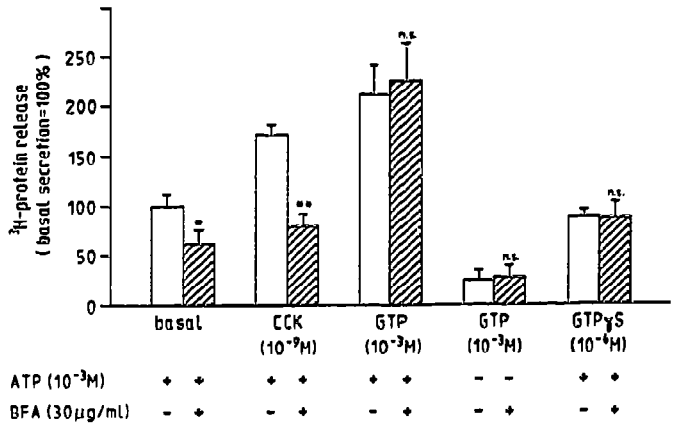


Fig. 3. Pulse-labelled permeabilized acinar cells were incubated with the substances as indicated. Total protein release was measured during a 120 min chase. Results are expressed as percentage of basal secretion (= 100%). Columns represent mean values ± SE of 3–7 experiments. $^*P < 0.05$, $^{**}P < 0.01$.

0.01, $n = 3$) (data not shown). When permeabilized acinar cells were incubated in an ATP-depletion system (no ATP, no ATP-regenerating system, no glucose, oligomycin 10 μM, sodiumazide 10 mM) GTP did not stimulate protein release, in fact protein release in the presence of GTP was drastically reduced (Fig. 3). Similar results were obtained in cells incubated with CCK in an ATP-depletion system (data not shown). BFA (30 μg/ml) significantly inhibited basal and CCK-stimulated protein secretion ($P < 0.05$ and $P < 0.01$, respectively), but had no effect in cells incubated with GTP (1 mM) or with GTPγS (100 μM) (Fig. 3).

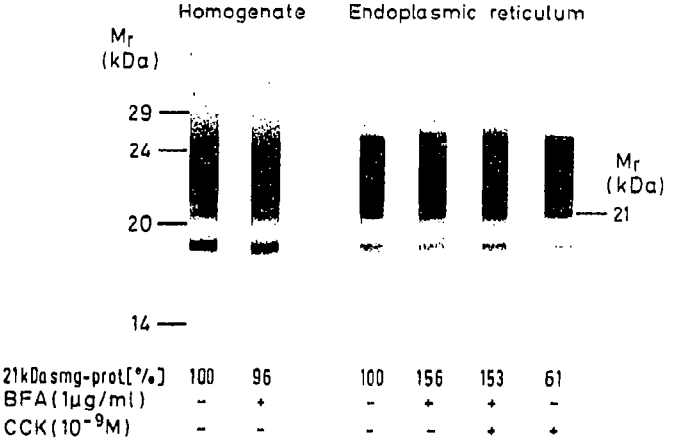


Fig. 4. Effect of BFA (1 μg/ml) and CCK (1 nM) on [α-^{32}P]GTP-binding to small molecular weight GTP-binding proteins. Isolated cells were incubated for 20 min with and without BFA and CCK, respectively. Homogenization, subcellular fractionation and the [α-^{32}P]GTP binding assay were performed as described in section 2. [α-^{32}P]GTP binding to small molecular weight GTP-binding proteins of the total cell homogenate and the subcellular fraction enriched in ER are shown. The protein bands of the 21 kDa GTP-binding protein were scanned by laser densitometry and the respective density expressed as percent of control (= 100%). The results are representative for five separate experiments.

These results implied that BFA might act on GTP-binding proteins. We therefore investigated the effect of BFA on the group of small molecular weight GTP-binding proteins, which are suggested to regulate intracellular vesicular transport. Isolated intact acinar cells were incubated for 20 min at 37°C with and without BFA (1 μg/ml) and subsequently fractionated. As shown in Fig. 4 (lane 1 and 2) no effect of BFA was observed in the total cell homogenate. However, in a subcellular fraction enriched in ER [α-^{32}P]GTP binding to a 21 kDa protein was enhanced in the presence of BFA (Fig. 4, lane 3 and 4). Previous studies of our group [15] have shown that [α-^{32}P]GTP binding to this 21 kDa protein is decreased when cells are preincubated with CCK (Fig. 4, lane 6). This CCK-effect was not observed in isolated acinar cells incubated with CCK (1 nM) and BFA (1 μg/ml), indeed [α-^{32}P]GTP binding to the 21 kDa protein was increased as in cells incubated with BFA alone (Fig. 4, lane 5). In permeabilized acinar cells BFA (30 μg/ml) had no effect on the 21 kDa GTP-binding protein in the presence of GTP (1 mM) or of GTPγS (100 μM) (data not shown).

Furthermore, we previously observed that vesicular membrane binding of the 21 kDa protein is regulated by the intravesicular pH established by a vacuolar-type H^+-ATPase [16]. However, BFA has no effect on the vacuolar-type H^+-ATPase of pancreatic acinar cells (data not shown).

4. DISCUSSION

We have demonstrated in this study that BFA inhibits basal and CCK-stimulated protein secretion in rat pancreatic acinar cells. About 10-fold higher concentrations of BFA were needed to inhibit protein release in isolated permeabilized cells as compared to intact cells, a phenomenon already described previously [17]. These results suggest that BFA might be concentrated in isolated intact cells by transport processes.

In the secretory pathway GTP-hydrolysis is required for vesicular transport between the ER and the Golgi complex, between different Golgi compartments and between the Golgi complex and the plasma membrane. The non-hydrolyzable GTP-analog GTPγS inhibits vesicular transport between ER and Golgi complex as well as between Golgi compartments (for review see [18]). In contrast, GTPγS increases secretion (exocytosis) of proteins which are already stored in secretory granules by stimulation of heterotrimeric G-proteins. GTP has little or no effect on secretion (exocytosis) of proteins which are already stored in secretory granules [19].

Using the pulse-chase technique which monitors vesicular transport from ER to final secretion (exocytosis included), we could show that GTP (1 mM) stimulates this event in permeabilized cells (Fig. 3). In our experiments where permeabilized cells were

metabolically inhibited no effect of GTP was observed. This implies an ATP-dependent GTP effect on intracellular protein transport. Furthermore GTPγS did not stimulate protein transport in our pulse-chase experiments, supporting the importance of GTP-hydrolysis for protein transport from ER to exocytosis.

The stimulation of intracellular protein transport by GTP seems to be due to activation of small molecular weight GTP-binding proteins. According to a model proposed by Bourne [20] small molecular weight GTP-binding proteins direct vesicles to specific target organelles in a GTP-bound active state. GTP hydrolysis would be required to trigger a conformational change and the inactive GDP form of the protein would then return to the cytosol to target another vesicle in a cyclic process [20]. BFA blocks vesicular transport from the ER to the Golgi complex and enhances [α-^{32}P]GTP binding to a 21 kDa protein band (Fig. 4). This suggests that the 21 kDa protein might be involved in the vesicular transport between these two compartments and that BFA might affect the function of the 21 kDa GTP-binding protein leading to inhibition of vesicular transport and accumulation of this protein in the ER-membrane.

Recently Donaldson and co-workers showed that guanosine nucleotides modulate the effect of BFA on a 110 kDa protein, which was identified as one of the major coat protein subunits of non-clathrin coated vesicles (β-COP) [17,21,22]. In the present study BFA did not inhibit protein transport in the presence of GTP (1 mM) or GTPγS (100 μM). Furthermore, no effect of BFA on the 21 kDa GTP-binding protein was observed when permeabilized cells were incubated with GTP (1 mM) or GTPγS (100 μM). Taken together evidence is accumulating that BFA acts on GTP-binding protein(s), including the 21 kDa GTP-binding protein in the ER, as a molecular target. The mechanisms by which guanosine nucleotides modulate the effect of BFA remain to be determined.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (Ze 237/3-1)

REFERENCES

[1] Doms, R.W., Russ, G. and Yewdell, J.W. (1989) J. Cell. Biol. 109, 61-72.
[2] Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S. and Klausner, R.D. (1989) Cell 56, 801-813.
[3] Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G. and Ikehara, Y. (1986) J. Biol. Chem. 261, 11398-11403.
[4] Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A. and Ikehara, Y. (1988) J. Biol. Chem. 263, 18545-18552.
[5] Balch, W.E. (1990) Trends Biochem. Sci. 15, 473-477.
[6] Streb, H. and Schulz, I. (1983) Am. J. Physiol. 245, G347-G357.
[7] Jamieson, J.D. and Palade, G.E. (1971) J. Cell Biol. 50, 135-158.
[8] Dehlinger-Kremer, M., Zeuzem, S. and Schulz, I. (1991) J. Membrane Biol. 119, 85-100.
[9] Streb, H., Bayerdörffer, E., Haase, W., Irvine, R.F. and Schulz, I. (1984) J. Membrane Biol. 81, 241-253.
[10] Laemmli, U.K. (1970) Nature 227, 680-685.
[11] Schnefel, S., Pröfrock, A., Hinsch, K.-D. and Schulz, I. (1990) Biochem. J. 269, 483-488.
[12] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
[13] Lapetina, E.G. and Reep, B.R. (1987) Proc. Natl. Acad. Sci. USA 84, 2261-2265.
[14] Thévenod, F., Kemmer, T.P., Christian, A.L. and Schulz, I. (1989) J. Membrane Biol. 107, 263-275.
[15] Zimmermann, P., Pröfrock, A., Zeuzem, S., Aktories, K. and Schulz, I. (1991) Pfluegers Arch. 418 (suppl. 1), (R 83 (abstract).
[16] Zeuzem, S., Zimmermann, P. and Schulz, I. (1991) Pfluegers Arch. 418 (suppl. 1): R83 (abstract).
[17] 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.
[18] Balch, W.E. (1989) J. Biol. Chem. 264, 16965-16968.
[19] Stutchfield, J. and Cockcroft (1988) Biochem. J. 250, 375-382.
[20] Bourne, H.R. (1988) Cell 53, 669-671.
[21] Donaldson, J.G., Lippincott-Schwartz, J. and Klausner, R.D. (1991) J. Cell Biol. 112, 579-588.
[22] Donaldson, J.G., Lippincott-Schwartz, J., Bloom, G.S., Kreis, T.E. and Klausner, R.D. (1990) J. Cell Biol. 111, 2295-2306.