Volume 332, number 1,2, 99–104 FEBS 13045 October 1993

Prevention of the induced synthesis and secretion of group II phospholipase A_2 by brefeldin A

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(Received)

Brefeldin A (BFA) has previously been shown to block protein secretion and to cause dismantling of the Golgi cisternae in many cultured cell lines. BFA was found to prevent the induced synthesis and secretion of 14 kDa group II phospholipase A_2 (PLA_2) in rat mesangial cells. Furthermore, BFA inhibited total protein synthesis although PLA_2 appeared to be more sensitive to the effect of this compound than total protein synthesis assessed by amino acid incorporation. BFA was unable to block protein synthesis or PLA_2 activity in the cell completely but secretion of enzymatic activity and PLA_2 protein into the cell culture media was totally inhibited.

14 kDa group II phospholipase A_2; Brefeldin A; Golgi structure; Inhibition of protein synthesis and secretion; Rat renal mesangial cell

1. INTRODUCTION

Group II phospholipase A_2 is believed to participate in inducing and maintaining inflammatory reactions, although the pro-inflammatory activity of group II PLA_2 has only been shown indirectly [1–6]. Previous studies, using cultured rat glomerular mesangial cells, demonstrated that the potent inflammatory cytokines interleukin-1β (Il-1β) and tumor necrosis factor induced the synthesis and secretion of group II PLA_2. Over 85% of this de novo-synthesized group II PLA_2 appears to be rapidly secreted from the cytokine-stimulated cells [5]. We tried to accumulate the enzyme in Il-1β-stimulated cells by blocking the secretion of group II PLA_2 in order to study the contribution of this enzyme to arachidonate release and prostaglandin synthesis. For this purpose we used a known inhibitor of protein secretion, brefeldin A (BFA). BFA was initially described as a reversible inhibitor of the intracellular transport of newly synthesized membrane and secretory proteins that did not affect protein synthesis or endocytosis [7–9]. It was shown later that these effects are due to disorganization of the Golgi complex the membranes of which apparently fuse with the endoplasmic reticulum [7,10].

In this manuscript we demonstrate that BFA profoundly alters the structure of the Golgi complex in mesangial cells. As expected, this blocked PLA_2 secretion, but Western blot analysis showed that this was not accompanied by an increase in the cellular level of PLA_2. Subsequent results showed that IL-1β-induced synthesis of group II PLA_2 was dose-dependently prevented by BFA. In addition, total protein synthesis was inhibited by BFA, although not to the same extent as cytokine-induced synthesis of group II PLA_2.

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2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant Il-1β was prepared by the Biotechnology Department of Ciba Geigy Ltd, Basel, Switzerland. [1-^{14}C]Linoleic acid and L-[4,5-^{3}H]leucine (45–70 Ci/mmol) were obtained from Amersham, Bucks., UK. 1-Acyl-2[1-^{14}C]linoleoylphosphatidylethanolamine was prepared biosynthetically by incubating rat liver microsomes with [1-^{14}C]linoleic acid and lysophosphatidylethanolamine, as previously described [5]. Nitrocellulose membranes were from Schleicher and Schull, Dassel, Germany. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulines (GAMAP) and pre-stained markers were obtained from Bio-Rad laboratories. Nitro blue tetrazolium and 5-bromo-4- chloro-3-indolyl phosphate, the Golgi marker, *Ricinus communis* agglutinin-TRITC, and BFA were obtained from Sigma, St. Louis, MO. BFA was stored as a stock solution of 1 mg/ml in ethanol at −40°C. Monoclonal antibodies against group II PLA_2 were prepared as described before [11]. Fluorescein isothiocyanate-labeled goat anti- mouse IgG (GAM-FITC) was from Nordic Laboratories, Tilburg, The Netherlands.

2.2. *Cell culture and incubation*

Mesangial cells were cultivated and characterized as has been described previously [12]. The cells were grown in RPMI 1640 supplemented with 11% (v/v) fetal calf serum, penicillin (100 U/ml), streptomycin (10 μg/ml) and insulin (0.66 U/ml). Only cells up to passage 27 were used for the experiments. Confluent mesangial cells cultured in 25 cm^2 tissue culture flasks were washed once with phosphate-buffered saline (PBS) and incubated with 5 ml DMEM containing 0.1 mg/ml of fatty acid-free bovine serum albumin with or without Il-1β or BFA at the indicated concentrations. The control and Il-1β-stimulated cells received an equivalent amount of ethanol to that received by the cells incubated with BFA. After the indicated time periods the supernatants were removed and assayed for PLA_2 activity. When the cells had to

be used for further analysis they were rinsed once with PBS and scraped with a plastic policeman into 50 mM HEPES, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 μM pepstatin A, 50 μM NaF, 250 μM Na_3VO_4, 10 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.02% azide. The cells were homogenized with 10 strokes of a Potter–Elvehjem motor-driven teflon pestle at 600 rpm and stored frozen until assayed.

2.3. *PLA_2 and protein assay*

PLA_2 activity was assayed using 0.2 mM *sn*-2-labeled [1-^{14}C]linoleoylphosphatidylethanolamine (specific activity 3,000 dpm/nmol) in 0.1 M Tris-HCl, pH 8.5, in the presence of 10 mM $CaCl_2$ and 0.05% Triton X-100 as previously described [5]. This assay employs the preference of group II PLA_2 for phosphatidylethanolamine and suppresses the activity of a high molecular weight PLA_2 which has a preference for *sn*-2-arachidonoyl phospholipids. Under these conditions the contribution of the high molecular weight PLA_2 to the total measured PLA_2 activity amounts to less than 5% in control cells and less than 0.5% in IL-1β-stimulated cells. We have previously shown that the culture medium contains only group II PLA_2 and no high molecular weight PLA_2 [5,6]. Protein was determined according to Bradford [13] with bovine serum albumin as a standard.

2.4. *Western blotting*

SDS-PAGE using 15% polyacrylamide gels was done according to Laemmli [14] at a constant voltage of 200 V. For immunoblotting the proteins were transferred to nitrocellulose at 2 mA/cm^2 for 1.5 h and immunodetected with monoclonal antibodies against PLA_2 (1:60 dilution) according to a standard procedure [5].

2.5. *Immunofluorescence*

Rat mesangial cells grown on glass coverslips were stimulated with the indicated agents. The coverslips were rinsed twice with PBS, fixed 30 min in 3% paraformaldehyde in PBS and washed twice with PBS. All incubation and washing steps were performed at room temperature. After permeabilization in 0.1% Triton X-100 for 5 min and quenching with 50 mM glycine in PBS for 5 min, the samples were washed twice with PBS containing 0.1% gelatin and 0.5% BSA (denoted as PBG). The coverslips were incubated with a monoclonal antibody against rat group II PLA_2 (1:5 dilution). After six 5 min washes in PBG, the coverslips were incubated for 1 h with both fluorescein isothiocyanate-labeled goat anti-mouse IgG (GAM-FITC, dilution 1:200) for detection of PLA_2, and tetramethylrhodamine isothiocyanate conjugated to the Golgi marker, *Ricinus communis* agglutinin (RCA 120-TRITC, dilution 1:100). After washing with PBG six times and once with distilled water, the preparations were embedded in Mowiol 4–88, 10% glycerol and 100 nM 1,4-diazobicyclo (2,2,2)-octane buffered with Tris, pH 8.3. The coverslips were examined with a Leitz orthoplane microscope (× 63 oil objective lens). The microscope was equiped with a double channel filter set with excitation lens at 488 nm and 514 nm and emission filters at 515 nm and 550 nm, thus allowing separation of the green (fluorescein) and red (rhodamine) images. The coverslips were photographed on Kodak Tri-X film.

2.6. *Incorporation of [^{3}H]leucine into cellular proteins*

Confluent mesangial cells grown in 22 mm diameter wells were washed once with PBS and incubated with 1 ml DMEM containing 0.1 mg/ml fatty acid-free bovine serum albumin. The cells were pulsed with 2 μCi/well of [4,5-^{3}H]leucine for 24 h. Il-1β and BFA were added to the medium at the concentrations indicated simultaneously with labeled leucine. Thereafter the medium was removed and the cells were washed two times with ice-cold PBS. The cell monolayer was removed from the bottom of the well by adding 2 ml/well of 8 mM EGTA PBS. The cells were pelleted by centrifugation at 2000 g for 5 min. The pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 1mM EGTA, 1 mM PMSF and sonicated. Aliquots of 50 μl and 30 μl from each sample were spotted on Whatmann 3MM filter papers. The filters were dried at room temperature. The protein was precipitated on the filter paper by washing them in 10% TCA for 15 min. Then, the filters papers were washed successively for 15 min each in 10%, 5% and 1% ice-cold TCA to remove traces of free [4,5-^{3}H]leucine. The filters were air-dried and transferred to scintillation vials. The radioactivity incorporated into proteins was measured by scintillation counting [15]. In pilot studies we tested this method (A) with the standard method of TCA precipitation and centrifugation as reference (B) [16]. The results showed that the DPM incorporated in control samples were almost identical in both methods (657,752 ± 73,046 and 716,922 ± 62,890 dpm/mg protein for A and B, respectively).

3. RESULTS

3.1. *Inhibition of group II-PLA_2 secretion from mesangial cells by BFA*

It has been reported previously that incubation of rat glomerular mesangial cells with Il-1β induced synthesis

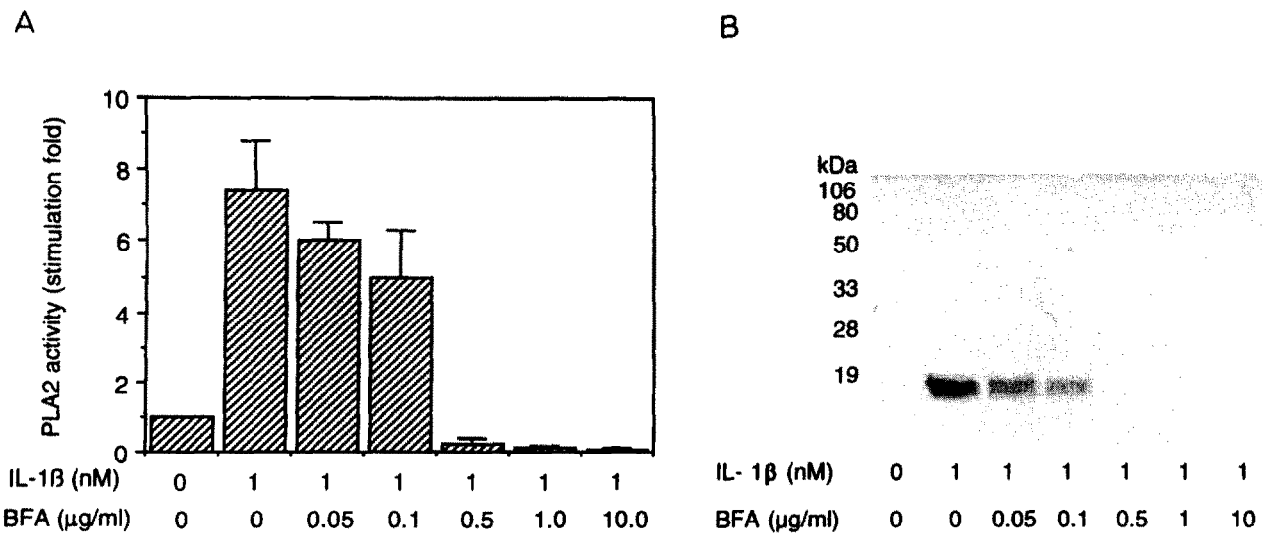

Fig. 1. Dose-dependent inhibition of IL-1β-induced secretion of PLA_2 by BFA. Confluent monolayers of mesangial cells were incubated for 24 h with the indicated concentrations of Il-1β and BFA. Thereafter, the culture supernatants were withdrawn and analyzed for PLA_2 activity (A) and PLA_2 protein by Western blotting and immunostaining (B). In panel A, data are expressed as the mean ± S.D. from three separate experiments. The value of PLA_2 activity for control was 0.395 ± 0.065 nmol/min/ml. In panel B, a representative experiment out of a series of three is depicted.

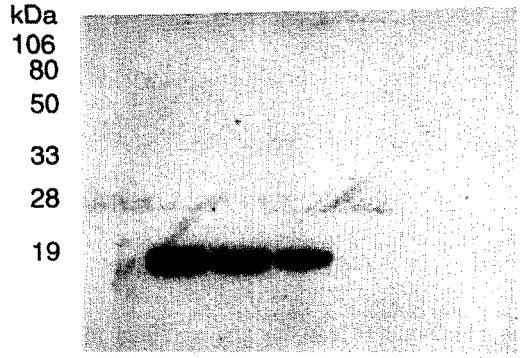

Fig. 2. Dose-dependent inhibition by BFA of the synthesis of PLA_2 upon IL-1β stimulation of mesangial cells. Confluent monolayers of mesangial cells were incubated for 24 h with the indicated concentrations of IL-1β and BFA. After the incubation period the media were removed and the cell layers were rinsed, scraped and resuspended. 20 μg of protein from cell homogenates was used for Western blotting and immunostaining as described in section 2.4.

and secretion of group II-PLA_2. Treatment of the mesangial cells with increasing concentrations of BFA gradually decreased the secretion of PLA_2 activity. In these experiments BFA was added together with the cytokine. When the concentration of BFA in the medium was 0.5 μg/ml, the Il-1β-induced secretion of PLA_2 activity was completely inhibited, resulting in PLA_2 activities even lower than control values (Fig. 1A). These results were supported by the immunoblot experiments (Fig. 1B). As can be seen in Fig. 1B, PLA_2 could not be detected on the immunoblots of the culture medium of unstimulated cells. After stimulation of the cells with IL-1β the protein became detectable in the culture supernatants, in full agreement with previous results which have also shown that this secretion followed de novo synthesis of PLA_2 protein [3,5]. The PLA_2 signals on immunoblots of the media from BFA-treated cells were gradually reduced and became undetectable at 0.5 μg/ml of BFA in accord with the values of PLA_2 activities in the culture supernatants.

3.2. *BFA prevents the induced synthesis of PLA_2 in mesangial cells*

In order to investiage whether the reduced levels of PLA_2 activity and PLA_2 protein in the culture supernatants were due to an inhibition of PLA_2 secretion and hence caused an accumulation of the induced PLA_2 in the cells, the PLA_2 protein levels in the cells were analyzed. Surprisingly, immunoblots from the treated cells showed that BFA did not cause an accumulation of PLA_2 in the cells. Rather, the drug prevented the de novo synthesis of PLA_2 protein, as induced by IL-1β, at the same concentrations that blocked the PLA_2 secretion (Fig. 2). However, although we did not detect cellular PLA_2 protein levels at 0.5 μg/ml, the PLA_2 activity could not be totally inhibited even at the highest concentrations of BFA (75 and 78% inhibition of PLA_2 activity from Il-1β-stimulated cells at 1.0 and 10.0 μg/ml) of BFA. In view of the intensities of the stained bands we cannot exclude the possibility that PLA_2 enzymes other than the immunoreactive group II PLA_2 contribute to these residual activities.

3.3. *Effects of BFA on the Golgi apparatus in mesangial cells: immunofluorescence localization of PLA_2 in BFA-treated cells*

We studied the effect of BFA on the Golgi apparatus and on group II PLA_2 levels in IL-1β-stimulated cells. In these experiments, the same concentrations of BFA as applied in previous experiments (0.05, 0.1, 0.5, 1.0, 10.0 μg/ml) were used. In accord with previous studies [18], in unstimulated cells negligible immunofluorescent labeling of PLA_2 was obtained (data not shown). As shown in Fig. 3, in IL-1β-stimulated cells intense fluorescence near the nucleus was observed for both the Golgi marker and the PLA_2. Treatment of rat mesangial cells with increasing concentrations of BFA caused marked changes in the morphology of the Golgi apparatus. Whereas in IL-1β-stimulated cells it showed a compact shape nearby the nucleus, at 0.1 μg/ml of BFA a circular staining around the nucleus was observed, and at 0.5 μg/ml all the cells had a totally disorganized Golgi, as shown by the appearance of a fine punctuate immunofluorescence pattern. This was even more pronounced at the highest concentrations (1.0 and 10.0 μg/ml), although at those levels many dead cells were found after a 24 h incubation with BFA and Il-1β. Phase-contrast micrographs of cells treated with up to 0.5 μg/ml of BFA showed similar shapes to those found in IL-1β-stimulated cells (data not shown). With regard to the staining of PLA_2 in BFA-treated cells, there was no apparent difference in the localization of the staining of PLA_2 found in IL-1β-stimulated cells and that in the cells treated with 0.05 μg/ml of BFA. As expected, the staining gradually decreased when BFA concentration increased, hence at 0.5 μg/ml of BFA we could hardly detect any staining.

3.4. *Effect of BFA on total protein synthesis*

The effect of varying concentrations of BFA on the incorporation of [4,5-^{3}H]leucine to the cellular proteins and PLA_2 was tested in mesangial cells. The results showed a different pattern for PLA_2 activity and protein synthesis (Fig. 4). Total protein synthesis remained unaffected at BFA concentrations up to 0.1 μg/ml and became about 50% inhibited at 0.5 and 1.0 μg/ml of BFA. By contrast, the IL-1β-induced synthesis of PLA_2 activity was more sensitive to BFA, both with respect to BFA concentration and magnitude of the inhibition (Fig. 4). However, again it was not possible to totally inhibit either protein synthesis or PLA_2 activity even at the highest concentration used (1.0 μg/ml BFA).

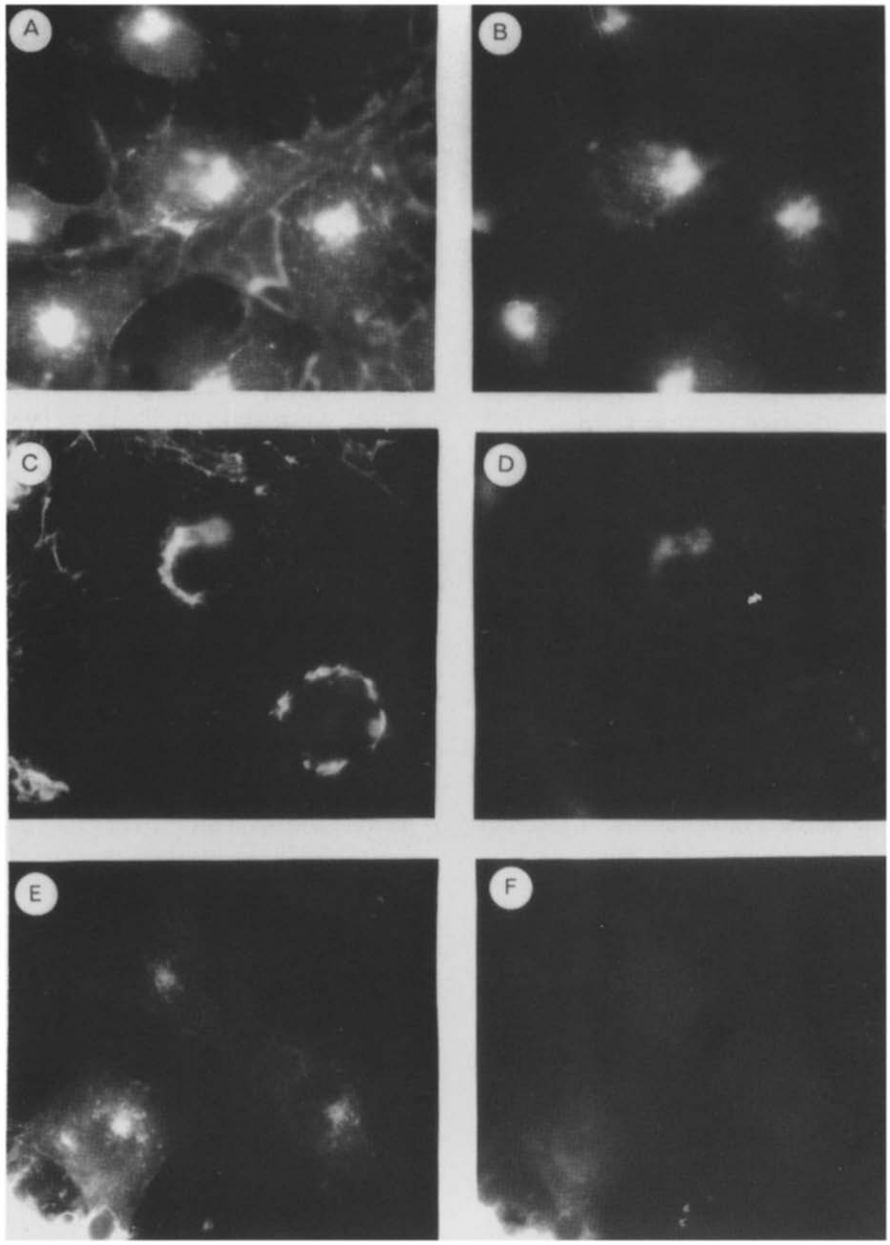

Fig. 3. Localization of group II PLA_2 and Golgi marker in stimulated cells treated with BFA. Rat mesangial cells grown on glass coverslips were treated with IL-1β (A,B), IL-1β plus 0.1 μg/ml BFA (C,D) or IL-1β plus 0.5 μg/ml BFA (E,F). Immunofluorescence was carried out as described in section 2.5. The Golgi localization, as detected with RCA120-TRITC, is shown in panels A, C and E, and the PLA_2 localization, detected with McAb-GAM-FITC complex, is shown in panels B, D and F.

4. DISCUSSION

The fungal metabolite, brefeldin A, has been used by many groups to study membrane traffic in the secretory pathway. BFA has been shown to block secretion and alter the structure of the Golgi complex [7] but little is known about the effect of this compound on other systems. We found that BFA prevented the induced synthesis and secretion of group II PLA_2 in mesangial cells. We expected a blockage of the secretion and consequently an accumulation of the enzyme in the cell. However, while secretion was blocked there was no accumulation but rather a decrease in the cellular PLA_2 protein levels (Fig. 2). Thus, the decrease in PLA_2 activity and protein levels in the culture supernatants could potentially have been caused solely by an inhibition of the induced PLA_2 synthesis without affecting protein secretion. However, there seemed to be two different effects of the drug as the PLA_2 activity in the medium, but not in the cells, could be totally blocked. It has been re-

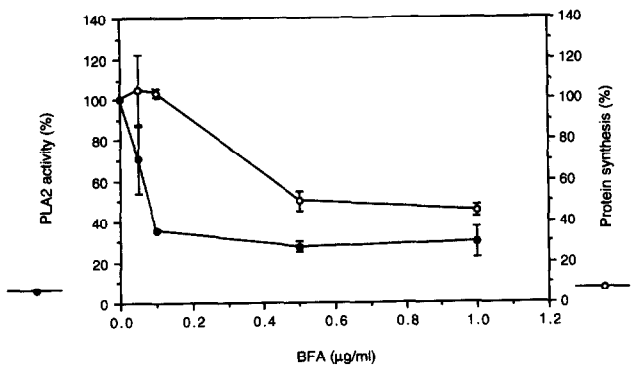

Fig. 4. Effect of BFA on total protein synthesis and PLA_2 activity in stimulated mesangial cells. Confluent monolayers of mesangial cells incubated for 24 h with 1 nM IL-1β and 2 μCi [4,5-^{3}H]leucine/well in the absence and in the presence of 0.05, 0.1, 0.5 and 1.0 μg/ml BFA. The cells were then assayed for protein synthesis (○) and PLA_2 activity (●) as described in sections 2.6 and 2.3, respectively. Data are expressed as the mean ± S.D. from three separate experiments (100% = 628,564 ± 35,440 dpm [4,5-^{3}H]leucine incorporated/mg protein for protein synthesis, and 1.54 ± 0.32 nmol/min/mg for PLA_2 activity).

ported that not all cell lines are equally sensitive to BFA, some of them even being totally insensitive [17]. Therefore, first of all, we tested the effect of BFA on the Golgi complex in rat mesangial cells and checked if BFA only prevented the induced synthesis of PLA_2, thereby decreasing the protein levels inside and outside the cell, or whether also disorganization of the Golgi complex, and hence inhibition of protein secretion, took place. Double immunofluorescence studies showed an obvious alteration of the morphology of the Golgi complex by BFA. The structure of this organelle began to be modified at 0.1 μg/ml of BFA, and at 0.5 μg/ml it was almost completely disassembled (Fig. 3). As was expected, at these concentrations (0.5–10.0 μg/ml) the PLA_2 activity in the culture supernatants was practically insignificant since the protein secretion was totally blocked (Fig. 1A). In accord with previous studies [18] PLA_2 was detected in the Golgi apparatus (Fig. 3B). BFA-treated cells showed a lower staining in comparison to cells receiving only IL-1β. Thus, the staining at 0.5 μg/ml is practically negligible (Fig. 3F), in full agreement with the inhibition of PLA_2 synthesis detected in the immunoblots (Fig. 2). Therefore, treatment of rat mesangial cells with BFA caused both the blockage of the protein secretion, due to the disassembly of the Golgi complex, and the inhibition of induced PLA_2 synthesis.

BFA has been widely used in cell biology and it has been reported that this compound does not affect protein synthesis [8,16,17,19,20]. However, while our studies were in progress Fishman and Curran have recently shown that BFA inhibits protein synthesis in rat glioma C6 cells and, to minor extent, also in other cell lines [21]. It may thus be that this additional effect of BFA is cell type-dependent. Therefore, we decided to test it in rat mesangial cells to investigate whether BFA had a specific effect in suppressing induced PLA_2 synthesis or whether this resulted from a general effect on total protein synthesis. In these experiments the cells were analyzed both for total protein synthesis and PLA_2 activity. BFA inhibited the [^{3}H]leucine incorporation into cellular proteins approximately 50% at 0.5 and 1.0 μg/ml, but no inhibition was observed at lower concentrations. However, PLA_2 activity was already inhibited over 60% at 0.1 μg/ml of BFA (Fig. 4).

Many secretory proteins are proteolytically processed after leaving the endoplasmic reticulum. This processing occurs in both the Golgi complex and in secretory vesicles and it is, to a large extent, highly specific [22]. Some authors have reported that BFA inhibited the proteolytic processing of envelope glycoproteins in cells infected with virus [23]. Whether proPLA_2 is processed proteolytically in the Golgi remains to be elucidated. Nevertheless, PLA_2 appears to be more sensitive to the effects of BFA, and thus at lower concentrations of this compound, in the first steps of the Golgi disassembly, this processing/secretion of PLA_2 was already blocked. As a result of this blockage, the cell could react by blocking the synthesis of this protein by a feed-back mechanism without affecting total protein synthesis. It would not be possible to detect this inhibition by measurement of total protein synthesis because the amount of PLA_2 is not significant with respect to the total amount of cellular protein. At higher concentrations of BFA (0.5, 1.0 μg/ml), the% inhibition of protein synthesis was closer to that found for PLA_2 activity, although the latter was always higher. Under these conditions the decrease in PLA_2 activity might be only due to the protein synthesis inhibition. Nevertheless, it should be pointed out that at these concentrations two effects are taking place simultaneously in the cell: the inhibition of the synthesis and the blockage of the secretion of PLA_2. Since after Il-1β stimulation over 85% of PLA_2 activity is secreted [5], we detect only 15% of the total enzymatic activity by assaying cell homogenates. However, in BFA-treated cells all the PLA_2 activity is detected because the protein secretion is blocked. The residual 30% activity in these cells was calculated against the 15% of activity remaining in IL-1β-stimulated cells not receiving BFA. Since these cells in total synthesize about 6-fold more PLA_2, the percentage residual activity would be only about 5% of that totally synthesized. Thus, the inhibition by BFA of the IL-1β-induced synthesis of PLA_2 may be as high as 95%.

In summary, next to the well-known effect of BFA on Golgi disassembly, this drug inhibits protein synthesis, although the synthesis of all proteins does not appear to be inhibited to the same extent. Those proteins processed/secreted via the Golgi complex, like PLA_2, may be more sensitive. The mechanism through which BFA inhibits protein synthesis remains unclear. It has been

shown by electron micrographs that in human lung fibroblast after incubation with BFA for 6 h, the endoplasmic reticulum is devoid of ribosomes [24]. This effect could explain an inhibition of total protein synthesis. Nevertheless, in rat mesangial cells, both total protein synthesis and PLA_2 activity could not be totally inhibited as would be expected if the endoplasmic reticulum lacked ribosomes. Some authors have postulated that disruption of intracellular trafficking/processing by BFA may lead to inappropiate processing or localization of some factors required for protein synthesis [21]. This may explain why BFA did not totally inhibit protein synthesis. Even though we can not discard a direct effect of BFA on protein synthesis it seems more likely that this effect was mediated by the Golgi disassembly, and in view of the results found, some secretory proteins that could be processed in the Golgi appear to be more sensitive to the effect of BFA on this complex.

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