Brefeldin A inhibits insulin-dependent receptor redistribution in HIRcB cells

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Abstract **Brefeldin A (BFA) is a potent inhibitor of intracellular vesicle traffic. We have investigated the effects of BFA on the traffic of the insulin receptor in HIRcB cells, a cell line derived from Rat-1 fibroblasts that over-expresses a normal human insulin receptor. We report here that insulin-dependent receptor redistribution is inhibited by BFA and that this drug has no effects on the insulin-independent redistribution of the receptor. Autophosphorylation of the insulin receptor and the stimulation of mitogen-activated protein kinase (MAPK) by insulin were not affected by treatment with the drug. The effects of BFA were further shown to require addition of the drug prior to the addition of insulin. BFA added 10 min after stimulation with insulin had no effects on the redistribution of the receptor. Dose–response studies demonstrated that the effects of BFA were half-maximal at a dose of 1 μg/ml and maximal at about 10 μg/ml. These findings suggest that BFA blocks an early step in the chain of events that lead to insulin receptor internalization without affecting the interactions of the receptor with insulin, the stimulation of the tyrosine kinase activity of the receptor by the hormone, or other insulin-regulated signalling pathways, such as the activation of MAPK.**

Key words: Brefeldin A; Insulin; Receptor; Endocytosis

1. Introduction

The insulin receptor (IR) is a heterotetrameric protein composed of two extracellular α-subunits and two transmembrane β-subunits [1]. Insulin binds to the α-subunits and initiates a chain of events that results in the autophosphorylation of the β-subunits and other intracellular substrates [2,3]. Stimulation of the tyrosine kinase activity of the receptor appears to be crucial to most biological effects of insulin [4–6]. One of the consequences of the binding of insulin to the receptor is the stimulation of the internalization of the hormone–receptor complex [7,8]. Internalization of this complex results in the degradation of the hormone [9–12]. Unliganded receptor is then recycled back to the surface of the cell or further degraded [13].

The internalization of the IR in HIRcB cells and other cell lines derived from Rat-1 fibroblasts has been extensively studied in recent years by ultrastructural and biochemical techniques [4,14–16]. Two independent pathways appear to exist in receptor-mediated insulin internalization: an ATP-dependent concentrative process using coated pits on the cell surface, and an ATP-independent process that uses non-coated plasma membrane invaginations [14]. The proportion of IR internalization by either pathway is ligand concentration dependent [14,16]. Coated pit-mediated internalization proceeds by a mechanism that involves several distinct steps: aggregation on the cell surface, migration to the coated pits and internalization [13,14]. Tyrosine kinase-deficient receptors were shown to aggregate normally but failed to concentrate in the coated pits and were not internalized [14]. The alternative, ATP-independent pathway of IR internalization has not been described in detail.

The isoprenoid fungal metabolite brefeldin A (BFA) is a potent inhibitor of intracellular vesicle assembly and traffic [17–20]. The effects of BFA include the disappearance of non-clathrin-coated buds and transport vesicles, the inhibition of constitutive secretion, and changes in the structure and function of organelles associated to the endocytic and exocytic pathways [17–20]. In spite of these remarkable effects on vesicle trafficking, BFA has not been shown to inhibit receptor-mediated endocytosis [21,22]. However, the effects of BFA on the internalization and processing of tyrosine kinase receptors have not been studied to date. Given the extensive work carried out in the characterization of the mechanisms of internalization of the insulin receptor in the Rat-1 fibroblast model [14–16,23], we decided to investigate the effects of BFA on the internalization of the insulin receptor in HIRcB cells. We report here that BFA blocks the insulin-dependent redistribution of the insulin receptor in a dose-dependent manner without affecting insulin receptor autophosphorylation or other signalling pathways associated to insulin, such as MAPK activation. We also show that the blockade of insulin-induced receptor redistribution requires addition of the toxin prior to exposure to insulin, suggesting that BFA's effects take place at a very early event during the generation of the signals that lead to receptor internalization.

2. Experimental

2.1. Cell culture conditions

HIRcB cells were a gift of Dr. D. McClain. These cells are derived from Rat-1 fibroblasts and over-express the human insulin receptor at very high levels (about 10^6 receptors/cell). Cells were cultured in 10 or 6 cm dishes using DMEM/F12 supplemented with 10% FBS and 100 nM methotrexate as described [4]. Cells were used within 5 days of subculture and were serum starved for 20 h in medium containing 0.1% BSA before treatment. These conditions maximize the levels of expression of insulin receptors on the surface of the cell and minimize basal levels of activity in terms of receptor autophosphorylation and MAP kinase activity.

2.2. Treatment with insulin and brefeldin A

Serum-starved cells were washed twice with fresh medium and incubated with BFA (3 or 10 μg/ml) for up to 15 min before the addition

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Abbreviations: BFA, brefeldin A; IR, insulin receptor; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PY, phosphotyrosine.

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of insulin. Cells were treated with insulin (20–100 nM) at 37°C in serum-free medium supplemented with 0.1% BSA.

2.3. Redistribution of the insulin receptor

The redistribution of insulin receptors was studied by a procedure based on the use of specific monoclonal antibodies that recognize the α- and β-subunits of the receptor. Internalized receptors were distinguished from those present on the cell surface on the basis of their sensitivity to mild trypsin treatment of the cells, as originally described by Levy and Olefsky [24], a procedure that has been used as a tool to distinguish cell surface from internalized receptors [4,23–26]. Mild trypsin treatment (1 mg/ml trypsin for 30 min at 4°C) cleaves the α-subunit of the receptor predominantly at one site, releasing two fragments: a 100 kDa fragment that contains the binding sites for insulin and for the monoclonal antibody 83-7, and a 20 kDa fragment that remains attached to the β-subunit [27]. Thus, the criterion used to detect intact receptors was the co-immunoprecipitation of α- and β-subunits: only those β-subunits attached to intact, non-proteolyzed α-subunits co-immunoprecipitate with mAb 83-7.

HIRcB cells were cultured as described above in 6 cm plates until confluent and serum starved overnight before use. Where indicated, 20 or 100 nM insulin and 3 μg/ml BFA were added to the plates for 30 min at 37°C. The cells were washed with PBS and DMEM/0.1% BSA (pH 4.0) to eliminate surface-bound insulin, and treated with trypsin (1 mg/ml) at 4°C for 30 min. Trypsin treatment was stopped by addition of 0.1 ml of a solution containing 0.4% Triton X-100, 25 mg/ml soybean trypsin inhibitor, 5 mg/ml bacitracin, 100 μg/ml leupeptin. Cells were solubilized in this medium for 20 min, and insoluble material removed by centrifugation. Antibody 83-7 (HIR α-subunit specific) was immobilized using goat anti-mouse IgG-agarose by incubation of hybridoma culture supernatants with the resin for 2 h followed by removal of unbound antibody by washing with PBS. Solubilized insulin receptors were immunoprecipitated by overnight incubation at 4°C with immobilized mAb 83-7. Insulin binding to the immunoprecipitated material was determined with [^{125}I]insulin (control experiments demonstrated that the insulin binding capacity of the immunoprecipitated receptors was not affected by trypsin treatment under the conditions described). The samples were then analyzed by SDS-PAGE and immunoblotted with either mAb CT-1 (IR β-subunit specific) or mAb 4G10 (anti-phosphotyrosine specific). A chemiluminescent method was used to detect the bound antibody, taking the precaution to adjust the exposure to within a linear response range as determined with a set of standards. The intensity of the 95 kDa band corresponding to the β-subunit of the insulin receptor was determined by densitometry and normalized to the insulin binding capacity of the immunoprecipitate.

2.4. Insulin binding

To validate the trypsin digestion/immunoprecipitation procedure used, the binding of insulin to the immunoprecipitated material was determined by standard competition binding procedures. Immunoprecipitated receptors were incubated with [^{125}I]insulin (0.1 nM) in the presence of increasing concentrations of unlabelled insulin (0.1–100 nM) for 1 h at room temperature in 50 mM HEPES (pH 7.5) containing 1 mM EDTA, 1 mM EGTA and 1 mg/ml bacitracin. Non-specific binding was determined in the presence of 10 μM unlabelled insulin. At the end of the incubation, the immunoprecipitated material was washed 4 times with immunoprecipitation buffer. Bound insulin was determined by gamma counting. Quantitation of insulin binding for the normalization of individual samples was carried out as follows. The immunoprecipitated material was divided into 3 equal parts and radiolabelled insulin was added to each to a final concentration of 0.1 nM. Unlabelled insulin was added to two of the samples (final concentrations: 2 nM and 10 μM, respectively). Binding was estimated as described above. Non-specific binding was estimated from the sample containing 10 μM unlabelled insulin. The samples were recovered after counting, boiled with Laemmli sample buffer and used to run the SDS-PAGE procedures described above to quantitative the levels of HIR β-subunits co-precipitating with the insulin binding activity.

2.5. Tyrosine phosphorylation of the insulin receptor

Autophosphorylation of the receptor was determined using material immunoprecipitated with 83-7 mAb. Briefly, the immunoprecipitates were resolved by SDS-PAGE in 9% acrylamide gels, blotted onto nitrocellulose, and phosphotyrosine-containing proteins were detected by immunoblotting using mAb 4G10.

2.6. MAPK activity

MAPK activity was determined using whole cell lysates after fractionation of the samples using FPLC procedures as described by Dent et al. [28]. Cells were solubilized in 50 mM HEPES (pH 7.5) containing 1% Triton X-100, 25 mM Na-fluoride, 2 mM Na-vanadate, 1 mM *p*-nitrophenylphosphate, 2 μg/ml leupeptin, 1 mg/ml soybean trypsin inhibitor, and 1 mM PMSF. After removal of the insoluble material by centrifugation, the equivalent of 1 mg of cell protein was fractionated by FPLC using a Mono Q ion-exchange column and a 0.1–0.3 M NaCl gradient. All samples were assayed for protein kinase activity using myelin basic protein (2 mg/ml) as a substrate and 100 μM [γ-^{32}P]ATP as the phosphate donor.

2.7. Other procedures

Protein concentrations were determined using a commercial kit (BCA assay, from Pierce). Chemiluminescence detection of protein bands in immunoblots was carried out as described by the manufacturer of the kit (Renaissance; NEN/Dupont).

2.8. Materials

Monoclonal antibodies 83-7 and CT-1, originally produced and characterized in the laboratory of Dr. K. Siddle, were a gift of Dr. Peter Isakson (Monsanto). These antibodies have been extensively described by Clark et al. [27]. Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnologies. Brefeldin A was purchased from Boehringer. ^{125}I-Labelled insulin and [γ-^{32}P]ATP were obtained from New England Nuclear. All other reagents were supplied by Sigma Chemical Co.

3. Results

The distribution of the insulin receptor (IR) between the cell surface and intracellular compartments was determined by exposure of cells to mild trypsin (1 mg/ml) treatment at 4°C for 30 min. As reported by other investigators, only receptors exposed to the extracellular medium are accessible to trypsin [4,23–26]. This treatment proteolyzes the α-subunit of the receptor in a unique site close to the C-terminus of the α-subunit and separates both subunits [27]. The larger fragment resulting from this treatment (100 kDa) contains both the mAb 83-7 and insulin binding sites, whereas the smaller fragment remains attached to the β-subunit of the receptor [27]. In agreement with this, we observed that mild trypsin treatment of the intact cells did not quantitatively alter the insulin binding capacity of the material precipitated with mAb 83-7: the total immunoprecipitable insulin binding activity from trypsinized cells was 112.2 ± 14% of the total obtained from untreated cells (mean ± S.D., $n = 8$ cell plates in each group). In contrast, trypsin treatment had a very significant effect in the amount of receptor β-subunits co-precipitated with the α-subunits (Fig. 1). This is consistent with the work of Clark et al., who suggested that mild trypsin treatment leaves the α-subunit of the receptor substantially intact [27]. Fig. 1 shows that treatment with 20 nM insulin increases the number of trypsin-resistant IR by about 3-fold. BFA abolished the effects of insulin on the redistribution of IR. In the absence of insulin the distribution of HIR was not affected by BFA.

The time–course of the insulin-dependent redistribution of the IR is shown in Fig. 2a. The levels of trypsin-insensitive receptors increased with time, reaching a maximum at about 30 min. Addition of BFA 5 min prior to insulin blocked redistribution of the receptor. Identical effects were observed with the addition of BFA 10 or 15 min before insulin (not shown).

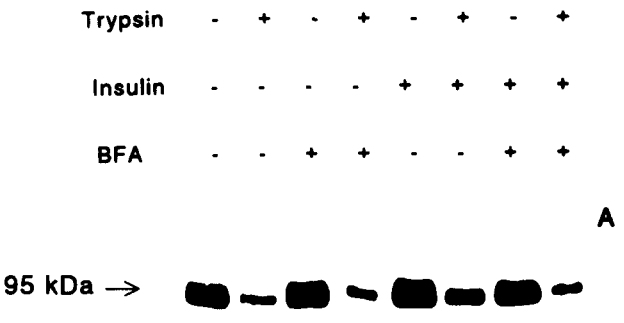
However, when insulin was added before BFA (Fig. 2b) the effects of the toxin were negligible. Fig. 2c shows the time–course of the internalization of the IR using anti-phosphotyrosine (mAb 4G10) antibodies to detect the tyrosine-phosphorylated β-subunit. The data obtained with mAb 4G10 were qualitatively very similar to those obtained with mAb CT-1, an observation that further supports the validity of the procedure used. These data and the data shown in Fig. 2d also demonstrate that BFA has no effect on the phosphorylation or dephosphorylation of the IR. We suggest, thus, that BFA interferes with an early event in the insulin signalling pathway leading to the internalization of the receptor, that this early event is not the auto-phosphorylation of the receptor, and that, once the initial signalling event that leads to receptor internalization has occurred, BFA has no further effects on the receptor redistribution process.

In order to determine whether or not the effects of BFA were confined to the redistribution of the IR, we also studied the effects of BFA on other insulin-regulated functions, such as the insulin-stimulated activation of MAPK. MAPK activation was not affected by preincubation of the cells with 5 μg/ml BFA. The MAPK activity, expressed as nmol of ^{32}P incorporated into protein per 60 min per mg of cell protein, was as follows: control, 4.56 ± 0.95; 5 μM BFA, 5.5 ± 1.1; 100 nM insulin, 29.2 ± 9.6; 100 nM insulin + 5 μM BFA, 31.2 ± 7.5 (mean ± S.E.M., $n = 3$). This suggests that receptor traffic and the activation of MAPK are regulated by intracellular signals that diverge early on in the signalling cascade initiated by insulin.

The insulin dose dependence of the redistribution of the receptor is shown in Fig. 3a and b. In these experiments, cells were stimulated with insulin at various concentrations for 30 min and the levels of trypsin-insensitive receptors were monitored as described. Typical results are shown in Fig. 3a (lanes 1–5). The data were analyzed by densitometry, normalized as described and summarized in Fig. 3b. As shown, the effect of insulin was half-maximal between 1 and 10 nM, consistent with the dose dependence of most insulin-modulated processes. The effects of BFA at maximal insulin concentrations (100 nM) are shown in Fig. 3a (lanes 6–11) and Fig. 3c. The half-maximal inhibitory dose was about 1 μg/ml, and complete inhibition of the insulin-induced redistribution of the receptor is observed at 10 μg/ml. These figures correlate very well with the concentrations of BFA necessary to disrupt assembly of the Golgi apparatus [19]. These observations were repeated 3 times with 2 independent lots of BFA and some lot-to-lot variations were observed. The correlation between the levels of BFA required to disrupt the Golgi and those found to inhibit IR internalization suggests a similar cause for both effects.

4. Discussion

The data we have presented in this paper demonstrate that short exposure to BFA does not affect the surface levels of IR, whereas exposure to insulin increases the levels of receptors in intracellular pools by about 3-fold. However, when cells are exposed to BFA prior to the addition of insulin, the effects of the hormone on the redistribution of the receptor are blocked. There are, in principle, three cellular events related to the insulin-induced surface redistribution of the IR that might be affected by BFA treatment. These are: (i) the internalization of the receptor; (ii) the recycling of internalized receptors to the surface of the cell; and (iii) the signalling mechanisms by which insulin modulates receptor internalization and/or recycling. The data shown in Figs. 1 and 2 provide significant support to the hypothesis that BFA interferes with the generation of an insulin-dependent signal rather than with receptor internalization or recycling processes per se. If the effects of BFA were related mainly to the processes involved in receptor internalization and/or recycling, BFA would be expected to alter the surface distribution of the receptor in the absence of stimulation by insulin, as shown for the IGF-II receptor [21]. Furthermore, Fig. 2a shows that if the effects of BFA were reduced to the inhibition of receptor traffic, then BFA should block receptor internalization, greatly accelerate the rate of recycling the receptor back to the surface or a combination of both processes. However, addition of BFA 10 min after insulin had no effects on the insulin-induced redistribution of the receptor. Fig. 2a–c show that the receptor redistribution process had not reached a steady state at the time of addition of BFA when the drug was added after insulin. Therefore, both receptor internalization and recycling were still active processes at the time of



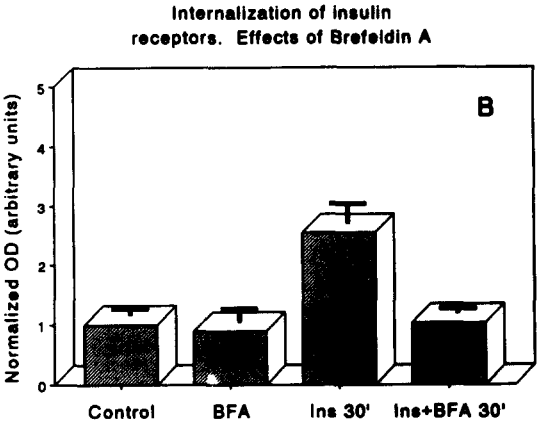


Fig. 1. The effects of brefeldin A on the internalization of the insulin receptor. BFA (3 μg/ml) was added to the cells 5 min prior to the addition of insulin. Cells were stimulated with 100 nM insulin for 30 min. (A) Representative experiment performed as described. (B) Summary of data obtained ($n = 9$ different plates in two independent sets of experiments for each treatment). ANOVA of the final results showed that the difference between insulin treatment alone and either control or insulin + BFA is significant ($P < 0.01$). As shown, BFA had no effects on the distribution of the receptor in unstimulated cells.

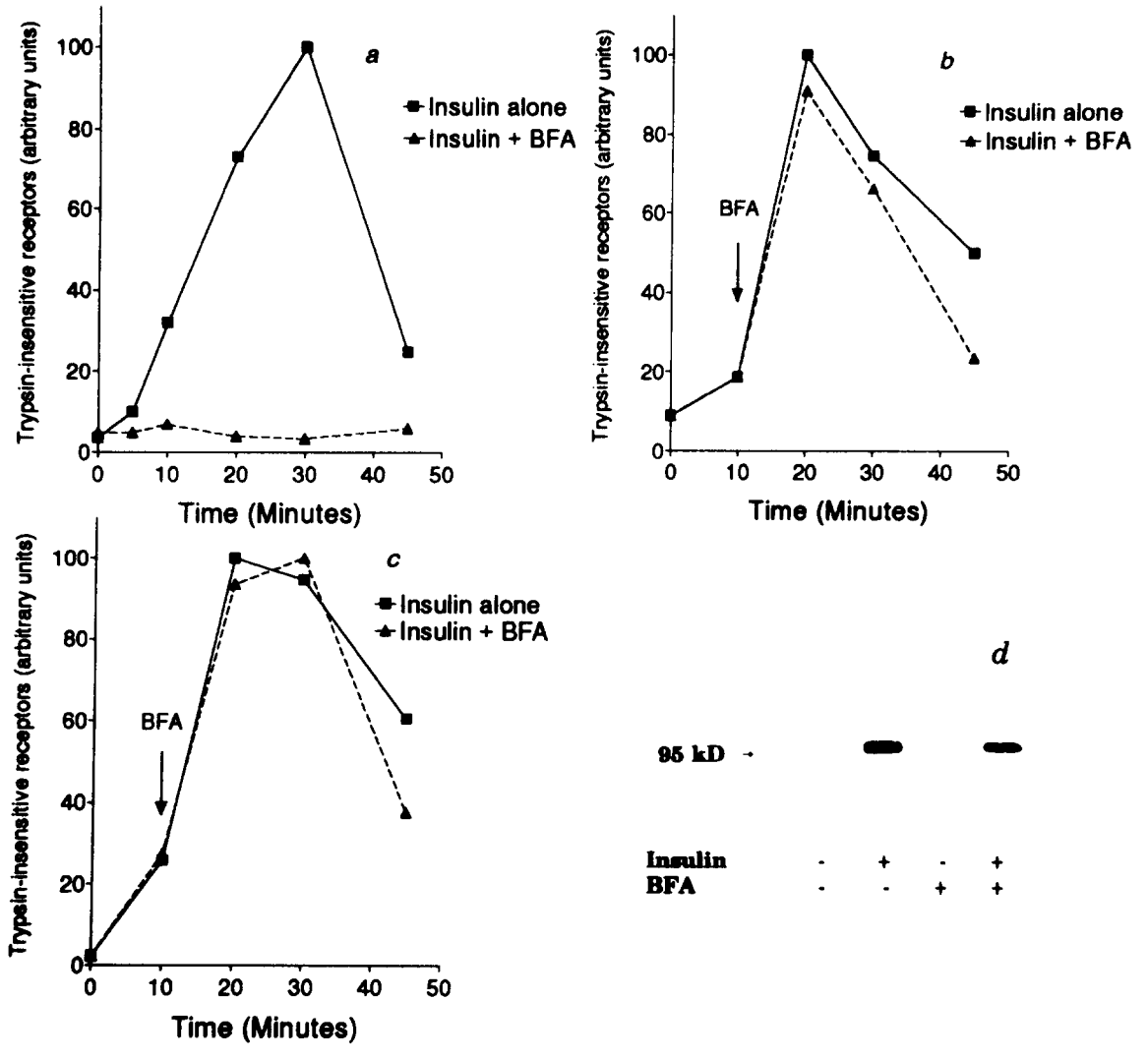


Fig. 2. (a) Time–course of the redistribution of the insulin receptor. These experiments were carried out as described in the legend for Fig. 1. The lower curve shows the effects of the addition of 3 μg/ml BFA 5 min before the addition of insulin. (b) Same as (a), with the exception that, when indicated (10 min after the addition of insulin), 3 μg/ml BFA was added to one set of plates. (c) Time–course of the internalization of the receptor obtained as described in (b) using anti-PY antibodies (mAb 4G10) to detect tyrosine-phosphorylated receptor β-subunits. (d) Cells were treated with insulin for 2 min where indicated and solubilized. For each condition, insulin receptors contained in 1 mg of cell lysate were precipitated with excess mAb 83-7 (see section 2). Therefore, equivalent amounts of receptor protein were analyzed. Samples were analyzed by SDS-PAGE and blotted. Blots were developed with anti-PY antibodies (mAb 4G10).

addition of the inhibitor. If BFA's effects were related to general, non-specific changes in the machinery of receptor trafficking, addition of BFA after initiation of the insulin-modulated events that lead to the redistribution of the receptor should have the same effects as addition of BFA before insulin. Thus, BFA would be expected to stop the internalization process, to accelerate the recycling process, or a combination of both. However, our data show that receptor redistribution was insensitive to the addition of BFA when the drug was added after stimulation of the cells with insulin. We suggest therefore that: (i) BFA's main effects in the receptor redistribution phenomenon are not due to the action of the drug on receptor internalization or recycling per se; (ii) BFA interferes with the signalling process by which insulin binding to the receptor modulates the subcellular distribution of receptors; and (iii) once receptor redistribution is triggered, BFA cannot interfere with the process.

It should be noted that BFA, in spite of its major effects on multiple membrane assembly and trafficking processes, has not been previously shown to inhibit receptor-mediated endocytosis. For instance, the IGF-II receptor in fibroblasts [21] and the transferrin receptor in epithelial cells [22] are internalized by processes that involve aggregation and accumulation in clathrin-coated pits but neither process is affected by BFA. Furthermore, it has been reasonably well established that although BFA causes the complete dispersion of Golgi-derived clathrin lattices it does not affect the assembly or function of clathrin-coated vesicles derived from the plasma membrane [34,35]. Thus, although the molecular basis for the BFA sensitivity of the IR internalization process is not clear at this time,

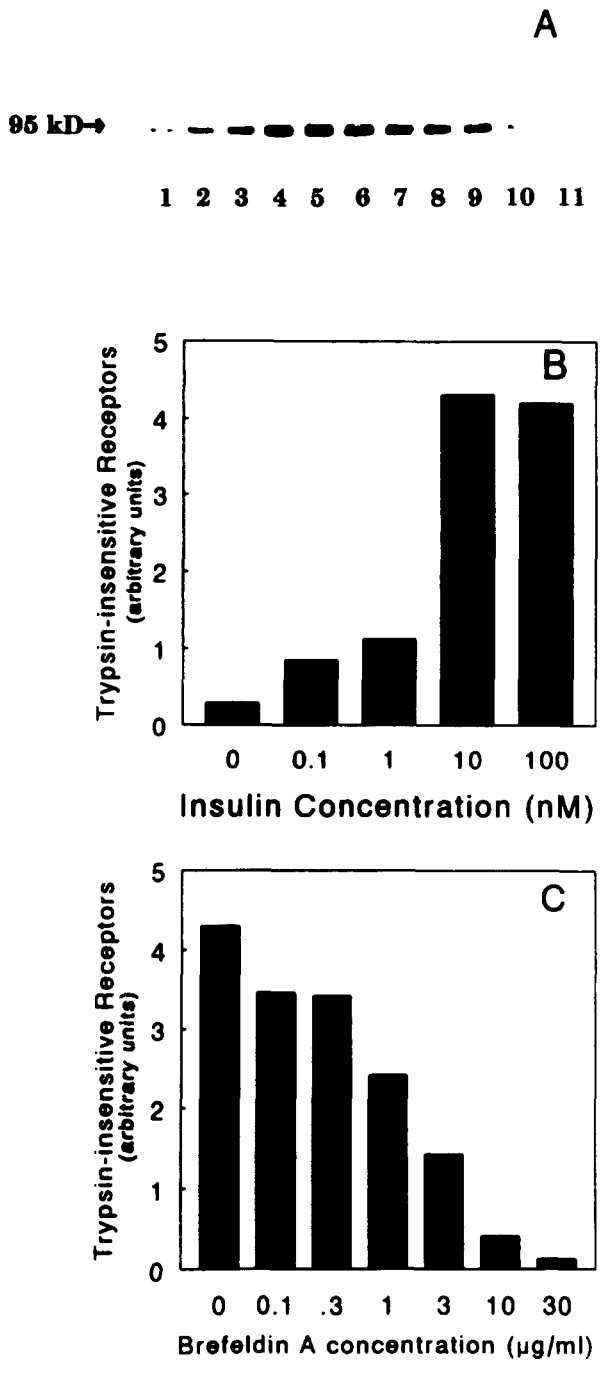


Fig. 3. Internalization of the insulin receptor as a function of the concentrations of insulin and BFA. (A) Western blot showing the levels of trypsin-resistant insulin receptors. Cells were treated with increasing concentrations of insulin (lane 1, untreated; lane 2, 0.1 nM insulin; lane 3, 1 nM insulin; lane 4, 10 nM insulin; lane 5, 100 nM insulin) or in the presence of BFA (lanes 6–11: 100 nM insulin, supplemented with: lane 6, 0.1 μg/ml BFA; lane 7, 0.3 μg/ml BFA; lane 8, 1 μg/ml BFA; lane 9, 3 μg/ml BFA; lane 10, 10 μg/ml BFA; and lane 11, 30 μg/ml BFA). The insulin receptors were precipitated with mAb 83-7 and the β-subunits detected with mAb CT-1 as described in section 2. (B) Summary of the data obtained by densitometry analysis of the blot shown in A, lanes 1–5. (C) Summary of the data obtained by densitometry analysis of A, lanes 6–11.

it is likely that the BFA-sensitive event precedes the assembly of the clathrin-coated vesicles. It should also be noted that IR internalization may occur by a second mechanism that does not involve clathrin coats, particularly at insulin concentrations in the nanomolar range, such as those used in our work [16]. BFA may interfere primarily with this mechanism, and not with the classical clathrin-coated pit system.

The mechanisms by which the endocytic apparatus detects the state of occupancy of the IR are not understood. Ultrastructural studies carried out with Rat-1 fibroblasts that overexpress normal and tyrosine kinase-deficient IR have shown that receptor aggregation per se is insufficient to trigger the internalization process [14]. IR internalization appears to require intact tyrosine kinase activity of the receptor β-subunit. This suggests that the signal that triggers internalization originates with the phosphorylation of some intracellular target. Our data show that BFA blocks IR internalization without altering the state of tyrosine phosphorylation of the receptor or the phosphorylation of the cellular targets that lead to the activation of MAPK. The question of what is the BFA-sensitive biochemical event in the mechanism of internalization of the IR thus remains unanswered. It should be noted that the only reasonably well-characterized biochemical effect of BFA is the blockade of the activation of small G proteins of the ADP-ribosylation factor (ARF) family, a class of small G proteins involved in many aspects of intracellular membrane traffic and assembly [29–33]. A potential scenario that derives from the observations reported here is one in which binding of insulin to the receptor leads to the activation of a member of the ARF family of G proteins and that this phenomenon is required for the activation of the internalization process. Work currently under way in our laboratory suggests that insulin can indeed stimulate the activation of ARF proteins in a reconstitution assay (K. Shome and G. Romero, in preparation). These findings, taken altogether, suggest that the internalization of the IR proceeds by a novel mechanism that involves a BFA-sensitive event, possibly the activation of a small G protein of the ARF family. We would also like to point out that after the initial submission of this manuscript evidence indicative of the involvement of a BFA-sensitive step in the mechanism by which insulin stimulates the recruitment of GLUT4 glucose transporters to the surface of rat adipocytes was reported by Lachaal et al. [36]. These findings, taken in conjunction with the data presented here, suggest that a BFA-sensitive event is required for the generation of insulin-mediated signals that modulate membrane trafficking processes.

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