Effect of Brefeldin A on the synthesis and transport of cell wall polysaccharides and proteins in pea root seedlings

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Abstract

The in vivo effect of Brefeldin A (BFA) on the synthesis and transport of cell wall polysaccharides and proteins in the roots of pea seedlings (Pisum sativum L. cv. Alaska) was investigated. BFA (10 µg ml⁻¹) inhibited the synthesis of cell wall matrix polysaccharides by approximately 43%. Under the same conditions, cellulose synthesis was inhibited by approximately 77%. The percentage of incorporation of L-[U-¹⁴C]leucine and L-[U-¹⁴C]proline into cytosolic, membrane and cell wall proteins was only slightly changed in the presence of BFA. In addition, the drug did not change the pattern of newly synthesized proteins in the three fractions as judged by SDS-PAGE fluorography. Double labelling of proteins and cell wall polysaccharides confirmed the above reported data. All these results showed that the synthesis and transport of proteins to the cell wall was only slightly affected by BFA under similar conditions to those which brought about a strong inhibition of the synthesis of matrix and cellulotic polysaccharides. BFA had no effect on the activity of membrane-bound and digitonin-solubilized mannan and glucosamin synthase isolated from the third internode of pea seedlings. This would exclude an effect of BFA at the level of the catalytic site of the synthases. The inhibition of polysaccharide synthesis by the drug was rapidly eliminated after its removal. It is concluded that the effect of BFA on the biosynthesis of cell wall polysaccharides could be caused by an interaction of the drug with the topological organization of the synthase complexes in the membranes. This effect would precede the action of the drug at the level of vesicle transport to the walls.

Key words: Brefeldin A, cell wall polysaccharides (synthesis and transport), Pisum sativum L., polysaccharide synthases, proteins (synthesis and transport).

Introduction

The Golgi bodies of both animal and plant cells are dynamic organelles which play a key role in processing, maturation and sorting of newly synthesized proteins, glycoproteins and proteoglycans. These are transported to specific membrane domains or they are secreted from the cell. The Golgi bodies are also involved in the recycling of receptors during endocytosis. In plant cells the Golgi apparatus is the site of synthesis and packaging of cell wall matrix polysaccharides such as pectins and hemicelluloses (Northcote, 1985). Biochemical and immunocytochemical studies have shown that the synthesis of matrix polysaccharides occurs in different cisternae of the Golgi stacks before they are packaged into secretory vesicles. Vesicles take part in the intra-Golgi transport of the polymers in a cis-to-trans Golgi direction (Zhang and Staehelin, 1992; Staehelin and Moore, 1995). Cellulose is polymerized at the plasma membrane. The enzymic complexes required for the synthesis of pectins, hemicelluloses, and cellulose are synthesized, at least in part, on the endoplasmic reticulum and then transported and sorted to the Golgi cisternae. Cellulose synthase complex is transported to the plasma membrane by secretory vesicles. Thus, the dynamic structural and functional organization of the plant cell wall during growth and differentiation depends on the activities of the enzymes associated with the endomembrane system.

Brefeldin A (BFA), a macrocyclic hydrophobic lactone isolated from a variety of fungi (Härry et al., 1963; Betina,
Fig. 2. Electron micrograph of a growing root hair of *Equisetum hyemale* showing Golgi vesicles in the hemisphere of the tip and Golgi bodies (GB) subapically. Bar=1 μm.

towards the edge of the pollen tube when nearing the vesicle-rich region and are absent from the vesicle-rich region proper (Miller *et al.*, 1996). Treatment with caffeine, a substance that greatly diminishes the calcium influx and abolishes the tip-focused calcium gradient (Pierson *et al.*, 1996), causes the actin filaments to redistribute in such a way that they are closer to the tip, thereby shortening the vesicle-rich region. When caffeine is removed, the vesicle-rich region re-establishes its normal size simultaneously with the removal of actin filaments from this area (Miller *et al.*, 1996). For further discussion of pollen tube actin, see Miller *et al.* (1996). Since pollen tubes and root hairs are both polar growing cells with similar morphology, organelle distribution and cytoplasmic streaming patterns, it would seem logical that the actin distribution within growing root hairs is similar to that of a pollen tube. Future experiments should address this issue.

**Spectrin-like antigen**

Animal spectrins are multifunctional molecules that belong to a family of proteins including actin binding protein 1, α-actinin, dystrophin, and fimbrin. These proteins have many binding sites with at least two actin filament binding sites, and binding sites for calcium and calmodulin (Hartwig, 1994). Spectrin and its associated proteins in red blood cells are now thought to provide organizational stability to a cell by controlling integral membrane protein distribution (Bennett and Gilligan, 1993; Devarajan and Morrow, 1996). Based on data from adrenal chromaffin cell secretion Hays *et al.* (1994) suggest that an increase of cytosolic calcium concentration ([Ca$^{2+}$]$_{i}$) dissociates actin from spectrin thereby allowing vesicle fusion.

Immunoblot analysis of young tomato leaves detected a 220 kDa protein labelled with human erythrocyte-spectrin (Michaud *et al.*, 1991), and Faraday and Spanswick (1993) detected a 230 kDa protein with antispectrin in the purified plasma membrane fraction of rice roots. In addition, De Ruiter and Emons (1993) detected spectrin-like epitopes in several tissues of maize and carrot, predominantly at the plasma membrane of cells growing along their whole length. This spectrin-like epitope is also localized at the tip of growing root hairs (Fig. 3), which is comparable to its localization at the tips of tobacco pollen tubes (Derksen *et al.*, 1995b) and the tips of growing hyphae of *Saprolegnia ferax* (Kaminskýj and Heath, 1995). Even though the nature of the plant epitope found in root hairs is still not known, its location suggests a role in exocytosis.

**Calcium gradient**

External Ca$^{2+}$ ions at the appropriate concentration and a Ca$^{2+}$ influx are required to elicit exocytosis in *Arabidopsis* root hairs (Schiefelbein *et al.*, 1992). These authors observed a net influx of Ca$^{2+}$ at the tips of growing root hairs with a calcium selective vibrating probe, whereas no influx was found at the sides of the hair nor into non-growing hairs. Selective vibrating probe analysis indicated Ca$^{2+}$ currents only in the tips of growing *Sinapis* root hairs (Herrmann and Felle, 1995; Felle and Hepler, 1997), which responded to changes in external concentrations of calcium by transient growth differences rather than an altered steady-state growth rate (Herrmann and Felle, 1995). The [Ca$^{2+}$]$_{i}$ increased in the apical area in the presence of increased external calcium and decreased when the external calcium concentration was lowered (Felle and Hepler, 1997). In the latter study non-growing hairs responded to changes in external calcium as well, but the [Ca$^{2+}$]$_{i}$ increase was uniform.
1926 Lanubile et al.

Brefeldin A, GDP-d-mannose, GDP-d-glucose and all other chemicals were purchased from Sigma Chemical Co., St Louis, Mo., USA. d-[U-14C]glucose (specific activity 10.5 GBq mmol⁻¹), l-[U-14C]proline (specific activity 9.32 GBq mmol⁻¹), l-[U-14C]leucine (specific activity 11.7 GBq mmol⁻¹), l-[4,5-3H]leucine (specific activity 3.15 TBq mmol⁻¹), l-[35S]methionine (specific activity ≥ 37 TBq mmol⁻¹), GDP-[U-14C]glucose (specific activity 8.40 GBq mmol⁻¹), GDP-[U-14C]mannose (specific activity 11.2 GBq mmol⁻¹) were from Amersham International, Amersham, Bucks, UK.

Plant material and radiolabelling experiments

Seeds of *Pisum sativum* L. cv. Alaska were soaked in running tap water (8 h), planted in damp vermiculite and grown for 2 d at 25 ± 1°C in darkness. Using a dim green light, uniform seedlings having primary roots 20-22 mm long were carefully washed in sterile bidistilled water and incubated with or without different concentrations of BFA in the presence of the radioactive tracers as specified for each experimental condition. The uptake of the radioactive tracer represents the difference between the initial radioactivity of the incubation mixture and the radioactivity remained at the end of incubation period of the seedlings.

Radioactive cell wall polysaccharide labelling

Three pea seedlings were incubated in water with or without BFA in the presence of 4 µCi of d-[U-14C]glucose at 25°C, in the dark, as specified for each experiment in tables and figures. At the end of the radiolabelling period, the roots were rapidly washed with an ice-cold unlabelled tracer solution and the root cap removed. Starting from the root apex, segments 1 cm long were cut from each root and homogenized with 2 ml Tenbroeck tissue grinder in 10 mM sodium-phosphate buffer pH 7.2, containing 10 mM imidazole, 1 mM benzamidine, 5 mM 6-aminosalicylic acid, 10 mM diethiothreitol, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The final volume of the homogenate was measured and aliquots were counted for radioactive material present in each homogenate. The homogenates were centrifuged at 800 x g for 10 min at 2°C in a model J2-21 centrifuge, rotor head J-20 (Beckman Instruments, Inc.,
Palo Alto, California, USA). The pellets, consisting of cell walls, were incubated in 0.05% sodium deoxycholate for 2 h, at 20°C and then washed with the homogenization buffer (twice) and acetone (twice) to obtain purified cell walls. The 800 × g supernatant was centrifuged at 100,000 × g for 60 min at 2°C in a Beckman preparative ultracentrifuge (model L8–55, rotor head SW 55) to obtain a supernatant (100,000 × g SN) and a pellet consisting of membrane and organelles. Each pellet was suspended in 75% ethanol containing 50 g l−1 glucose and centrifuged at 8000 × g for 5 min at 2°C (five times) to remove all free sugars. This fraction was the membrane fraction.

Purified cell walls and membranes were hydrolysed with 3 N HCl in sealed tubes at 120°C, for 1 h. The hydrolysates were dried, resuspended in water and counted for radioactivity. The cell wall residue was dissolved in 3% (w/w) H2SO4 and hydrolysed at 120°C, for 1 h. The hydrolysate was neutralized with a 15% (v/v) solution of methyl-di-n-octylamine in chloroform (excess of amine was removed by washing with five changes of chloroform), taken to dryness, resuspended in water and counted for radioactivity (cellulose).

The 100,000 × g SN was treated with 60% ethanol (final concentration) and centrifuged in a J2–21 centrifuge, rotor head J-20 for 10 min at 10,000 × g. The pellet was washed five times with 60% ethanol (v/v). The pellet and aliquots of the 10,000 × g supernatant were counted for radioactivity in order to determine radioactive soluble polysaccharides and cytosolic pool, respectively.

Radioactive protein labelling
Six pea seedlings were incubated in water with or without 10 µg ml−1 BFA for 1 h, at 25°C, in the dark. During the last 30 min of incubation, 8 µCi of L-[U-14C]proline, 8 µCi of L-[U-14C]leucine, 750 µCi of L-[3H]methionine were added to the incubation medium. At the end of the radiolabelling period, membranes and purified cell walls were isolated. To evaluate the radioactivity incorporated into cytosolic proteins, the 100,000 × g SN was concentrated (Centricon 10, Amicon) and the proteins were precipitated with 80% acetone at −20°C overnight. Membrane proteins were obtained from membranes depleted of lipids as described by Dalessandro et al. (1985). The proteins were precipitated with 80% acetone (final concentration) at −20°C overnight. Purified cell walls were resuspended and digested in phenol:acetic acid:H2O 2/1/1, (w/v/v) at 70°C for 30 min (Fry, 1988) to extract non-covalently/ionically bound cell wall proteins (PAW extract). After centrifugation (8700 × g, 10 min) the proteins present in the supernatant were precipitated with 0.1 M ammonium acetate in methanol overnight. The 8000 × g pellet, consisting of cell wall and covalently bound proteins, was hydrolysed with 3 N HCl in sealed tubes at 120°C, for 1 h (HCl hydrolysate). PAW extract and HCl hydrolysate were dried, resuspended in water and counted for radioactivity.

Cytosolic, membrane and cell wall proteins were analysed by SDS-PAGE electrophoresis (Laemmli, 1970) on a 13% polyacrylamide gel. Protein bands were stained with Coomassie brilliant blue R-250. Labelled proteins were detected by fluorography on Hyperfilm-β-max (Amersham).

**Double radiolabelling of proteins and cell wall polysaccharides**
Six pea seedlings were incubated in water with or without 10 µg ml−1 BFA for 1 h, at 25°C, in the dark. During the last 30 min of incubation, 8 µCi of D-[U-14C]glucose and 100 µCi of L-[4,5-3H]leucine were simultaneously added to the medium. After radiolabelling, the cytosolic pool, soluble polymers, membrane and cell wall polysaccharide and proteins were isolated.

**Mannan and glucomannan synthesis**
The effect of BFA (10 and 50 µg ml−1 for 30 min) on the in vitro synthesis of mannan and glucomannan was studied by using membrane-bound and digitonin-solubilized enzymes isolated from the third internode of pea seedlings. Enzymic preparations, incubation procedure and isolation of radioactive polymers were performed as reported by Piro et al. (1993). Enzymic assays were performed by using membrane-bound or digitonin-solubilized enzymes preincubated or not in the presence of BFA.

**Radioactivity counting procedure**
Radioactivity counting procedure was performed as described by Piro et al. (1993), using a 1217 Rack Beta liquid scintillation spectrometer (LKB). Counting efficiency was approximately 90%.

**Results**
All experiments used 2-d-old pea seedlings which were incubated for short times with and without BFA in the presence of D-[U-14C]glucose, L-[U-14C]leucine, L-[U-14C]proline or both D-[U-14C]glucose and L-[4,5-3H] leucine. Segments (1 cm) of decapitated roots were used to estimate the incorporation of radioactive sugars and amino acids into cell wall polysaccharides and proteins. Incorporation data are expressed as percentages of the total radioactivity measured in the homogenate of the root segments.

BFA at 10 and 20 µg ml−1 inhibited D-[U-14C]glucose uptake in pea seedlings by approximately 16% and 50%, respectively, up to 1 h of incubation (Fig.1A). A more pronounced inhibition by BFA was observed in the total amount of radioactive material found in the homogenates obtained from decapitated root segments isolated from BFA-treated seedlings (Fig.1B). The incorporation of radioactive sugars into matrix polysaccharides was inhibited by BFA (10 µg ml−1) by approximately 17% and 30% after 30 and 60 min, respectively. BFA at 20 µg ml−1 markedly inhibited the incorporation of radioactive sugars into matrix polysaccharides from 15 min of incubation (Fig.1C). At 10 and 20 µg ml−1 of BFA, the biosynthesis of cellulose, expressed as a percentage of radioactive glucose incorporated into the cellulose, was drastically inhibited after 15 min of incubation. BFA at 10 µg ml−1 inhibited cellulose synthesis more rapidly than matrix polysaccharides.

To test how BFA affects the synthesis and transport of cell wall polysaccharides to the wall, pea seedlings were incubated for 1 h with and without BFA (10 µg ml−1) and after 30 min of incubation, D-[U-14C]glucose was added in the medium. In these experimental conditions the inhibitory effect of BFA on the synthesis of cell wall polysaccharides had already started before the addition...
Fig. 1. Effect of BFA on: uptake of D-[U-14C]glucose by pea seedlings (A), radioactive material present in the homogenate (B), radioactive sugars incorporated into matrix polysaccharides (C) and cellulose (D) in 1 cm long pea root segments. 2-day-old pea seedlings (three) were incubated in water with or without BFA (10 and 20 ng ml⁻¹) in the presence of 4 µCi of D-[U-14C]glucose for 15, 30 and 60 min. At the end of incubation period, the seedlings were rapidly washed in a solution of cold glucose and depleted of root cap. Starting from the root apex, a segment 1 cm long was cut from each root and processed to analyse the amount of radioactivity present in the homogenate and incorporated into cell wall polysaccharides. (■) Water, (▲) BFA 10 ng ml⁻¹, (●) BFA 20 ng ml⁻¹. The data are from one representative experiment of three.

Table 1. Effect of BFA on radioactive 100 000 × g SN and radioactive polysaccharides found in membrane and purified cell wall

<table>
<thead>
<tr>
<th>Radioactivity (% of the homogenate)</th>
<th>H₂O</th>
<th>10 ng ml⁻¹ BFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 000 × g SN</td>
<td>73.9</td>
<td>87.0</td>
</tr>
<tr>
<td>Membrane</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Cell wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix polysaccharides</td>
<td>16.6</td>
<td>9.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>7.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Total radioactivity of the homogenate (kBq)</td>
<td>20.4</td>
<td>20.1</td>
</tr>
</tbody>
</table>

Pea seedlings. The inhibitory effect of BFA on the matrix polysaccharides in membranes and cell walls was 6% and 43%, respectively. Under the same conditions, cellulose was inhibited by approximately 77%. The lack of any accumulation of radioactive matrix polysaccharides in membranes suggested that an inhibition of matrix polysaccharide synthesis occurred before an inhibition of their transport to the wall.

Pea seedlings were incubated for 1 h with BFA (10 ng ml⁻¹), and during the last 30 min of incubation, L-[U-14C]proline or L-[U-14C]leucine was added to the medium. BFA inhibited the uptake of L-[U-14C]proline and L-[U-14C]leucine by approximately 60% and 50%, respectively. A similar inhibition was found for the total amount of radioactive material present in the homogenates. However, the percentage of incorporation of L-[U-14C]leucine and L-[U-14C]proline into cytosolic, membrane and cell wall proteins was only slightly changed in the presence of BFA (Table 2). In particular the percentage of L-[U-14C]leucine and L-[U-14C]proline incorporated into both non-covalently/ionically bound (PAW extract) and covalently bound (HCl hydrolysate) cell wall proteins was slightly stimulated. The synthesis and transport of newly synthesized proteins to the cell wall was only slightly affected by BFA under similar conditions to those which brought about a strong inhibition of the synthesis of matrix and cellulosic polysaccharides.

Confirmation that the biosynthesis of cell wall polysaccharides was inhibited by BFA without affecting the synthesis and transport of cell wall proteins was obtained when pea root seedlings were incubated for 1 h with BFA and during the last 30 min D-[U-14C]glucose and L-[4,5-3H]leucine were added simultaneously to the incubation medium. The percentage of [3H]material...
Table 2. Effect of BFA on the incorporation of L-[U-14C]proline or L-[U-14C]leucine into cytosolic, membrane and cell wall proteins extracted from pea root segments

Cytosolic, membrane and cell wall proteins were isolated from 1 cm long root segments obtained from six pea seedlings incubated in water with or without 10 μg ml⁻¹ BFA for 1 h, at 25 °C, in the dark. During the last 30 min of incubation 8 μCi of L-[U-14C]proline or 8 μCi of L-[U-14C]leucine were added to the medium. Incorporation is expressed as a percentage of the total amount of radioactivity measured in the homogenate. The data are from one representative experiment of three.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Radioactivity (% of the homogenate)</th>
<th>L-[U-14C]proline</th>
<th>L-[U-14C]leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O</td>
<td>10 μg ml⁻¹ BFA</td>
<td>H₂O</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>18.4</td>
<td>17.4</td>
<td>19.0</td>
</tr>
<tr>
<td>Membrane</td>
<td>12.4</td>
<td>11.8</td>
<td>11.9</td>
</tr>
<tr>
<td>Cell wall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAW extract</td>
<td>1.8</td>
<td>2.2</td>
<td>3.1</td>
</tr>
<tr>
<td>HCl hydrolysate</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>51.3</td>
<td>14.7</td>
<td>60.3</td>
</tr>
</tbody>
</table>

Table 3. Effect of BFA on the incorporation of D-[U-14C]glucose and L-[4,5-3H]leucine into soluble, membrane and cell wall polymers (proteins and polysaccharides) extracted from pea root segments

1 cm long pea root segments were isolated from six pea seedlings incubated in water with or without 10 μg ml⁻¹ BFA for 1 h, at 25 °C, in the dark. During the last 30 min of incubation 8 μCi of D-[U-14C]glucose and 100 μCi of L-[4,5-3H]leucine were simultaneously added to the medium. Incorporation is expressed as a percentage of the total amount of the radioactivity measured in the homogenate. The data are from one representative experiment of three.

<table>
<thead>
<tr>
<th>Radioactivity (% of the homogenate)</th>
<th>10 μg ml⁻¹ BFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-[U-14C]glucose</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
</tr>
<tr>
<td>Cytosolic pool</td>
<td>40.9</td>
</tr>
<tr>
<td>Soluble polymers</td>
<td>1.1</td>
</tr>
<tr>
<td>Membrane</td>
<td>7.2</td>
</tr>
<tr>
<td>Cell wall</td>
<td></td>
</tr>
<tr>
<td>PAW-extract (proteins)</td>
<td>2.1</td>
</tr>
<tr>
<td>HCl hydrolysate (matrix polymers)</td>
<td>6.8</td>
</tr>
<tr>
<td>H₂SO₄ hydrolysate (cellulose)</td>
<td>3.9</td>
</tr>
<tr>
<td>Total radioactivity of the homogenate (kBq)</td>
<td>31.9</td>
</tr>
</tbody>
</table>
Fig. 2. Pattern of cell wall, membrane and cytosolic proteins extracted from BFA-treated and untreated pea root seedlings. Cell wall, membrane and cytosolic proteins were isolated from 1 cm long root segments obtained from 30 pea seedlings incubated in water with or without 10 \(\mu\)g ml\(^{-1}\) BFA for 1 h, at 25 °C in the dark. During the last 30 min of incubation 750 \(\mu\)Ci of L-[\(^{35}\)S]methionine were added in the medium. The amount of radioactive proteins on each lane is approximately 250,000 dpm. Standard protein molecular-weight markers (kDa) are on the left.

Discussion

The results show that in pea root seedlings BFA rapidly and markedly inhibits the synthesis of both cellulose and cell wall matrix polysaccharides without altering the synthesis and transport of newly synthesized proteins into the walls. Therefore under these experimental conditions the inhibitory effect of BFA on cell wall polysaccharides cannot be due to an inhibition of vesicular traffic to the wall, but to an indirect or direct effect on the activity of polysaccharide synthase complexes located at the membranes.

Driouich et al. (1993) working with sycamore suspension-cultured cells showed that, although the newly synthesized cellular proteins in sycamore cells were only slightly inhibited by BFA, their secretion into the culture medium was inhibited. The incorporation of \([^{3}H]\)xylose from UDP-d-[\(^{3}H]\)xylose and \([^{3}H]\)fucose into the hemicelluloses extracted from the cell wall of BFA-treated sycamore cells was also inhibited. The close correlation between the inhibitory effect of BFA on protein secretion into the culture medium and the reduced amount of radioactive sugars incorporated into the hemicelluloses extracted from the walls was taken as proof that BFA brought about its effects by an inhibition of the secretory pathway. However, using the same experimental conditions they showed that the incorporation of \([^{3}H]\)mannose, \([^{3}H]\)xylose from UDP-d-[\(^{3}H]\)xylose and \([^{3}H]\)fucose into the oligosaccharide chains of glycoproteins secreted within the cell or to the culture medium was differentially inhibited by BFA. They suggested that the drug acted on the synthesis and/or processing of N-linked glycans. However, Driouich et al. (1993) gave no explanation for the fact that the amount of newly synthesized glycoproteins secreted into the culture medium calculated as percentage with respect to newly synthesized cellular glycoproteins remained unchanged or was even stimulated in BFA-treated sycamore cells compared to that of control cells. Thus, if the inhibitory effect of BFA was mainly due to an inhibition of the vesicular traffic, as postulated by Driouich et al. (1993), it is difficult to explain why BFA did not alter but even stimulated the secretion of unglycosylated or incorrectly glycosylated proteins into the culture medium.

The results of this study show that, in pea root seedlings, cellulose and cell wall matrix polysaccharides are inhibited by BFA under experimental conditions in which it is possible to verify that (a) the synthesis and transport

<table>
<thead>
<tr>
<th>BFA</th>
<th>Membrane-bound enzyme</th>
<th>Digitonin-solubilized enzyme</th>
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<tbody>
<tr>
<td></td>
<td>Mannan-synthase</td>
<td>Glucomannan-synthase</td>
</tr>
<tr>
<td>—</td>
<td>3.80</td>
<td>0.40</td>
</tr>
<tr>
<td>10 (\mu)g ml(^{-1})</td>
<td>3.70</td>
<td>0.45</td>
</tr>
<tr>
<td>50 (\mu)g ml(^{-1})</td>
<td>3.75</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Table 4. Effect of BFA on membrane-bound and digitonin-solubilized mannan and glucomannan synthase isolated from the third internode of pea seedlings

Mannan- and glucomannan-synthase activity was measured as the amount of either \([^{14}\)C]mannose or \([^{14}\)C]glucose incorporated in the \([^{14}\)C]polymer. For the mannan-synthase activity the reaction mixture contained: 0.20 nmol of GDP-d-[\(^{14}\)C]mannose (approximately 1500 Bq), 2.5 nmol of GDP-d-mannose, BFA (10 or 50 \(\mu\)g ml\(^{-1}\)) and membrane enzymic preparation (180 \(\mu\)g of protein) or digitonin-solubilized enzymes (50 \(\mu\)g of protein). For the glucomannan-synthase activity the reaction mixture contained: 0.27 nmol of GDP-d-[\(^{14}\)C]glucose (approximately 1500 Bq), 1.0 nmol of GDP-d-glucose, 5.0 nmol of GDP-d-mannose, BFA (10 or 50 \(\mu\)g ml\(^{-1}\)) and membrane enzymic preparation (180 \(\mu\)g of protein) or digitonin-solubilized enzymes (50 \(\mu\)g of protein). Reaction time at 27 °C was 30 min. The data are from one representative experiment of three.
BFA: cell wall and protein synthesis

1931

IWWIMUWmmatM ^1

0 incubation time (min) 60

I I I I

15 20 30 60

recovery time (min)

H

2

O

BFA 10 |g ml-1

D-[U-14C] glucose

Fig. 3. Recovery of the biosynthesis of cell wall polysaccharides from pretreatment with BFA in pea root seedlings. 1 cm long pea root segments were isolated from 3 pea seedlings incubated with or without 10 \( \mu \)g ml\(^{-1}\) BFA for 60 min, in the dark at 25 °C. The recovery from the effect of BFA was studied by transferring seedlings in water for 15, 20, 30, and 60 min and adding 4 \( \mu \)Ci of D-[U-14C]glucose as radioactive tracer in the last 10 min of incubation as reported in the scheme. (A) Cellulose, (•) matrix polysaccharides. The data are from one representative experiment of three.

of newly synthesized proteins into the wall was not affected by the drug (Tables 2, 3); (b) the patterns of newly synthesized cytosolic, membrane-bound and cell wall proteins isolated from BFA-treated and control roots were similar (Fig. 2); (c) the inhibition of cellulose synthesis started at a concentration of BFA which did not affect the synthesis and transport of cell wall matrix polysaccharide into the wall. These data show that the inhibitory effect of BFA on cell wall polysaccharide synthesis started before any effect on the vesicular traffic of proteins to the walls. In support of this idea, Kimura et al. (1993) have demonstrated that the application of BFA to suspension-cultured rice cells inhibited the synthesis of O-linked saccharide chains (recognized by peanut lectin (PNA) and Ulex europaeus lectin-I (UEA-I)) of Golgi membrane glycoproteins. It was also shown that both the protein composition of Golgi membrane and the intracellular transport of \( \alpha \)-amylase molecules were not affected by BFA. It has also been reported that as a consequence of BFA treatment an altered \( O - \) and \( N - \)glycosylation occurred in mammalian and plant cells (Chawla and Hughes, 1991; Collins and Mottet, 1992; Sampath et al., 1992; Driouich et al., 1993; Gomez and Chrispeels, 1993).

The cellulose synthase complex of the plasma membrane is not only involved in the synthesis of \( \beta \)-glucan chains but is also implicated in their association into micro- and macro-fibrils. The complex has catalytic subunits for the synthesis of \( \beta \)-glucan chains and a highly organized number of structural and regulatory proteins which bring about the crystallisation of \( \beta \)-glucan chains into cellulose I (Northcote, 1991; Delmer and Amor, 1995). It was found that BFA, given simultaneously with a radioactive tracer, rapidly inhibited cellulose synthesis (Fig. 1D). It was also observed that \textit{in vitro} BFA did not change the activity of the membrane-bound and digitonin-solubilized mannan and glucosomannan synthase isolated from the third internode of pea seedlings. In animal systems also, BFA does not affect the sugar nucleotide transporters or the glycosyltransferases (Misumi et al., 1986). All the evidence indicates that the inhibitory effect of BFA on both cellulose and matrix polysaccharides formation is not at the catalytic site of the synthases.

Little is known about the structural and functional organization of polysaccharide synthase complexes in membranes but it is probable that, similar to the cellulose synthase complex, different proteins such as donor substrates, transferases and acceptor molecules are involved in the synthesis of cell wall matrix polysaccharides. These proteins must interact very closely with the phospholipids of membranes since \textit{in vitro} membrane-bound polysaccharide synthases lose their activity very rapidly in the presence of phospholipases (Piro et al., 1993). Since BFA is known to be a lipophilic molecule which interacts with membranes, the effect of BFA on the biosynthesis of cell wall polysaccharides could be caused by an interference of the drug with the topological organization of the synthase complexes in the membranes. This effect would precede the action of the drug at the level of vesicle transport of proteins to the walls. This would explain the differential sensitivity to the drug, at the Golgi apparatus, toward the synthesis of cell wall polysaccharides and the machinery of vesicular transport of proteins to the wall. A differential action of BFA on membrane compartments has been reported in animal and plant cells (Hunziker et al., 1991; Pelham, 1991; Gomez and Chrispeels, 1993).

The synthesis of glycosaminoglycan chains occurs in
most eukaryotic cells (Hardingham and Fosang, 1992). In
condrocytes of chondrosarcoma cells from the swarm rat,
the disruption of vesicular traffic by BFA rapidly inhibited
the synthesis of chondroitin sulphate, the major structural
components of proteoglycan aggrecan. However, the hyal-
uronic synthease complex, at the plasma membrane, was
much more stable and hyaluronan synthesis continued at
the same rate up to 8 h. The recovery of chondroitin
sulphate synthesis from the action of BFA after its removal
occurred in the presence of cycloheximide so that its
synthesis probably reassembled from previously existing
proteins (Calabro and Hascall, 1994a, b). These results, in
contrast to our results, indicated that BFA had no effect
on the glycosyltransferases involved in the chondroitin
sulphate and hyaluronan synthesis. It must be noted,
however, that the morphology and function of the Golgi
apparatus differ between plant and animal cells. It is also
established, as has already been indicated, that the action
of BFA varied not only with the concentration of the drug
applied to the tissue but even between different species of
plants. Differences between the action of BFA on plant
and animal cells might therefore be expected. It is possible
that a more complex organization of polysaccharide synth-
ases including the glycosyltransferases, is present on plant
membranes compared to that of animal cells. No poly-
saccharide synthases have been purified and characterized
from higher plants whereas several glycosyltransferases
have been purified and characterized in animal systems
(Paulson and Colley, 1989; Kleene and Berger, 1993).

The rapid inhibition of cellulose and matrix polysaccha-
ride synthesis by BFA in pea-root seedlings is paralleled
by a rapid recovery of the inhibitory effect when BFA is
removed so that BFA probably interacts non-covalently
with molecules such as lipids, proteins or both in an
hydrophobic environment. This would explain why the
effects of BFA on membranes are not only multi-targeted
(Pelham, 1991; Klausner et al., 1992), but also cell-type
specific (Hunziker et al., 1991; Satiat-Jeunemaitre and
Hawes, 1994). The many different responses to BFA both
biochemically and morphologically, may depend on the
unique lipid and protein composition of membrane com-
partments in plant and animal cells.

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