RESEARCH PAPER

1-Butanol targets the Golgi apparatus in tobacco BY-2 cells, but in a different way to Brefeldin A

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Abstract

The effects of 1-butanol on the organelles of the early secretory pathway in tobacco BY-2 cells have been examined, because this primary alcohol is known to interfere with phospholipase D an enzyme whose activity contributes to COPI-vesicle formation. Since the fungal lactone Brefeldin A (BFA) also prevents COPI-vesicle production by the Golgi apparatus, the sequential and simultaneous application of these two inhibitors was also investigated. 1-Butanol, but not 2-butanol caused rapid changes in the morphology of the BY-2 Golgi apparatus resulting in extended curved cisternae. By contrast with BFA-treated cells, ER cisternae did not attach laterally to these structures, and ER–Golgi fusion hybrids were not obtained with 1-butanol. However, immunofluorescence microscopy revealed that 1-butanol, like BFA, elicited the release of the GTPase ARF1 from Golgi membranes. Washing out the butanol resulted in re-attachment of ARF1 and a recovery of Golgi stack morphology. BY-2 cells treated sequentially with 1-butanol then BFA (each 30 min), did not reveal any BFA-typical changes in Golgi structure. Cells treated first with BFA, then 1-butanol retained the typical ER–Golgi sandwich morphology induced by BFA, but were larger. When 1-butanol and BFA were added together (for a 30 min period), even larger Golgi aggregates were formed with, again, no ER attachments. Thus, although both inhibitors had the Golgi apparatus as their principle cytological target and both interfere with coatamer attachment, they differ in their ability to induce an interaction with the ER.

Key words: ARF1, Brefeldin A (BFA), 1-butanol, BY-2 cells, Golgi structure.

Introduction

Phospholipase D (PLD; EC 3.1.4.4) hydrolyses phosphatidylethanolamine (PC) to produce phosphatidic acid (PA) and choline (Frohman and Morris, 1999). Whereas there is only one PLD gene in yeast and two in mammals (Liscovitch et al., 2000), higher plants possess 12 PLD genes (Wang, 2000; Elias et al., 2002). Of these, two (PLDf1 and PLDf2) have the PX (Phox) and PH (pleckstrin homology) domains typical of the yeast and mammalian enzymes. Both PLD and PA seem to be very important for a number of developmental processes in plants, for example, pollen tube growth, root hair growth and patterning, fruit ripening, seedling development, and leaf senescence (Munnik, 2001; Potocky et al., 2003; Testerink et al., 2004; Wang, 2005). Some of these effects may result from the participation of PLD/PA in hormone-mediated signalling pathways (Wang et al., 2000; Munnik, 2001; Romanov et al., 2002; Zhang et al., 2004; Li and Xue, 2007).

There is quite a large body of literature pointing to a role for PA in the maintenance of Golgi structure and function in mammalian cells (see Roth et al., 1999; De Matteis et al., 2005, for reviews). The importance of PA is reflected in the subcellular distribution of PLDs. By interacting with ADP-ribosylation factor (ARF-1), PLD1 has been shown to be recruited to Golgi membranes (Chen et al., 1997; Freyberg et al., 2001). PLD2 also associates with the Golgi apparatus, being located to the periphery where vesiculation occurs (Freyberg et al., 2002). Many of these studies use 1-butanol treatment to reduce the availability of PA, which is achieved through the transphosphatidylation activity of PLDs leading to the production of phosphatidylbutanol rather than PA. However, this effect is only possible when millimolar concentrations are present in order successfully to compete with...
water in the transphosphatidylation reaction (Roth et al., 1999). The Golgi apparatus rapidly fragments when mammalian cells are treated with 1% 1-butanol, and equally quickly reassembles when the primary alcohol is washed out (Siddhanta et al., 2000; Radulescu et al., 2007).

It is well established that COPI-vesicles are formed by the sequential recruitment of ARF1-GTP and the heptameric protein complex coatamer to Golgi membranes (Scheekman and Orci, 1996). Less well-known is that COPI proteins bind preferentially to membranes enriched in the acidic lipids PA and phosphatidylinositol (4,5) bisphosphate (PIP2) (Kiistakis et al., 1996). Although these lipids may additionally assist in altering the curvature of the membrane as coatamer is recruited, without ARF1 vesiculation is not possible (Spang et al., 1998). Nevertheless, the availability of these lipids does seem to be necessary for efficient COPI-vesicle production. Interestingly, there also appears to be a feedback loop between PIP2, PLD, and ARF1 (Roth et al., 1999; Siddhanta et al., 2000): PIP2 is a cofactor for PLD activity (Pertile et al., 1995), and it also stimulates ARF1 binding to Golgi membranes (Randazzo, 1997). However, ARF1 also acts as an activator for PLD (Kiistakis et al., 1995). Thus, inhibiting the formation of PA has both direct and indirect effects on coatamer recruitment and COPI-vesicle formation. It should also be mentioned that PA is not only required for COPI-vesicle formation, but also for later events in the secretory pathway of mammalian cells (Chen et al., 1997; Siddhanta and Shields, 1998), in particular, vesicle release at the trans Golgi (Siddhanta et al., 2000). Therefore, vesiculation of any kind in the Golgi apparatus stops as a result of 1-butanol treatment.

Information on particular cellular targets for PLD/PA action in plant cells is slowly becoming available. On the one hand, there is evidence for an interaction between PA and the cytoskeleton (Gardiner et al., 2001, 2003; Motes et al., 2005). In the case of the actin, PA binds to and inactivates a capping protein (ATCP) which enhances actin polymerization; conversely, inhibition of PA synthesis results in a disorganized actin network (Huang et al., 2006). On the other hand, recently published data point to a crucial role for PLD/PA in secretory and endocytic membrane trafficking in plants. Working with germinating pollen tubes, Monteiro et al. (2005) have shown that the inhibition of PA synthesis caused a serious reduction in the number of secretory vesicles accumulating at the growing tip, resulting in an inhibition of growth and loss of polarity. Li and Xue (2007) demonstrated that PLD2 and PA were essential for endocytic uptake and recycling of the auxin efflux transporter PIN2 in Arabidopsis roots. In addition, these authors showed that overexpression of PLD2 diminished considerably the cellular response towards Brefeldin A (BFA), a well-known perturbing of vesicle-mediated intracellular trafficking (Nebenführ et al., 2002; Geldner, 2004).

Using 1-butanol to inhibit PLD activity, it is shown here that, in suspension-cultured tobacco BY-2 cells, it is the Golgi apparatus which is the most severely affected of the endomembranes by the lack of PA. The effects are rapid and reversible, and appear to be related to the cessation of COPI-vesicle formation as judged by the release of Golgi-bound ARF1 into the cytosol. Golgi stacks undergo drastic morphological changes as a result of a 30 min 1% 1-butanol treatment, but these are not identical to those elicited by BFA, although BFA also leads to a release of ARF. Although prior treatment with 1-butanol prevents the formation BFA-induced ER–Golgi fusion hybrids, when added together with BFA, 1-butanol gives rise to large interconnected Golgi stack aggregates.

Materials and methods

Tobacco BY-2 (Nicotiana tabacum var. Bright Yellow 2) cells stably transformed with the Golgi marker GONST1-YFP (obtained from Dr Paul DuPree, Cambridge, UK; see also Baldwin et al., 2001) were cultivated by shaking in the dark in Murashige and Skoog’s medium at 25 °C on an orbital shaker (100 rev min⁻¹). The suspension-cultured cells were maintained in the log phase by subculturing weekly into fresh medium at a dilution of 1:50. Cells from 3-old cultures were treated with 1% (v/v) 1-butanol or BFA (10 μg ml⁻¹) for 30 min, 60 min, and 120 min.

Live cell imaging

A control sample of BY-2 cells was removed from the cultures before addition of the inhibitors. Further samples were removed after 30 min, 60 min, or 120 min 1-butanol or BFA treatments. Cells were allowed to settle down in an area bordered with a frame of plastic isolating tape and covered with a cover slip (24×32 mm). Cells were observed under a Zeiss Axiosvert CLSM 510 Meta microscope using a single tracking mode and a C-Apochromat 63×/1.2 W water immersion objective with a 1 Airy Unit (121 μm) pinhole. An excitation wavelength of 514 nm was used. The fluorescence was detected with the Meta detector in an emission range between 530–550 nm.

Determination of Golgi stack number

Living cells were observed under the same conditions as described in ‘Live cell Imaging’. Golgi stacks were 3-D reconstructed from 50–80 serial images with an x, y, z scaling of 0.14×0.14×0.48 μm using ImageJ (1.37v), saved as tiff-files and loaded into Huygens Essential 3.3.0p3 (Scientific Volume Imaging BV, Netherlands). The number of Golgi stacks was then determined using the ‘Object Analyzer’ tool. Threshold, Seed (% thr), and Garbagevol were set individually for each cell using the 3-D reconstruction (performed with the Zeiss LSM Image Browser 3.5.0.376) as an optical standard.

Immunofluorescence labelling with ARF1 antibodies

Control and 1-butanol-treated samples were fixed with 1.5% (v/v) glutaraldehyde in culture medium for 15 min at room temperature. Further processing was performed exactly as given previously in Ritzenhauer et al. (2002). For immunostaining, cells were first incubated with ARF1 antibodies (Pimpl et al., 2000) at a primary dilution of 1:100 in phosphate-buffered saline for 16 h, and after washing were then incubated for 2 h with Alexa-fluor 543 goat anti-rabbit IgG (Molecular Probes Leiden, Netherlands) diluted 1:100.
Cells were observed under the LSM 510 Meta microscope using a multi-tracking mode and a C-Apochromat 63×/1.2 W water immersion objective with a pinhole of 1 Airy Unit (133 μm/138 μm). Excitation wavelengths of 514/543 nm were used. The fluorescence was detected with the Meta detector in an emission range between 529–550 nm and 582–625 nm.

Electron microscopy

Samples were removed for electron microscopy at the times indicated, pelleted and resuspended in a primary fixative containing glutaraldehyde and picric acid as given in Ritzenthaler et al. (2002). All other processing steps were identical to those given in Ritzenthaler et al. (2002). Sections were observed in a Philips CM 10 electron microscope operating at 80 kV.

Results

1-Butanol elicits changes in the structure and morphology of the BY-2 Golgi apparatus which are different to those induced by BFA

The addition of 1-butanol to a final concentration of 1% (a typical concentration employed in studies on mammalian cells, for example, Radulescu et al., 2007), to log-phase growing tobacco BY-2 cells expressing the fluorescent Golgi marker GONST1-YFP (Tse et al., 2004) did not bring about any obvious change in the distribution of Golgi stacks over a 2 h period (Fig. 1A–C). This is in clear distinction to BFA (10 μg ml⁻¹), which rapidly causes Golgi stacks to collect around the immediate peri-nuclear vicinity and to form large aggregates (Fig. 1G–I). Although Golgi stack numbers per cell varied considerably according to cell size, their numbers remained essentially unchanged after 1-butanol treatment, in distinct contrast to BFA (Fig. 1D–F; Table 1).

At the level of the electron microscope, significant changes in the ultrastructure of the Golgi stacks in cells treated with 1% butanol for 30 min were registered. Stacks were no longer flat, but curved with cisternae extended up to twice their normal length (Fig. 2C). As a result of this curvature, Golgi stacks often gave the appearance of concentric cisternal circles (Fig. 2D). Fusions between adjacent stacks were also often seen (Fig. 2C). Similar aberrant Golgi structures have frequently been reported in

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Fig. 1. Comparison of the effects of 1-butanol (1%) and BFA (10 μg ml⁻¹) on the distribution and number of the Golgi stacks in tobacco BY-2 cells expressing GONST1-YFP. (A, D) Untreated cell; (B, E) cell treated for 60 min with 1-butanol; (C, F) cell treated for 2 h with 1-butanol; (G) cell treated for 30 min with BFA; (H) cell treated for 60 min with BFA; (I) cell treated for 2 h with BFA. (A–C, G–I) Median optical sections. (D–F) 3-D reconstructions.
the plant literature, especially in cells subjected to energy
depletion (e.g. Robinson and Ray, 1977). This structural
response was most different to that induced by BFA over
the same time period, which involves the production of
ER–Golgi hybrid structures in which trans-like Golgi
cisternae become sandwiched between ER cisternae (see
Ritzenthaler et al., 2002; Robinson and Ritzenthaler, 2006).
Control incubations with 2- and 4-butanol (1%) did not
generate any structural changes in the Golgi apparatus of
BY-2 cells (Fig. 2B).

1-Butanol releases ARF1 from BY-2 Golgi stacks, and
its effects are reversible

In order to determine whether COPI vesicle production
was prevented by 1-butanol, immunofluorescence label-
ing was performed with ARF1 antibodies (Pimpl et al.,
2000) on GONST1-YFP BY-2 cells. Control cells showed
an almost perfect colocalization of the ARF1- and
GONST1-YFP signals (Fig. 3A–C). Treatment with 1-
butanol (1%) caused the ARF1 to dissociate from the
Golgi stacks and give rise to a diffuse cytosolic signal
after 60 min (Fig. 3D–I). Release of Golgi-associated
ARF1 begins after 15 min, but is more clearly recognized
after 30 min 1-butanol treatment (Fig. 3F–I). Thus, the
kinetics of ARF1 release is similar to that observed when
BFA is given to BY-2 cells. BY-2 cells treated with 1%
butanol for 30 min and then briefly washed by 2-fold cen-
trifugation and resuspension in fresh culture medium,
showed after 2 h the reattachment of ARF1 to GONST1-
YFP labelled Golgi stacks (Fig. 3J, K). Cells fixed and
processed for electron microscopy after this treatment
procedure revealed Golgi stacks which were indistinguish-
able from control cells (data not shown). Therefore like
BFA, 1-butanol causes Golgi cisternae to enlarge pre-
sumably by preventing COPI vesicle formation for which
ARF1 is required.

Table 1. Effects of 1-butanol (1% v/v) and BFA (10 μg ml⁻¹)
on the numbers of Golgi stacks in tobacco BY-2 cells

<table>
<thead>
<tr>
<th>Time of treatment (min)</th>
<th>BFA</th>
<th>1-Butanol</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cell 1</td>
<td>Cell 2</td>
</tr>
<tr>
<td>0</td>
<td>615</td>
<td>631</td>
</tr>
<tr>
<td>30</td>
<td>102</td>
<td>130</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>74</td>
</tr>
<tr>
<td>120</td>
<td>97</td>
<td>114</td>
</tr>
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</table>

a Golgi stack numbers were determined on CLSM 3-D reconstruc-
tions of individual cells using the Huygens Essential 3.3.0p3 ‘Object
Analyzer’ tool.

Fig. 2. Comparison of the effects of 1-butanol and 2-butanol (1%) on the ultrastructure of the BY-2 Golgi apparatus. (A) A Golgi stack from an
untreated cell clearly showing parameters of polarity from cis (bottom) to trans (top); (B) a Golgi stack from a cell treated with 2-butanol for 30 min;
(C, D) Golgi stacks perturbed by treatment with 1-butanol for 30 min.

Sequential and synergistic effects of 1-butanol and
BFA on the Golgi apparatus of BY-2 cells

Since 1-butanol and BFA have different effects on the
structure of the Golgi apparatus of BY-2 cells, it was de-
cided to see if the distinctive features induced by BFA
were still possible to induce after cells had initially been
subjected to 1-butanol. As seen in Fig. 4A, this was not the case: an attachment of ER cisternae to the curved, enlarged cisternae formed as a result of 1-butanol treatment was not observed. On the other hand, when BY-2 cells were treated with 1-butanol subsequent to BFA, the typical ‘ER–Golgi sandwich’ was still observed and did not disassemble (Fig. 4B). In fact, the length of the Golgi cisternae in these hybrid structures measured on the average some 40 ± 6% longer. It was then decided to determine which had the more dominant effect on Golgi structure by presenting 1-butanol and BFA simultaneously to BY-2 cells. Intriguingly, while the face-wise attachment

Fig. 3. 1-butanol causes the release of ARF1 from Golgi stacks. (A–C) Untreated BY-2 cells showing colocalization of ARF1 (red signal) to Golgi stacks (GONST1-YFP – green signal). (D, E) Cells treated for 30 min or 60 min with 1-butanol (1%). The red (ARF1) signal is lost from the Golgi stacks, and accumulates in the cytoplasm. (F–I) Gradual loss of the ARF1 signal during 1-butanol treatment. (J, K) Recovery from a 30 min 1-butanol treatment after 2 h washout. Golgi stacks with associated ARF1 are again visible.
of ER cisternae to Golgi cisternae was prevented by this combined treatment, the curved Golgi stacks typical for 1-butanol were not observed (Fig. 5). Instead, impressive Golgi fusion profiles were obtained with either large numbers of cisternae (Fig. 5A), or with individual cisternae linking together up to three stacks (Fig. 5B). The cisternae in these aggregates lacked a clear polarity and had a more trans-like appearance and staining intensity. Moreover, intercisternal filaments were visible between most cisternae.

**Discussion**

Our results on BY-2 cells strongly support a key role for PA in Golgi function in plant cells, in the sense that 1-butanol treatment leads to a dissociation of Golgi-bound ARF1 and therefore to the cessation of COPI-vesicle formation. As with mammalian cells, 1-butanol treatment also results in severe structural modifications of the Golgi apparatus which are different from those caused by BFA. However, the changes induced by 1-butanol treatment in the Golgi apparatus of BY-2 cells are not the same as those exhibited by mammalian cells: short-term exposure to 1-butanol does not lead to the complete fragmentation of the Golgi apparatus in BY-2 cells. Instead, the Golgi cisternae which survive this treatment grow in length considerably and have a uniform trans-like morphology. This effect is even exacerbated by the simultaneous addition of BFA. It is assumed that the growth of the cisternae is a consequence of a continued flow of membrane from the ER in the absence of a COPI-based retrograde transport.

The two Golgi inhibitors are, nevertheless, clearly not identical in their action. Ritzenthaler et al. (2002) and Nebenführ et al. (2002) tried to explain the BFA-induced fusion of ER and Golgi cisternae in terms of unspecific SNARE–SNARE interactions. They based their interpretation on an earlier paper by Elazar et al. (1994) which pointed to the sequestration of SNARE molecules as being the underlying fusion potential of COPI vesicles. Prevention of COPI-vesicle formation through BFA treatment would, therefore, cause cognate SNAREs to distribute themselves randomly in the membranes of the Golgi apparatus and ER, culminating in unspecific membrane fusions. However, this interpretation is insufficient to...
explain the curious sandwiching of residual trans-like Golgi cisternae by the ER which is typical of short-term BFA treatments in BY-2 cells. Moreover, if these effects are solely the consequence of the inhibition of an ARF1-mediated recruitment of coatomer, why are they not elicited by 1-butanol which also appears to interfere with COPI-vesicle formation? An obvious answer is not clear, but clearly reducing the availability of PA is not enough to cause the redistribution of Golgi membranes into the ER, neither in BY-2 cells nor in mammals.

Because of their rapid and distinctive response to BFA, BY-2 cells are regarded by some plant scientists as model organisms for studies on the secretory pathway in plants. Immunolabelling with ARF1 and various COPI antisera are restricted to the Golgi apparatus in this organism (Pimpl et al., 2000; Robinson and Ritzenthaler, 2006), and the formation of a so-called ‘BFA-compartment’ in response to BFA treatment is difficult to register (Ritzenthaler et al., 2002). However, it should be pointed out that these features are by no means shared by all higher plants. With Arabidopsis, for example, there is no effect of BFA on Golgi structure or membrane association of ARF1, or the COPI subunit γ-COP (Geldner et al., 2001, 2003). Instead, BFA targets an ARF-GEF located on a recycling endosome and results in the formation of large ‘BFA-compartments’ (Steinman et al., 1999; Geldner et al., 2003). This significant difference appears to relate to the existence of BFA-sensitive as against BFA–insensitive Golgi-localized ARF-GEFs in tobacco and Arabidopsis (Richter et al., 2007). Bearing this in mind, it would therefore be most interesting to investigate at the ultrastructural level the effects of 1-butanol on PIN-recycling in Arabidopsis roots as recently reported on by Li and Xue (2007).

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References


