Effects of brefeldin A on sphingomyelin transport and lipid synthesis in BHK21 cells

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1. Addition of brefeldin A (BFA) to BHK cells incubated for 4 h with [3 H]acetate led to a 3–4-fold increase in incorporation of label into sphingomyelin, monoglucosylceramide and cholesterol ester compared with untreated controls. There was a similar increase in incorporation of [3 H]choline into sphingomyelin. The level of cholesterol ester increased 3-fold when BFA was added to cells labelled to equilibrium with [3 H]acetate, but no statistically significant changes in the levels of other lipids were seen. 2. BFA appeared to act by diverting incorporation of acetate into sphingolipids and cholesterol ester at the expense of phosphatidylcholine (decreased by up to 15%), cholesterol (decreased by 30–40%) and triacylglycerol (decreased by 35–50%). 3. Forskolin (100 μ M) prevented the changes in labelling induced by 0.25 μ g of BFA/ml, but in the presence of 1 μ g of BFA/ml it had no effect on sphingomyelin

and triacylglycerol labelling and only partly blocked the effects of BFA on labelling of cholesterol and cholesterol ester. 4. None of the labelled sphingomyelin was degraded in BFA-treated cells which were subsequently exposed to an extracellular sphingomyelinase, showing that all the newly synthesized sphingomyelin remained inside the cells. Determinations of phospholipid phosphorus in unlabelled cells confirmed that, in the presence of BFA, no newly synthesized sphingomyelin was able to reach the cell surface, supporting the idea that sphingomyelin normally depends on vesicular transport for its passage to the plasma membrane. 5. The results are consistent with the hypothesis that cholesterol synthesis and esterification processes in BHK cells are sensitive to the plasma-membrane deficit of sphingomyelin caused by BFA.

INTRODUCTION

The fungal toxin brefeldin A (BFA) prevents vesicular transport of proteins to the cell surface by promoting retrograde transport of Golgi components back to the endoplasmic reticulum [1,2]. This effect of BFA, which results in the fusion and dispersion of these normally discrete organelles, is apparently related to its action in detaching a specific 110 kDa protein (β COP) from the Golgi membrane [3,4], although BFA may have additional effects on other compartments, including the *trans*-Golgi network and endosomes [5,6]. BFA also blocks the synthesis of certain complex glycolipids [7–9], and this has been explained on the basis that BFA prevents the vesicular transport of simpler glycolipids from their site of synthesis in the Golgi to the *trans*-Golgi network, which is presumed to be the site of the terminal glycosylations forming mature gangliosides.

There are several reports indicating that synthesis of sphingomyelin, which like glycolipids is mainly localized at the cell surface, occurs in the *cis*-Golgi compartment [10–15], and there is some evidence that it then undergoes vesicular transport to the surface [16–18]. However, we have recently shown that the sphingomyelin of BHK cells is divided into two metabolically distinct pools, the larger one at the surface and another accounting for about one-third of the total at an intracellular site which does not mix with the surface pool [19]. We have pointed out that the evidence for localization of sphingomyelin synthesis in the *cis*-Golgi is more likely to relate to the internal pool than to the surface pool of sphingomyelin in BHK cells.

We have now examined the effects of BFA as a known inhibitor of vesicular transport on the synthesis of sphingomyelin and its delivery to the cell surface. Our results show that BFA does prevent transport of sphingomyelin to the surface, but also

demonstrate that it greatly increases synthesis of sphingomyelin, glucosylceramide and cholesterol ester at the same time as diminishing labelling of cholesterol, triacylglycerol and phosphatidylcholine.

MATERIALS AND METHODS

Cell culture and incubation

BHK 21 cells were cultured in 3.5 cm-diam. Falcon dishes with 2 ml of Glasgow Minimal Essential Medium (MEM) supplemented with 5% foetal-calf serum and tryptose phosphate broth as described previously [20]. For short-term labelling experiments subconfluent cells were incubated for up to 6 h in the presence of 20 μ Ci of [3 H]acetate (NEN Dupont)/dish or 5 μ Ci of [3 H]choline (Amersham International)/dish, with or without addition of BFA (1 μ g/ml). After replacement of the medium with serum-free Glasgow MEM, treatment with *Bacillus cereus* sphingomyelinase (Sigma Chemical Co.; 0.1 unit/ml for 20 min) was used routinely to measure the amount of labelled sphingomyelin which was present on the cell surface. To terminate the incubations, the medium was removed and 1.9 ml of methanol/chloroform (2:1, v/v) was added. In other experiments, incorporation of [3 H]acetate was measured when the amount of BFA was varied between 0 and 5 μ g/ml and cells were extracted after incubation for 4 h.

The ability of forskolin (100 μ M, added from a 100 mM stock in dimethyl sulphoxide; Sigma) to block the effects of BFA (either 0.25 or 1 μ g/ml) was tested. Forskolin was added 1 h before addition of [3 H]acetate and BFA. Cells were then incubated for 4 h and extracted as above.

Cells were also labelled to equilibrium with 5 μ Ci of [3 H]acetate or 2 μ Ci of [3 H]choline per dish for 48 h, under conditions where

Abbreviation used: BFA, brefeldin A.

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they had almost reached confluence after approximately four doublings. Cells were washed twice and incubated in non-radioactive medium for 2 h before washing again. The BFA ($2 \mu\text{g/ml}$) was added to the medium in half of the dishes, and incubation was continued for a further 4 h. At the end of this period, the medium was removed and a sample taken for scintillation counting before extraction of the cells with chloroform/methanol as above.

Experiments were also carried out using unlabelled cells grown almost to confluence in 10 cm Falcon dishes so that determinations of phospholipid phosphorus could be made [21]. Dishes were incubated for 5 h with or without addition of BFA ($2 \mu\text{g/ml}$), and half of the samples were treated with sphingomyelinase (0.1 unit/ml) for the last 20 min of the incubation.

Lipid analysis

The extracts of cell lipid were transferred to glass test tubes, and 0.6 ml of chloroform and 1.1 ml of 0.9% NaCl were added. The mixture was vortex-mixed and centrifuged to give two phases in accordance with the procedure of Bligh and Dyer [22]. In the case of the choline-labelled cells, the radioactivity in the upper (aqueous) phase (corresponding to the aqueous fraction of the cells) was also measured and a sample was analysed for water-soluble choline metabolites by paper chromatography [19]. The lower (chloroform) phase containing the lipids was dried under vacuum in a desiccator. Phospholipids and neutral lipids were separated by t.l.c. as described previously [20,23] and identified by comparison with standards (Sigma). In order to resolve reliably triacylglycerol from cholesterol ester and diacylglycerol from cholesterol, neutral lipid plates were also run in the solvent hexane/diethyl ether/acetic acid (155:45:2, by vol.). R_F values in this solvent were 0.92 for cholesterol ester, 0.58 for triacylglycerol, 0.28 for fatty acid, 0.10 for cholesterol, and 0.05 for diacylglycerol. The distribution of radioactivity in cholesterol ester was analysed by eluting the spot with 1 ml of methanolic NaOH and then hydrolysing the material for 2 h at 60°C . After acidification and extraction into light petroleum (b.p. $60\text{--}80^\circ\text{C}$), the dried residue was re-run in the same solvent and the spots corresponding to cholesterol, cholesterol ester, fatty acid and fatty acid methyl ester were counted for radioactivity. Under the conditions used, > 95% of the cholesterol ester radioactivity was recovered in cholesterol, non-esterified fatty acid and fatty acyl methyl ester.

In some experiments, total lipid extracts were subjected to alkaline methanolysis [24] to degrade glycerolipids, and the sphingolipids which remained were separated on t.l.c. plates in solvents consisting of chloroform/methanol/water (35:15:2, by vol.) or chloroform/methanol/acetic acid/water (75:45:12:4, by vol.). Spots were stained with iodine vapour and identified by comparison with authentic standards, which included sphingomyelin, galactosylceramide, lactosylceramide and ganglioside GM3 (Sigma). Spots identified by iodine staining were transferred to Pico Prias counting vials (Canberra Packard) to which were added 0.2 ml of methanol/water/acetic acid (5:3:2, by vol.) and 2 ml of Ultima Gold scintillation fluid. Samples were counted for radioactivity in a Canberra Packard 2500 scintillation counter.

RESULTS

When cells were incubated with [^3H]acetate for up to 4 h, radioactivity was incorporated into lipids as shown in Figure 1. Inclusion of BFA in these incubations did not significantly affect

total incorporation of label, but caused dramatic changes in the distribution of radioactivity (Figure 2, Table 1). Thus after 4 h there were large increases in label entering sphingomyelin, monoglucosylceramide and cholesterol ester (which were labelled 3–4 times as much as in control cells) and corresponding decreases in labelling of cholesterol (by 30–40%) and triacylglycerol (by 35–50%). A decrease in labelling of phosphatidylcholine (up to 15%) was also seen, but no significant changes in labelling were observed in phosphatidylserine, phosphatidylinositol or phosphatidylethanolamine. A 40% fall in labelling of ceramide was also seen consistently. The rise in radioactivity in sphingomyelin, monoglucosylceramide and cholesterol ester was approximately balanced by the fall in radioactivity in cholesterol, phosphatidylcholine and triacylglycerol. These effects of BFA were seen at concentrations as low as $0.25 \mu\text{g/ml}$ and were maximal at $1 \mu\text{g/ml}$ (Figure 2).

Addition of forskolin ($100 \mu\text{M}$) blocked the changes in labelling produced by $0.25 \mu\text{g}$ of BFA/ml (Table 2). However, in the presence of $1 \mu\text{g}$ of BFA/ml, forskolin had little effect on the changes in labelling of triacylglycerol or sphingomyelin, and its effect on changes in labelling of cholesterol and cholesterol ester was decreased by over 70%. Addition of forskolin alone had no significant effect on labelling of the lipids.

Incorporation of [^3H]choline into sphingomyelin was also increased 3–4-fold in cells exposed to BFA (Figure 3), although no changes were seen in labelling of either phosphatidylcholine or the water-soluble fraction of the cells. Neither were any significant changes seen in the individual choline metabolites (free choline, glycerol-3-phosphocholine, phosphocholine or CDP-choline) in the water-soluble fraction, where 95% of the radioactivity chromatographed as phosphocholine (results not shown). In the experiment shown in Figure 3, the total radioactivity in the cells at 6 h was 457437 ± 16259 d.p.m. in controls and 522896 ± 20125 d.p.m. in BFA-treated samples (statistically significant, $P < 0.02$). Thus the increment of label in sphingo-

Figure 1 Incorporation of [^3H]acetate into lipids of BHK cells

Cells were incubated for up to 4 h with [^3H]acetate, and lipids were extracted and analysed as described in the Materials and methods sections. Values shown are means $\pm$ S.D. for triplicate samples in one experiment which was repeated on two further occasions with substantially the same results. ●, Phosphatidylcholine; ○, sphingomyelin; ▲, cholesterol.

Figure 2 Effect of BFA concentration on incorporation of [^3H]acetate into lipids of BHK cells

Cells were incubated for 4 h with [^3H]acetate in the presence of different concentrations of BFA. Lipids were extracted and analysed as described in the Materials and methods section. Values shown represent means $\pm$ S.D. for triplicate samples in one experiment, which was repeated on two further occasions with virtually the same results. (a) ●, Phosphatidylcholine; ○, sphingomyelin; ▼, cholesterol. (b) ▽, Triacylglycerol; ▲, cholesterol ester; ■, phosphatidylethanolamine.

Table 1 Incorporation of [^3H]acetate into BHK cells exposed to BFA and/or sphingomyelinase

Incorporation of [^3H]acetate was measured at 4 h in the absence or presence of 2 μg /ml of BFA/ml. Sphingomyelinase (SMase; 0.1 unit/ml) was added to half of the samples during the last 20 min of the incubation as described in the Materials and methods section. Values represent means $\pm$ S.D. of the percentage of the total counts from quadruplicate samples. The results shown here were confirmed in three other experiments under the same conditions. ND, not determined. Significantly different from controls: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Abbreviations used in this and subsequent Tables: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; CHOL, cholesterol; CHOL. EST., cholesterol ester; CER, ceramide; DAG, diacylglycerol; TAG, triacylglycerol; GCER, monoglycosylceramide; NEFA, non-esterified fatty acid; FAME, fatty acid methyl ester.

	Control	+ SMase	+ BFA	+ BFA + SMase
SM	4.9 $\pm$ 0.1	3.1 $\pm$ 0.4**	21.1 $\pm$ 0.9***	21.6 $\pm$ 1.3
PC	43.6 $\pm$ 0.6	43.6 $\pm$ 1.5	37.5 $\pm$ 1.8*	35.5 $\pm$ 1.4
PS/PI	3.6 $\pm$ 0.1	4.2 $\pm$ 0.4	4.4 $\pm$ 0.4	4.5 $\pm$ 0.7
PE	9.1 $\pm$ 0.6	9.0 $\pm$ 0.1	9.1 $\pm$ 0.5	9.1 $\pm$ 0.3
CHOL	23.7 $\pm$ 2.2	22.8 $\pm$ 1.9	13.6 $\pm$ 1.7**	15.2 $\pm$ 0.5
CER	4.2 $\pm$ 0.7	6.9 $\pm$ 0.5**	2.4 $\pm$ 0.2**	3.5 $\pm$ 0.3
TAG	7.3 $\pm$ 1.3	8.6 $\pm$ 0.5	5.7 $\pm$ 1.2	6.1 $\pm$ 1.3
GCER	1.2 $\pm$ 0.1	1.1 $\pm$ 0.2	4.0 $\pm$ 0.2***	3.7 $\pm$ 0.3
CHOL. EST.	0.6 $\pm$ 0.1	ND	2.4 $\pm$ 0.3***	ND
Total d.p.m. ...	34 645 $\pm$ 3833	43 191 $\pm$ 8022	33 007 $\pm$ 2751	33 998 $\pm$ 3308

Table 2 Effect of forskolin in reversing BFA-induced changes in incorporation of [^3H]acetate

Cells were incubated for 4 h in 3.5 cm dishes in the presence of [^3H]acetate and BFA and/or forskolin as described in the Materials and methods section. Results are given as percentages of the total lipid radioactivity and are expressed as means $\pm$ S.D. from triplicate determinations in one experiment which was repeated twice with substantially the same results. Abbreviations as in Table 1.

Forskolin (μM) ...	0	0	0	100	100	100
BFA ($\mu\text{g}/\text{ml}$) ...	0	0.25	1	0	0.25	1
SM	6.3 $\pm$ 0.3	10.7 $\pm$ 0.2	16.4 $\pm$ 0.3	7.9 $\pm$ 0.5	7.4 $\pm$ 0.4	18.9 $\pm$ 0.3
PC	35.6 $\pm$ 0.9	35.1 $\pm$ 0.8	33.3 $\pm$ 0.4	36.3 $\pm$ 0.5	36.4 $\pm$ 0.6	33.4 $\pm$ 0.6
PS/PI	8.5 $\pm$ 0.3	9.6 $\pm$ 0.3	8.6 $\pm$ 0.2	7.4 $\pm$ 0.5	7.9 $\pm$ 0.1	8.8 $\pm$ 0.3
PE	9.5 $\pm$ 0.3	10.5 $\pm$ 0.4	9.7 $\pm$ 1.0	9.2 $\pm$ 0.3	10.4 $\pm$ 0.8	10.2 $\pm$ 0.8
CHOL	18.9 $\pm$ 0.3	11.7 $\pm$ 0.9	12.3 $\pm$ 0.6	18.1 $\pm$ 2.8	18.9 $\pm$ 0.4	14.6 $\pm$ 1.3
TAG	8.2 $\pm$ 0.1	6.5 $\pm$ 0.2	3.5 $\pm$ 0.1	9.3 $\pm$ 1.6	7.1 $\pm$ 0.4	3.4 $\pm$ 0.1
CHOL. EST.	0.7 $\pm$ 0.1	3.7 $\pm$ 0.2	3.7 $\pm$ 0.2	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	1.6 $\pm$ 0.1

Figure 3 Incorporation of [^3H]choline by BHK cells in the presence or absence of BFA

Cells were incubated with [^3H]choline for up to 6 h in the absence (●, ▲) or presence (○, △) of BFA (5 $\mu\text{g}/\text{ml}$) and lipids were extracted and analysed as described in the Materials and methods section. Values shown represent means $\pm$ S.D. for triplicate samples in one experiment which was repeated on two further occasions with virtually the same results. (a) Phosphatidylcholine; (b) sphingomyelin.

myelin seen in the presence of BFA appeared to represent a net increase in uptake of choline into the cells and not a redistribution of label.

Treatment of control cells with sphingomyelinase at the end of the period of incubation with [^3H]acetate caused about a 40% decrease in sphingomyelin radioactivity and an equivalent rise in ceramide radioactivity (Table 1). However, in cells treated with

Table 3 Effects of BFA and sphingomyelinase on sphingomyelin content of BHK cells

Cells approaching confluence in 10 cm dishes were incubated for 5 h with or without BFA in complete growth medium. After replacement of the medium with 5 ml of serum-free Glasgow MEM, 0.5 unit of sphingomyelinase (SMase) was added to half of the dishes, and lipids were extracted and analysed as described in the Materials and methods section. Values represent (a) means $\pm$ S.D. of mol% of total phospholipids from triplicate samples; (b) nmol of total phospholipid or sphingomyelin. Surface sphingomyelin is calculated as the amount which is broken down by sphingomyelinase. The results shown here were confirmed in three other experiments under the same conditions. Control (0 h) refers to samples which were extracted immediately before the 5 h incubation period. PL is the total phospholipid in the samples (nmol of phosphorus). SM(T) is total sphingomyelin in cells; SM(S) is surface sphingomyelin calculated as SM(T) minus sphingomyelin remaining after treatment with sphingomyelinase. Significantly different from sphingomyelinase-treated control: * $P < 0.01$. Abbreviations as in Table 1.

	Control (0 h)	Control (5 h)	+ SMase (5 h)	+ BFA (5 h)	BFA + SMase (5 h)
(a) Percentage of total phospholipid					
SM	7.5 $\pm$ 0.1	7.5 $\pm$ 0.3	2.8 $\pm$ 0.2	7.9 $\pm$ 0.1	4.3 $\pm$ 0.2*
PC	48.0 $\pm$ 1.0	48.1 $\pm$ 0.6	52.1 $\pm$ 0.3	44.9 $\pm$ 0.3	47.8 $\pm$ 0.6
PS/PI	13.1 $\pm$ 0.8	13.4 $\pm$ 0.2	14.1 $\pm$ 0.2	14.5 $\pm$ 0.2	15.4 $\pm$ 0.2
PE	26.5 $\pm$ 0.2	27.1 $\pm$ 0.9	27.1 $\pm$ 0.2	28.4 $\pm$ 0.3	27.9 $\pm$ 0.9
CL	4.8 $\pm$ 0.2	3.9 $\pm$ 0.1	3.9 $\pm$ 0.2	4.7 $\pm$ 0.3	4.4 $\pm$ 0.2
(b) nmol of phospholipid					
PL	185 $\pm$ 7	258 $\pm$ 10	256 $\pm$ 12	246 $\pm$ 11	240 $\pm$ 15
SM(T)	13.9	19.3	7.2	19.4	10.3
SM(S)	8.8	12.1		9.1	

BFA there was no significant loss of label from sphingomyelin and only a small increase in labelled ceramide after the sphingomyelinase treatment. This demonstrated that the sphingomyelin showing enhanced labelling was almost entirely localized to internal pools, and implied that BFA inhibited the appearance at the surface of the bulk of the newly synthesized sphingomyelin.

This conclusion was confirmed by experiments to measure the quantity of sphingomyelin which reached the surface with or without addition of BFA. Determinations of phospholipid phosphate on unlabelled cells demonstrated that sphingomyelin accounted for 7.5% of total phospholipid in untreated cells, equivalent to 13.9 nmol of sphingomyelin in the experiment shown in Table 3. Treatment with exogenous sphingomyelinase showed that 63%, or 8.8 nmol, was located on the cell surface. Further incubation for 5 h led to an approx. 40% increase in the total amount of phospholipid and correspondingly in sphingomyelin, the level of which rose to 19.3 nmol in control and BFA-treated cells. In control cells breakdown remained at 63% (12.1 nmol) (Table 3). However, after 5 h exposure to BFA only 48% (9.1 nmol) of the cell sphingomyelin were degradable by sphingomyelinase, an amount virtually unchanged from the original value. Thus no new sphingomyelin had reached the surface during the 5 h incubation with BFA.

BHK cells labelled to equilibrium with [3 H]acetate and incubated in non-radioactive medium (Table 4) showed a distribution of phospholipid radioactivity which resembled the mass distribution as measured by phosphate determinations (Table 3). Although no direct determinations of mass were made on neutral lipids, it was assumed that the masses of each of these lipids bore the same relationship to their radioactivity as for the phospholipids. When the cells were treated with 5 μ g of BFA/ml

Table 4 Distribution of radioactivity in BHK cells labelled to equilibrium with [3 H]acetate or [3 H]choline and exposed to BFA in non-radioactive medium

Cells were incubated for 48 h with [3 H]acetate or [3 H]choline, washed with non-radioactive medium and treated with 2 μ g of BFA/ml for 4 h as described in the Materials and methods section. Values represent (a) means $\pm$ S.D. of percentages of total lipid radioactivity from quadruplicate samples and (b) the percentage of total phospholipid label present in the individual phospholipids. For the choline-labelled cells, the radioactivity in the medium and in the intracellular aqueous fraction (CELL AQU.) were also measured. The total d.p.m. per dish $\pm$ S.D. is quoted. The results shown here were confirmed in three other experiments under the same conditions. Significantly different from controls: * $P < 0.01$. Abbreviations as in Table 1.

	Control		+ BFA	
	(a)	(b)	(a)	(b)
Acetate label				
SM	6.3 $\pm$ 0.3	9.6	6.6 $\pm$ 0.2	10.1
PC	32.5 $\pm$ 1.1	49.6	32.4 $\pm$ 1.1	49.8
PS/PI	7.8 $\pm$ 0.6	11.9	8.2 $\pm$ 0.3	12.6
PE	15.5 $\pm$ 0.2	23.7	14.8 $\pm$ 0.5	22.7
CL	3.4 $\pm$ 0.3	5.2	3.1 $\pm$ 0.4	4.8
CHOL. EST.	1.1 $\pm$ 0.1		3.0 $\pm$ 0.3*	
CHOL	22.4 $\pm$ 0.8		20.4 $\pm$ 0.7	
CER	0.38 $\pm$ 0.07		0.26 $\pm$ 0.03	
NEFA	0.25 $\pm$ 0.03		0.24 $\pm$ 0.02	
DAG	0.8 $\pm$ 0.1		1.0 $\pm$ 0.1	
TAG	6.1 $\pm$ 0.4		7.7 $\pm$ 0.2	
CELL AQU.	2.7 $\pm$ 0.2		2.6 $\pm$ 0.2	
Total d.p.m. ...	50205 $\pm$ 3341		50236 $\pm$ 4258	
Choline label				
SM	8.8 $\pm$ 0.5		9.4 $\pm$ 0.5	
PC	39.8 $\pm$ 1.7		40.3 $\pm$ 1.0	
CELL AQU.	38.5 $\pm$ 1.9		40.0 $\pm$ 0.8	
Medium	12.9 $\pm$ 0.6		11.3 $\pm$ 0.8	

for 5 h there was no change in total lipid radioactivity compared with controls incubated for the same period, and there were no marked changes in the distribution of the label among the major lipids (Table 4). However, the slight but statistically insignificant increase in sphingomyelin radioactivity in treated cells was consistently observed in all experiments. This small effect was accompanied by a 3-fold increase in radioactivity in cholesterol ester and a corresponding decrease in cholesterol radioactivity which was, however, not generally statistically significant. When radioactivity was measured in the cholesterol and fatty acid portions of the cholesterol ester, it was found that cholesterol accounted for about 65% of the total radioactivity, whereas fatty acid accounted for the remaining 35% (Table 5). The increment in radioactivity in the fatty acid portion of the cholesterol ester observed in cells treated with BFA was too large to be accounted for by changes in radioactivity in either non-esterified fatty acid or the aqueous fraction of the cells.

DISCUSSION

At concentrations comparable with those at which it blocks protein translocation [2], BFA had two rather dramatic effects on cell lipids: it completely inhibited access of newly synthesized sphingomyelin to the plasma membrane surface (Tables 1 and 4) and induced remarkable changes in the short-term labelling of BHK cell lipids with [3 H]acetate (Figure 2, Tables 1 and 2).

Treatment with sphingomyelinase showed that in the presence of BFA little radioactive sphingomyelin reached the surface, and

Table 5 Distribution of radioactivity in the cholesterol and fatty acyl moieties of cholesterol ester of cells incubated with or without BFA

Cholesterol ester isolated by t.l.c. from [^3H]acetate-labelled cell samples treated for 2 or 4 h with or without BFA in the same experiment as that shown in Table 3 was subjected to alkaline methanolysis as described in the Materials and methods section. The radioactivity (d.p.m.) in the original samples before methanolysis was compared with that recovered in cholesterol, cholesterol ester, non-esterified fatty acid and fatty acyl methyl esters after methanolysis and separation by t.l.c. Results are means of d.p.m. from duplicate determinations which varied by less than 5%. The proportion of radioactivity found in the fatty acid moiety of cholesterol ester was calculated as radioactivity in NEFA + FAME as a percentage of that in NEFA + FAME + cholesterol. In three similar experiments, the mean value was found to be 35.7 ± 0.6 . Abbreviations as in Table 1.

	Incubation period				
	0	2 h	2 h + BFA	4 h	4 h + BFA
Before methanolysis	1140	1395	2075	2346	4787
After methanolysis					
CHOL. EST.	46	63	60	62	94
FAME	182	210	313	337	698
NEFA	202	248	379	466	896
CHOL	689	806	1195	1386	2868
Total	1096	1327	1947	2251	4556
(NEFA + FAME)%	35.8	36.2	36.7	36.7	35.7

consequently that most of the increased synthesis of sphingomyelin was in an internal compartment of the cells (Table 1). The inhibition by BFA of sphingomyelin transport to the cell surface was confirmed by determinations of phospholipid phosphorus on unlabelled cells (Table 3). Although the total amount of sphingomyelin after 5 h incubation with BFA (19.4 nmol) was virtually the same as in the controls (19.3 nmol), the amount of surface sphingomyelin in BFA-treated cells was only 9.1 nmol, similar to the original amount (8.8 nmol) in the cells at the start of the incubation.

The failure of newly synthesized sphingomyelin to reach the surface of BFA-treated cells thus endorses previous suggestions of a vesicular mechanism for the intracellular transport of sphingomyelin [18,25]. If sphingomyelin were transported by a protein carrier similar to that well established for cholesterol [26], then BFA should not prevent it from reaching the cell surface [27].

In addition to its effect on vesicular transport of sphingomyelin in BHK cells, treatment with BFA shifted incorporation of [^3H]acetate away from cholesterol, phosphatidylcholine and triacylglycerol into sphingomyelin, monoglycosylceramide and cholesterol ester (Figure 2 and Table 1). There was no overall change in incorporation into lipid, suggesting that BFA changed the balance of the lipid-synthesis pathways in favour of the synthesis of sphingolipids at the expense of that of phosphatidylcholine, triacylglycerol and cholesterol. Incorporation of choline into sphingomyelin was increased by a similar proportion to incorporation of acetate in the presence of BFA (Figure 3), suggesting that the whole molecule was being synthesized and not simply the ceramide backbone or the *N*-acyl-linked fatty acid.

Although the effect of BFA on synthesis of sphingomyelin was dramatic, a 5 h incubation of cells with BFA had little effect on sphingomyelin mass as assessed by determinations of phospholipid phosphorus (Table 3) or labelling of cells to equilibrium with [^3H]acetate or [^3H]choline (Table 4). Nevertheless, all these experiments showed a small increase in total

sphingomyelin which was not statistically significant in individual experiments (Table 4). From estimates of choline specific radioactivity, it was calculated from the data of Figure 3 that the maximum increase in sphingomyelin synthesis in BFA-treated cells after 5 h would only represent about 5% of the total mass, which would be difficult to detect. There was no indication that BFA stimulated turnover of sphingomyelin by increasing its degradation, since no loss of label from sphingomyelin could be detected in cells which had been labelled to equilibrium and then incubated with BFA in non-radioactive medium (Table 4).

The most prominent effect of BFA on the synthesis of neutral lipids was the increase in labelling of cholesterol ester, from 0.6% to 2.4% of total label (Table 1). A similar effect of BFA in increasing cholesterol esterification has been reported recently in some other cell types [28]. That report also noted that in macrophages, but not CaCo_2 or HepG $_2$ cells, BFA decreased synthesis of triacylglycerol as in BHK cells (Figure 2). The BFA-induced fall in labelling of cholesterol in our experiments (Tables 1 and 2 and Figure 2) can be ascribed only partly to the utilization of cholesterol for synthesis of cholesterol ester; the additional deficit in labelling of cholesterol indicates an inhibition of cholesterol synthesis. A preferential utilization of acetate for fatty acid synthesis rather than synthesis of cholesterol seems unlikely, since the labelling of the phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol was not affected by BFA (Table 1).

In cells labelled to equilibrium with [^3H]acetate (Table 4), the increase in radioactivity of cholesterol ester from 1% to 3% of the total lipid counts is likely to represent an equivalent rise in relative mass of cholesterol ester. This rise was largely accounted for by an equivalent fall in radioactivity of cholesterol, although the decrement in cholesterol labelling was not statistically significant in all experiments. This would be consistent with a recent report [28] showing that BFA may have a general effect in causing esterification of cholesterol by stimulation of acyl-CoA: cholesterol acyltransferase. However, analysis of the distribution of label in cholesterol ester (Table 5) shows that 36% of the total radioactivity of cholesterol ester in BFA-treated cells was present in fatty acid, not very different from the mass proportion in cholesterol palmitate. This suggests a specific radioactivity of the fatty acyl moiety similar to that of cholesterol. Under the chase conditions used here, there was little radioactivity in non-esterified fatty acids (Table 4), as expected from the prolonged incubation (up to 6 h) of labelled cells in unlabelled medium, which would have considerably decreased the specific radioactivity of non-esterified fatty acid and fatty acyl-CoA. Therefore these molecules are unlikely to be the immediate precursors of the fatty acyl moiety of the labelled cholesterol ester. Since both phosphatidylcholine and triacylglycerol lose radioactivity as the radioactivity of cholesterol ester increases in the presence of BFA (Figure 2), it is possible that these lipids act as the donors of fatty acid in a process analogous to the lecithin:cholesterol acyltransferase reaction.

Forskolin, which can block and reverse the morphological effects of BFA on endoplasmic reticulum and Golgi apparatus [29], has been reported to inhibit partially BFA-induced cholesterol esterification [28]. We found that forskolin caused almost complete inhibition of the effects of a low concentration of BFA (Table 2) on lipid labelling. However, it had little effect when incubated with a higher concentration of BFA (1 $\mu\text{g/ml}$).

It is not known by which mechanism BFA causes such a large increase in sphingolipid synthesis, but diversion into an internal pool of sphingomyelin normally destined for the cell surface should not cause a change in the total cellular content of this lipid. The increased intracellular synthesis of sphingomyelin

and changes in labelling of other lipids may occur as a consequence of the radical fusion of endoplasmic reticulum/Golgi elements promoted by BFA. The inhibition of the BFA effects on lipids by forskolin, which also blocks the morphological changes [29], may support this hypothesis. A similar explanation has been presented by Wieland and his collaborators, who recently published data comparable with our own on the enhancement of sphingomyelin labelling in CHO cells treated with BFA [30].

Recent evidence localizes the site of synthesis of sphingomyelin to the *cis*-Golgi [12–15]. Ceramide synthesis is considered to be spatially separated from sphingomyelin synthesis, and has recently been shown to be localized in the endoplasmic reticulum [31]. It is significant that BFA causes a consistent decrease in ceramide relative mass (Table 4) and in its labelling (Table 1). The inference is that the BFA-induced fusion of endoplasmic reticulum and Golgi increases the utilization of ceramide for sphingolipid synthesis, possibly because sphingomyelin precursor and synthetic activity are now in the same compartment. This in turn might release feed-back inhibition of the serine-palmitoyl-CoA ligase, the rate-limiting enzyme in sphingolipid synthesis [32]. Thus palmitoyl-CoA utilization for synthesis of sphingolipids would be increased at the expense of that for glycerolipid synthesis; ceramide synthesis would be increased, but steady-state levels lowered, because of the quicker turnover into sphingolipids. In this model, vesicular transport of ceramide to the enzymes converting it into sphingolipids would be the overall rate-determining step in the synthesis of sphingolipids.

The reciprocal effects of BFA on sphingomyelin and cholesterol synthesis observed here (Figure 2) fit in with previous indications that cholesterol metabolism is sensitive to the level of plasma-membrane sphingomyelin [33–35]. Thus a deficit in surface sphingomyelin caused by sphingomyelinase activity [33–35] or by BFA (the present work) leads not only to inhibition of cholesterol synthesis but also to increased esterification of cholesterol. These effects would both tend to decrease the amount of free cholesterol in the cells, particularly in plasma membranes. Such data support the concept that cells maintain strict limits on the ratio of sphingomyelin to cholesterol in their membranes and respond to a decrease in the amount of sphingomyelin in the plasma membrane by decreasing cholesterol synthesis and/or by increasing esterification of cholesterol. Cholesterol itself does not appear to depend on a vesicular transport pathway in order to reach the plasma membrane [27], so that the observed effects of BFA on cholesterol metabolism may be secondary to effects on the vesicular transport of sphingomyelin.

It is interesting to note from Tables 3 and 4 that treatment of cells with BFA, which prevents sphingomyelin equivalent to about 0.8% of total lipid from reaching the surface in 4 h, causes an increase in cholesterol ester which is approximately equivalent to 2% of total lipid. Taking the molecular mass of sphingomyelin as about 770 and that of cholesterol ester as about 690, it appears that a plasma-membrane deficit of 1 mol of sphingomyelin results in esterification of about 3 mol of cholesterol. This is close to the normal ratio of sphingomyelin and cholesterol in plasma

membranes [36] and suggests that this ratio may be maintained in part by cholesterol esterification in response to the level of sphingomyelin in the plasma membrane.

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