Studies on the synthesis and transport of endogenous sphingomyelin in BHK-21 cells

by

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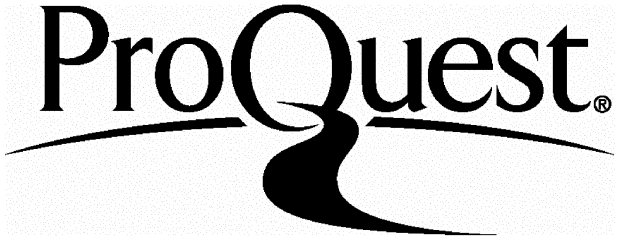

Abstract

The synthesis and intracellular transport of sphingomyelin in BHK-21 cells has been studied in the presence of brefeldin A (BFA) and monensin which are inhibitors of vesicular transport through the Golgi apparatus. Both drugs inhibit delivery of newly synthesised sphingomyelin to the cell surface, albeit by different mechanisms. BFA increases overall synthesis of sphingomyelin, but prevents vesicular transport of newly synthesised sphingomyelin to the *trans* Golgi network and the cell surface. Monensin seems to specifically inhibit synthesis of plasma membrane sphingomyelin by blocking vesicular transport of ceramide past the *medial*-Golgi.

Ceramide can be produced at the plasma membrane by degradation of surface sphingomyelin by an extracellular sphingomyelinase. In untreated cells this ceramide is almost completely utilised for the resynthesis of plasma membrane sphingomyelin. Neither BFA nor monensin interfere with resynthesis of sphingomyelin from plasma membrane ceramide. However, resynthesis of sphingomyelin is largely prevented in mitotic and ATP-depleted cells in which endocytosis and vesicular transport are suppressed.

These findings are conceptualised by a model which assumes in contrast to the prevailing orthodoxy, that the major site for the synthesis of plasma membrane sphingomyelin is not in the *cis-/medial*-Golgi, but distal to the *trans*-Golgi in an endosomal compartment on the plasma membrane recycling pathway.

The depletion of plasma membrane sphingomyelin by either drug or sphingomyelinase treatment causes an increased esterification of cholesterol and, in the case of BFA, a decrease of cholesterol synthesis. These observations are consistent with the previous findings that cholesterol synthesis and esterification processes in cells are influenced by the plasma membrane content of sphingomyelin. It is suggested that the correct balance between plasma membrane cholesterol and sphingomyelin is created at the putative endosomal site of sphingomyelin synthesis after delivery of newly synthesised cholesterol to this site.

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I warmly thank David Allan for enduring all the enthusiastic (and sometimes ferocious) scientific discussions. Beyond showing me that one can think about lipids in other ways than fat, David proved to be a valuable friend in a sad situation of my life.

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Publications originating from this work

1. Kallen K.-J., Quinn P., Allan D., Biochem.J. (1993) 289, 307-312, "Effects of brefeldin A on sphingomyelin transport and lipid synthesis in BHK21 cells"

2. Kallen K.-J., Quinn P., Allan D., Biochim.Biophys.Acta (1993) 1166, 305-308, "Monensin inhibits synthesis of plasma membrane sphingomyelin by blocking transport of ceramide through the Golgi: evidence for two sites of sphingomyelin synthesis in BHK cells"

3. Allan D., Kallen, K.-J., Prog. Lipid Res. (1993) 32, 195-219, "Transport of lipids to the plasma membrane in animal cells"

4. Kallen K.-J., Quinn P., Whatmore J., Allan D., Biochim.Biophys.Acta (1994) 1191, 52-58, "Synthesis of surface sphingomyelin in the plasma membrane recycling pathway of BHK-cells"

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Abbreviations

α2-M	α2-macroglobulin
ACAT	acyl-CoA:cholesterol acyltransferase
ADP	Adenosine 5'-diphosphate
ARF	ADP-ribosylation factor
BFA	brefeldin A
BHK cells	baby hamster kidney fibroblasts
C5-DMB	N-[5-(5,7-dimethyldipyrromethene boron difluoride)-1-pentanoyl]
C6-NBD	N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-aminocaproate
CER	ceramide
CDP	cytidine 5'-diphosphate
CGN	*cis*-Golgi network
CHOL	cholesterol
CHOL EST	cholesterol ester
CL	cardiolipin
CURL	compartment of uncoupling of receptor and ligand
DAG	diacylglycerol
DMSO	dimethylsulphoxide
DOG	2-deoxyglucose
DTT	dithiothreitol
EGF	epidermal growth factor
EGTA	ethylene diamino tetraacetic acid
ERGIC	endoplasmic reticulum Golgi intermediate compartment
FAME	fatty acid methyl ester
FCS	foetal calf serum
GAP	GTPase activating protein
GDP	Guanosine 5'-diphosphate
GEF	guanosine nucleotide exchange factor
GluCer	monoglucosylceramide
GMEM	minimal essential medium, Glasgow modification
GPI	glycosylphosphatidylinositol
GRF	guanosine releasing factor
GTP	Guanosine 5'-triphosphate
Hep-G_2 cells	human hepatoma G_2 cells
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HRP	horseradish peroxidase
IP_3	inositol trisphosphate
LDH	lactate dehydrogenase
LPC	lysophosphatidylcholine
MDBK	Madin-Darby bovine kidney cells
M6PR	mannose 6 phosphate receptor
NADH	nicotinamide adenine dinucleotide
NaCN	sodium cyanide
NaN_3	sodium azide
NEFA	non-esterified fatty acids

NEM	N-ethylmaleimide
NL	neutral lipids
NSF	NEM sensitive fusion protein
NRK	normal rat kidney cells
PC	phosphatidylcholine
PCCP	phosphatidylcholine : ceramide choline phosphotransferase
PE	phosphatidylethanolamine
PIPES	piperidine sulphonate
PIP_2	phosphatidylinositolbisphosphate
PKC	protein kinase C
PLD	phospholipase D
PM	plasma membrane
PS	phosphatidylserine
RNA	ribonucleic acid
SM	sphingomyelin
SMase	sphingomyelinase
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
SPT	serine-palmitoyl-CoA ligase
TGN	trans-Golgi network
TMA-DPH	1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene)
t.l.c.	thin layer chromatography
VSV	vesicular stomatitis virus

"When I did my PhD twenty-five years ago I considered sphingolipids the world's most boring molecules. Now I find myself spending the rest of my working life studying them"

David Allan, University College London

A. Introduction

A.1. Sphingolipid research revived

Some hundred years after its discovery as a major, ether-soluble fraction of brain lipids [326] research into sphingomyelin (SM) seems to have lost its esoteric aura in the last decade. The considerable number of reviews on sphingolipids and sphingomyelin published in the last few years [88,107,148,153,202,304] shows that the above cited researcher is not alone with regard to his newly developed interest in the cellular metabolism and function of sphingolipids and specifically, sphingomyelin.

Besides glycerophospholipids and cholesterol, sphingolipids are major lipid components of mammalian membranes. This is particularly obvious in the nervous system of mammalian organisms where sphingolipids and sphingomyelin are present at very high concentrations in the myelin sheath of peripheral neurons [140]. Aberrations of sphingolipid metabolism can cause substantial neurological defects [243,258], but their precise role in health and disease of the nervous system, in particular in demyelinating diseases, still has to be elucidated.

Nevertheless, it has become clear that sphingolipids, including sphingomyelin, are vital components of virtually all mammalian cells [2,314]. Glycosphingolipids appear to be involved in cell-cell adhesion and probably the modulation of cell growth and transmembrane signalling [31,107]. Some have also been identified as tumour antigens [86]. The implications of these observations and the cell biological details thereof are still largely unknown. Recently, it was suggested that breakdown products of plasma membrane sphingomyelin, in particular ceramide, might provide the basis of a novel second messenger system (see A.7.) [147,148]. Sphingomyelin

has also attracted greater interest, since the experimental evidence suggests that cellular sphingomyelin is predominantly located in the plasma membrane [163,345] where it is almost entirely restricted to the outer leaflet of the lipid bilayer [5,8,59]. Depending on the cell type varying amounts of sphingomyelin are found intracellularly where it is confined to the luminal leaflet of organelle membranes [4,202,250]. Since transbilayer movement of sphingomyelin could as yet not be demonstrated [375], it is generally assumed that intracellular transport of sphingomyelin occurs only by vesicular means and not via cytosolic proteins [345]. It has also been pointed out [345] that insight into the mechanisms of membrane bulk flow and vesicular transport could be achieved by exploiting these properties of sphingomyelin.

The following section tries to summarize present knowledge of the biochemical and biophysical properties of sphingomyelin as well as the more recent findings concerning the cell biological significance of this most abundant sphingolipid.

A.2. Biophysical characteristics of sphingomyelin

A.2.1. Structure of sphingomyelin

The fundamental structure of sphingomyelin is shown in Figure A-1. Essentially the molecule consists of three parts: a characteristic long chain (18 carbon atoms) sphingoid base (sphingosine), a fatty acyl chain, which is connected to the sphingosine chain by an amide linkage, and a polar phosphorylcholine headgroup. Mammalian sphingosines are either D-erythro-sphingosine, typified by a *trans*-4,5 double bond, or D-erythro-sphinganine (-dihydrosphingosine) in which the double bond is hydrogenated [140,202]. The fatty acyl chain can either be of normal length (C_{14}-C_{20}) or a very long chain (C_{22}-C_{30}) explaining why sphingomyelin and other sphingolipids often run as doublets on thin-layer chromatography (t.l.c.) [140]. In both cases saturated and *cis*-monounsaturated moieties dominate, whereas polyunsaturated fatty acids are notably absent in sphingolipids and sphingomyelin. Brain sphingolipids show an interesting peculiarity in that more than half of the very-

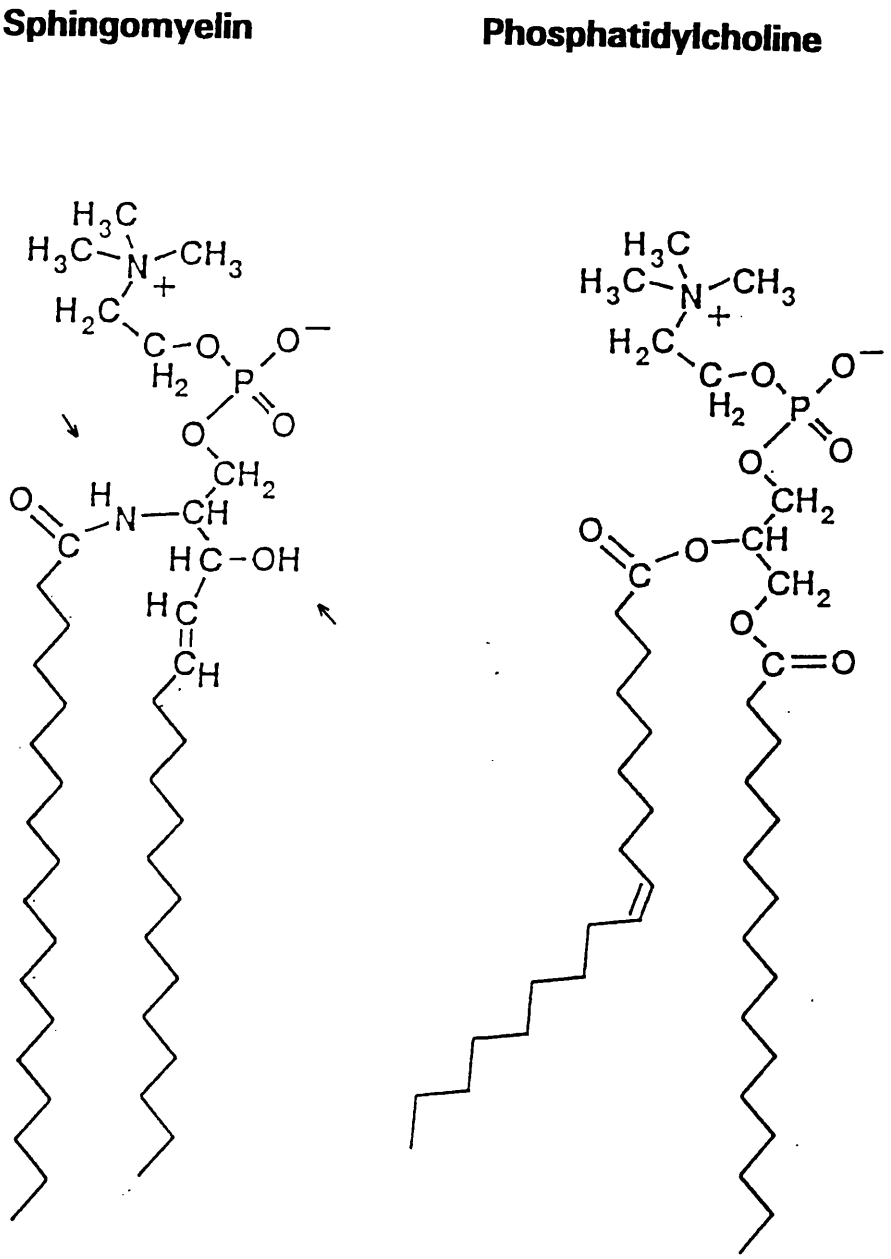


Figure A-1: Structure of sphingomyelin and phosphatidylcholine. (modified from Koval & Pagano [153]). The structure of a representative sphingomyelin molecule (a) is contrasted with the structure of a representative phosphatidylcholine molecule (b). Note the ability of sphingomyelin to act as donor (see arrows) and acceptor of hydrogen bonds. Also note, that in contrast to phosphatidylcholine sphingomyelin does not contain *cis*-double bonds meaning that there is no kink formation in the fatty acyl moiety of sphingomyelin.

long fatty acyl chains are α-hydroxylated [140]. The very-long chain fatty acids and their α-hydroxylated derivatives are conspicuously absent from lipids other than sphingolipids which suggests that the cell operates a mechanism to restrict incorporation of these fatty acids into sphingolipids [314]. Plant, (phyto-) sphingolipids differ from the mammalian form in that they contain an additional hydroxy group in the C_4 position of the sphingosine backbone [140].

A fundamental difference between sphingomyelin and other phospholipids is the ability of sphingomyelin to act as an acceptor and donor of hydrogen groups in hydrogen bonding [153]. In principal, this structural peculiarity should make possible self-aggregation of sphingolipids. NMR studies in artificial membrane systems, which detected phase separation of glycosphingo- and glycerophospholipids at glycosphingolipid concentrations exceeding 20 mol%, may lend some experimental support to this theoretical prediction [213]. A physiological role for the alleged tendency of sphingolipids to self-aggregate might be to facilitate the sorting of sphingolipids to different membrane domains in polarized cells [347].

A.2.2. Biophysical properties of sphingomyelin

In contrast to other membrane phospholipids sphingomyelin does not undergo transbilayer migration [375]. Moreover, comparative studies in a number of different erythrocyte species showed that the sphingomyelin content of the plasma membrane in each case is negatively correlated with the transbilayer mobility of glycerophospholipids [22]. This might be related to the observation that sphingomyelin, a cylindrical molecule, hinders the formation of non-bilayer, hexagonal H_{II}-phases which appear to be involved in an increased transbilayer mobility of phospholipids [53]. Thus sphingomyelin could be involved in the regulation of lipid dynamics, a function classically attributed to cholesterol [28,56,373]. In this context it is interesting to note the higher affinity of cholesterol for sphingomyelin than other phospholipids [57,179,373]. Stereotactic and thermodynamic considerations by Vandenheuvel [355] suggest that this is due to stronger van der Waals forces and hydrogen bonding between cholesterol and sphingomyelin than between cholesterol and other phospholipids. This reasoning

seems to have been accepted by other authors [57,179,373].

The ratio of sphingolipid to cholesterol could also be of crucial importance for the barrier function of membranes. Disruption of the barrier function of the epidermis by acetone is first followed by a surge in cholesterol synthesis, then by increased sphingolipid synthesis [119]. Depletion of cholesterol in erythrocyte membranes cholesterol increases their water permeability [58].

A.2.3. The sphingomyelin - cholesterol connection

Several other observations support the concept of a functional significance for the interaction between cholesterol and sphingomyelin. The exchange rate of cholesterol molecules between different liposomes is markedly influenced by the relative sphingomyelin content of the respective liposomes [240,325], indicating that the sphingomyelin content of a membrane is important for the accommodation of cholesterol. Work by Slotte and his colleagues point to a similar mechanism acting in mammalian cells. Here, enzymatic degradation of plasma membrane sphingomyelin by bacterial sphingomyelinases (S.aureus or B.cereus) leads within ten minutes to increased esterification of plasma membrane cholesterol [293] and redistribution of cholesterol to a pool where it is resistant to an external oxidase [296]. Both processes are consistent with a shift of cholesterol from a plasma membrane pool to an intracellular pool. However, contrary to earlier impressions, new results indicate that only part of the oxidase-resistant plasma membrane cholesterol pool appears to become intracellular [personal communication by Isabella Pörn, University of Turku, Finland]. The remainder might stay in the plasma membrane where this "extra" cholesterol might form a separate phase from phospholipids. Such a concept has been put forward to explain the higher fragility of sphingomyelinase-treated human erythrocytes which becomes particularly apparent at lower temperatures [8]. Digestion of surface sphingomyelin also causes a reduction in cholesterol synthesis [106,297].

The significance of sphingomyelin-cholesterol interaction for eukaryotic cells is also illustrated by the enrichment of sphingomyelin and cholesterol in *caveolae*, invaginations of the plasma membrane that seem to be involved in potocytosis

[40,264] (see A.6.). Interactions of sphingomyelin and cholesterol also have implications for pathological conditions, e.g. Niemann-Pick disease or atherosclerosis (see A.8.), where alteration of the metabolism of either of these lipids is accompanied by disturbances in the metabolism of the other. Thus the evidence available so far appears to give some support to an early theoretical prediction by Patton [232] that the amount of cholesterol in cellular membranes is determined by their sphingomyelin content.

A.3. Biosynthesis of sphingomyelin

A.3.1. Synthesis of ceramide

Ceramide (CER) is the common precursor of sphingomyelin and all other sphingolipids. Synthesis of ceramide (Figure A-2) starts with the condensation of palmitoyl-CoA and serine to 3-keto-sphinganine (dehydrosphingosine) [140]. In mouse liver cells, subcellular fractionation has located this reaction to the ER [185]. The reaction is catalysed by the *serine-palmitoyl-CoA-transferase* (SPT), an enzyme with a high specificity for palmitoyl-CoA, which requires pyridoxal-5'-phosphate, Mn^{++} and NADPH as necessary cofactors [140]. The SPT catalyses an irreversible reaction unique to sphingolipid metabolism which is regarded as the rate limiting step of ceramide and hence sphingolipid synthesis [202]. The importance of the SPT is thus comparable to that of *phosphatidic acid phosphatase*, the rate limiting enzyme of glycerophospholipid synthesis, which also resides in the ER [26].

The reaction product of the SPT, 3-keto-sphinganine, is reduced to sphinganine (dihydrosphingosine) by an NADPH-dependent reaction [140]. Apparently the reductase has a much higher activity than the SPT as 3-keto-sphinganine is not detectable as a metabolic intermediate *in vivo* [205]. Some controversy prevails as to the exact nature of the next reactions. Most textbooks suggest oxidation of sphinganine to *trans*-4,5-sphingosine by a FAD requiring enzyme as the next step [312]. By contrast, several studies [222,310] using double-labelled ($^{3}H/^{14}C$) sphinganine suggested that sphinganine is first acylated to dihydroceramide, which is subsequently dehydrogenated to ceramide. However, this

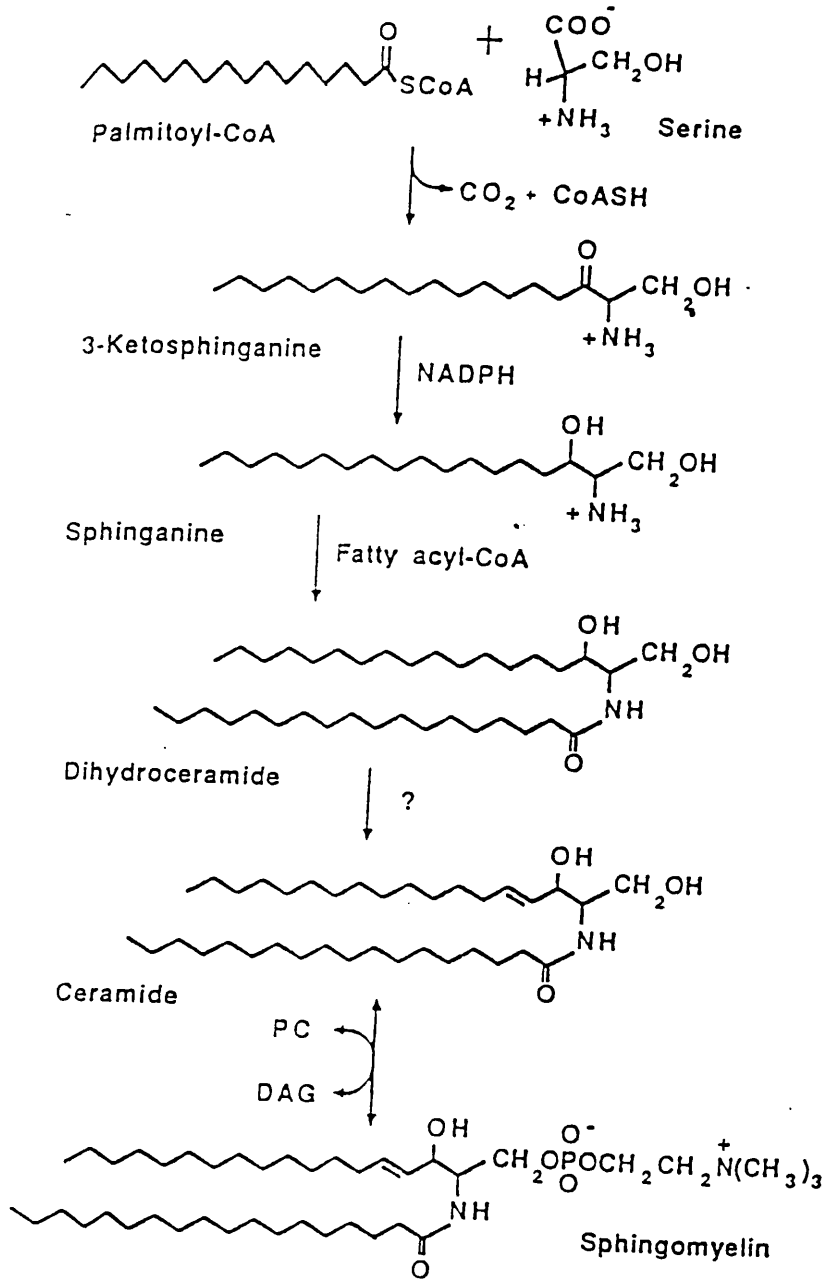


Figure A-2: The pathway of *de novo* synthesis of sphingomyelin. The figure shows a scheme for the sequence of reactions in biosynthesis of sphingomyelin as recently suggested by Merrill & Jones [202]. Known cofactors of the different reaction steps are cited in the text. More recent evidence [see 148,202 and ref.therein] favours acylation of sphinganine to dihydroceramide and subsequent oxidation thereof over the older sequence cited in most textbooks (sphinganine → sphingosine → ceramide). The question mark denotes that this issue has not conclusively been decided. None of the enzymes involved has as yet been purified.

putative ceramide dehydrogenase is not well-characterized in enzymological terms, let alone purified [202].

N-acylation of sphinganine or sphingosine to (dihydro)ceramide occurs by CoA-dependent [1,305,330] and CoA-independent pathways [211,286]. The latter pathway appears to be specifically localized to brain, requires Mg^{++}, a pyridine nucleotide, addition of heat-stable and heat-labile factors from brain cytosol [287,288]. The CoA-independent pathway appears to favour very-long-chain fatty acids [283] which are more abundant in brain sphingolipids [140]. Four different, tissue-specific enzymes with differing preferences for normal-chain, very-long-chain fatty acids and the α-hydroxy derivatives thereof appear to catalyse the synthesis of ceramide from sphingoid bases [140].

The reactions described above, responsible for *in vivo* synthesis of ceramide are very rapid which explains why intermediates are normally unmeasurable in tissue or cell culture extracts [202]. Due to these technical difficulties the exact details of these reactions are still obscure. The low concentrations of early sphingolipid metabolites are quite understandable since the free long-chain intermediates of ceramides are cytotoxic [201] and have been found to be potent inhibitors of protein kinase C [108,204]. Recently, early sphingolipid metabolites have been implicated in the regulation of cell proliferation, cell differentiation and apoptosis [208, see A.7.].

A.3.2. Addition of the phosphocholine headgroup to ceramide

Following an original suggestion by Diringer et al.[61] several groups [189,190,331,361] were able to confirm that phosphatidylcholine is the donor of the phosphocholine moiety of sphingomyelin in a reaction catalysed by the enzyme *phosphatidylcholine : ceramide choline phosphotransferase* (*PCCP*):

$$PC + Cer \rightarrow SM + DAG$$

Many biochemistry textbooks, however, still cite the older concept that the phosphocholine moiety of sphingomyelin is derived from CDP-choline by analogy with the synthesis of phosphatidylcholine (PC) from diacylglycerol (DAG) and CDP-

choline [312]. The *PCCP* was extensively characterized by Marggraf and Kanfer [186,191] in plasma membrane samples from Ehrlich ascites cells. Using ^{3}H-ceramide as labelled precursor they measured Michaelis-Menten constants of 1.0 mM and 0.35 mM for the substrates ceramide and phosphatidylcholine respectively. A V_{max} of 35 nmol $\cdot$ mg^{-1} $\cdot$ h^{-1} and an activation energy of 17.2 $\pm$ 0.2 kcal/mol were estimated for the reaction. $MnCl_2$ might be a stimulating cofactor of the reaction [191]. Analysis of the kinetics of the reaction prompted the authors to postulate a two step 'ping-pong-mechanism' for the *PCCP* reaction [186]:

$$\text{enzyme} + \text{PC} \rightarrow \text{enzyme-phosphocholine} + \text{DAG} \quad (1)$$

$$\text{enzyme-phosphocholine} + \text{CER} \rightarrow \text{enzyme} + \text{SM} \quad (2)$$

This implies that PC and sphingomyelin compete for the free enzyme, whereas ceramide and diacylglycerol (DAG) form a second pair of competitors for the intermediate enzyme complex. The K_i of diacylglycerol is 0.13 mM, marking diacylglycerol as a strong inhibitor of the PCCP reaction. Removal of free diacylglycerol from the assay may explain the higher activity of the PCCP in the presence of defatted bovine albumin [191]. The ability of diacylglycerol to inhibit the *PCCP* could constitute the metabolic basis for the observation that the cellular levels of sphingomyelin and phosphatidylcholine appear to be inversely related to each other [148] leaving the sum of the two phospholipids fairly constant for a given cell type. Down-regulation of the *phosphatidic acid phosphatase* the key enzyme of glycerophospholipid synthesis [26] would thus lead to elevated activity of the PCCP by lowering the concentration of DAG. Interestingly free fatty acids (other than palmitoyl-CoA) which activate phosphatidic acid phosphatase by increasing the association of this molecule with the endoplasmic reticulum [26] have an inhibitory effect on the SPT [206,207].

Substrate molecules of the PCCP must reside in the same membrane as the enzyme, and in fact, the availability of endogenous ceramide appears to be the rate limiting factor of the reaction [186]. The activity of the PCCP is 10 times higher for unsaturated than saturated phosphatidylcholines [184,186]; phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol inhibit the transfer reaction. The enzyme

seems to be less fastidious with regard to its ceramide substrate since a diversity of ceramide analogues, e.g. with shortened sphingosine and acyl chains [34,129,356] or fluorescent labels in the N-acyl position [175,227], can be utilized by the PCCP. However, studies in rats and Hep-G_2 cells demonstrated that a higher percentage of radioactivity was recovered in sphingomyelin when radioactively-labelled saturated ceramides rather than monounsaturated analogues were added [307]. Cells with high plasma membrane turnover appear to have a higher activity of the enzyme [186].

An additional, different pathway for sphingomyelin synthesis might exist in liver and brain [184,195]. Malgat et al.[184] found that plasma membrane and microsomal fractions of these cells are able to produce ceramide-phosphoethanolamine from phosphatidylethanolamine and ceramide by a transfer reaction similar to that described above. Direct methylation of ceramide-phosphoethanolamine to sphingomyelin could be demonstrated, but the pathway seems to be of only minor physiological significance.

A.3.3. Intracellular location of sphingomyelin synthesis

The activity of the ceramide phosphocholine transferase was originally considered to reside in the plasma membrane of cells [186,334,361]. In contrast to these reports Lipsky and Pagano [175] reported that blockage of transport through the Golgi by monensin inhibited appearance on the cell surface of fluorescent-labelled sphingomyelin which had been synthesized intracellularly from NBD-ceramide. This was interpreted to indicate synthesis of sphingomyelin at a *cis-/medial*-Golgi site. There is now widespread agreement among different authors that the main site of sphingomyelin synthesis is on the luminal side of the *cis-* or *medial*-Golgi compartment [90,129,143,176,187], although it was tentatively suggested that a minor proportion of sphingomyelin ($\sim 10\%$) could be synthesized in the plasma membrane [90]. Little notice, however, has been taken of work by Malgat et al.[184] who observed synthesis of sphingomyelin in both plasma membrane and microsome fractions of liver and rat brain. This observation suggests the existence of two sphingomyelin synthesis sites in mammalian cells. The finding that labelling of plasma membrane sphingomyelin with ^{3}H-choline takes much longer than labelling

of intracellular sphingomyelin [250] could support this view.

Similarly to sphingomyelin, synthesis of glucosylceramide has been located to the *cis-/medial*-Golgi fraction of rat liver and CHO cells respectively [89,130]. However, glucosylceramide synthesis was also found in a late Golgi-compartment [130] and in a fraction that might correspond to the intermediate compartment [89]. In contrast to sphingomyelin, synthesis of glucosylceramide appears to take place on the cytosolic face of the analyzed membrane compartments. Thus synthesis of sphingomyelin and glucosylceramide is separated from that of their immediate precursor ceramide which is assumed to occur in the ER [185].

A.4. Distribution of sphingomyelin among various cellular membranes

The relative sphingomyelin content of different cellular membranes varies considerably [see 153 and references therein]. Plasma membrane and the related organelles, endosomes and lysosomes, have high concentrations of sphingomyelin (about 15-25 mol%), whereas the sphingomyelin concentration in Golgi membranes is cited to be somewhat lower ($\sim$10 mol%). Mitochondria and nuclear membranes contain only very little sphingomyelin (0-5 mol% respectively). The figures for sphingomyelin content of the endoplasmic reticulum (ER) are somewhat controversial. Renkonen and coworkers measured a concentration of 10 mol% in the ER of BHK cells [256], whereas other authors [see 153] found figures from 0-4%, mostly based on subcellular fractionation of rat hepatocytes. These figures have been claimed to prove the existence of a sphingomyelin gradient from ER to plasma membrane [33,202,345]. A similar gradient on the secretory pathway is believed to exist for cholesterol [345], since the cholesterol-sensitive drug filipin appears to show enhanced binding to distal Golgi-stacks [52,224]. Although this concept is widely accepted there is a considerable amount of experimental evidence [21,87,117,121,241] that argues against its universal validity.

In plasma membranes, sphingomyelin is confined to the outer leaflet of the lipid bilayer [5,8], so it is possible to assess the amount of cell surface sphingomyelin by degradation with exogenous sphingomyelinases. Depending on the cell type the proportion of sphingomyelin located in the plasma membrane varies

considerably: values from 35% in rat hepatocytes [153] to 90% in human skin fibroblasts [163] have been reported. In BHK cells the plasma membrane pool, representing 60-70% of total sphingomyelin, labels with kinetics distinctly different from the intracellular pool [250]. In spite of the rapid endocytosis and recycling of plasma membrane seen in these cells the two pools do not mix even after prolonged incubation. Since up to 10% of endoplasmic phospholipids might consist of sphingomyelin [256] it has been speculated that the internal pool of sphingomyelin might reside in a large compartment, e.g. the endoplasmic reticulum. However, the precise location of the internal pool of sphingomyelin is still a mystery.

A.5. Protein transport in cells

In the last decade many of the fundamental mechanisms underlying transport of proteins from the ER to the plasma membrane have been elucidated [103,200,267]. Similar advances have been made with regard to the endocytosis and recycling of proteins and receptors at the plasma membrane [99,105,233]. Some of the key features of these pathways will be summarized here, and the particular problems posed by lipid transport will be outlined in the following section.

A.5.1. The secretory pathway

In eukaryotic cells, endoplasmic reticulum (ER), Golgi apparatus (or Golgi complex) and plasma membrane constitute a conceptual functional unit, the "secretory pathway" [230]. The "secretory pathway" organizes the transport, modification and targeting of newly synthesized proteins and lipids on their way from the ER through the Golgi complex to their various cellular or extracellular destinations and thus the generation and maintenance of different cellular organelles [80,200,267].

In contrast to older concepts that defined the Golgi apparatus as the multi-layered stack of flattened cisternae in a juxtanuclear position in the cell [36], its definition has been widened to include two additional tubulovesicular structures:the *cis*-Golgi-network (CGN) located between ER and Golgi stack and the *trans*-Golgi

network (TGN) distal to the stack [154,200,267]. Resident ER-proteins appear to be retrieved in the CGN by mechanisms that are becoming clearer now (see A.5.2.) [124,168,234,236], whereas the TGN is assumed to direct proteins leaving the Golgi to their respective targets, e.g. plasma membrane, lysosomes or in the case of regulated secretion to secretory granules [101,200]. In contrast to this accepted view, Landinsky and coworkers concluded that sorting of molecules to their distinct targets might occur in the *trans*-Golgi cisternae or at least before the formation of tubules from the TGN [159]. Similarly, a tubulovesicular cisterna between ER and Golgi was recently discovered and defined as the intermediate compartment by a specific 53 kD marker protein, p53 [115]. Whereas some authors accept this compartment to be different from the CGN [259], others regard CGN and the intermediate compartment as essentially the same structures [267].

Most post-translational modification of proteins appears to take place in the Golgi stack [101]. Since these reactions have been distinguished by subcellular fractionation or immunocytochemistry, it is assumed that the stack can be further divided into at least three subcompartments, the *cis*-, *medial*- and *trans*-Golgi, the *cis*-Golgi being closest to the ER [80]. However, the different subcellular fractions have as yet not been correlated with distinct sets of cisternae [200,12]. Despite recent advances [245] there is also no agreed solution to the problem of how constitutive proteins of the Golgi apparatus and CGN could be retained there in spite of the enormous membrane flow through these compartments [217,33].

Palade [230] suggested that protein flow through the secretory pathway is mediated by carrier vesicles ($\sim$75 nm diameter [267]) shuttling proteins from one compartment to the next. These vesicles carry a protein coat and an intricate protein machinery exits to regulate the numerous budding and fusion events required by the extensive flow of vesicles through the secretory pathway. Insight into the molecular details of this process (for reviews see [68,155,266,267]) has been gained during the past few years and the following picture begins to emerge:

1. Budding of coated vesicles from Golgi membranes appears to depend on binding of several coat proteins ('COPs', comprising α-, ß-, ß'-, γ-, δ-, ϵ-(a doublet) and ζ-COP with molecular weights of 160 kD, 110 kD, 100-102

kD, 98 kD, 61 kD, 35 - 36 kD and 20 kD respectively) [111,112,158,309,366]. The COPs are also present as a complex of cytosolic proteins, termed coatomer (650 - 700 kD) [366]. Assembly of the coated vesicle requires *en bloc* incorporation of the coatomer into the coat [158]. ß-COP shows homology to ß-adaptin [73,280], whereas ζ-COP is similar to AP17 and AP19 [158], components of the two clathrin adaptor complexes. γ-COP has recently been identified as the mammalian homologue of the yeast SEC21 gene product, which is involved in ER to Golgi transport [309].

2. The assembly of the protein coat is ATP-dependent [267] and requires a small GTP-binding protein, the ADP-ribosylation factor (ARF, 21 kD) [223]. ARF appears to be the first representative of a new class of small GTP-binding proteins [68]. Myristoylation of the amino terminal end and binding of GTP (but not GDP) enable ARF to insert non-specifically into lipid bilayers and to induce binding of the coat proteins [68,267]. Accumulation of coated vesicles in the presence of GTPγS implies that detachment of the COP-proteins requires hydrolysis of GTP [267]. Experiments with a GTPase-defective ARF mutant demonstrated that this hydrolysis occurs through ARF [318]. The nucleotide status of ARF is affected by the activity of guanine nucleotide exchange factor (GEF) [66,116] and a GTPase activating protein (GAP) [68]. Heterotrimeric G-proteins have been found to exert an inhibitory effect on vesicular traffic at different stages of the secretory pathway [19,277,311]. This could be mediated by influencing ARF binding to Golgi membranes as suggested by the antagonism of the ßγ-subunit and pertussis-toxin to ARF effects [67,99,156].

3. After detachment of the coat proteins a number of proteins cooperate in the fusion of the uncoated vesicle to the target membrane. A key component of the fusion machinery is a tetrameric N-ethyl maleimide-sensitive fusion protein (NSF, 300 kD) that possesses ATPase activity [267,369]. Binding of the hydrophillic NSF to Golgi membranes depends on a family of soluble NSF attachment proteins (α-, β-, γ-SNAP with M_r of 35 kD, 36 kD and 39

kD respectively) [368]. The 'SNAPs' have characteristics of amphipaths [267] and binding of the NSF is preceded by binding of the SNAPs to a specific integral membrane receptor, termed SNARE (SNAP-receptor) [266]. Hydrolysis of ATP by NSF disrupts this SNARE complex and is followed by membrane fusion [266]. In yeast the existence of gene products functionally corresponding to NSF and α-SNAP, sec18p and sec17p respectively, has been demonstrated [368,369].

4. In nerve endings three different SNAREs were isolated by affinity purification [303]. Homologues of these proteins have also been found in other tissues [266]. One of the neuronal SNAREs, VAMP, was only found in vesicle membranes, whereas the other two SNAREs, syntaxin and SNAP-25, were restricted to the plasma membrane [17]. The 'SNARE-hypothesis' assumes that two types of SNARES exist, a vesicular one originating from the source organelle (v-SNARE) and one typical of the target membrane (t-SNARE). As a highly specific, high affinity binding could be demonstrated between VAMP and its plasma membrane counterparts [302], interaction between several organelle/ membrane specific v- and t-SNAREs could conceivably provide the basis for targeting and docking of vesicles. The 'SNARE-hypothesis' was recently developed further to explain the topology and dynamics of intracellular membranes, e.g. stacking of Golgi cisternae and changes thereof during mitosis [thorough discussion of the whole topic in 266].

5. Additionally, a number of small GTP-binding proteins, belonging to the *rab*-subfamily and unrelated to ARF, are also involved in vesicular transport through the secretory [98,238,242] and endocytic pathways [35,337]. Bound to the cytoplasmic face of organelles and vesicles they are located at distinct stations on the secretory pathway (Figure A-3) [103]. Prenylation of the hypervariable C-terminal end is necessary for function of *rab*-proteins [13,182,238]. Although *rab*-proteins are clearly necessary for docking and fusion, their precise role in this process is unknown. It has recently been

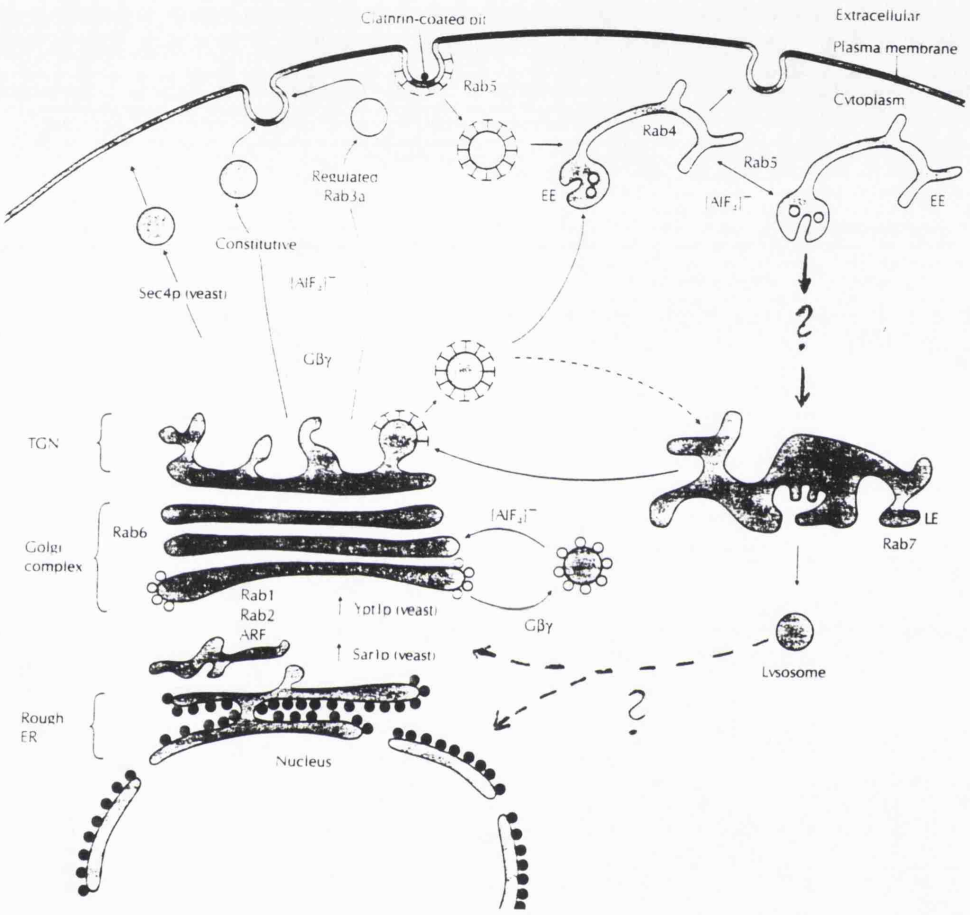


Figure A-3: Main vesicular traffic pathways in mammalian cells The picture shows (slightly modified) the secretory and the endocytic pathway as recently suggested [103]. Small GTP-binding proteins have been localized to specific compartments as far as the experimental evidence reaches. The question mark between early endosomes (EE) and late endosomes (LE) leaves open whether endocytic compartments are preexisting or maturing structures. A transport pathway for ceramide between lysosomes and ER/early-Golgi may exist [152,153], but little is known about its characteristics.

hypothesized that they act as regulators of SNARE interaction [239].

In addition to their role in vesicular intra-Golgi transport coat proteins are also involved in transport between ER, intermediate compartment and *cis*-Golgi [155]. In fact around 70% of membrane bound COPs were found on the *cis*-side of the Golgi [155]. As yet it is not clear to which extent COPs are involved in TGN → plasma membrane or endosomal traffic. In contrast NSF-dependent fusion has been documented in endosome-endosome fusion [60] as well as in vesicular transport between ER and Golgi [20] and intra-Golgi transport [267].

Clearly the final picture of the processes involved in the secretory pathway will be even more complicated than the scheme sketched above. Thus phospholipase D (PLD) might have a role in membrane traffic via interaction with ARF [44] and activation of protein kinase C (PKC) wich stimulates phospholipase D (PLD) apparently enhances binding of ß-COP and ARF to Golgi membranes [55]. These results imply that membrane receptors can modulate flow through the secretory pathway. Recently, the finding that intra-Golgi transport can occur independent of coated vesicles in-*vitro* systems added another complexity to understanding transport on the secretory pathway [322]. However, Elazar et al.[77] showed that removal of ARF and coat proteins from their in vitro assay system
leads to fusion of Golgi stacks, but prevents budding of vesicles. Thus the vesicle coat's primary role might be to prevent uncontrolled fusion with consequent disassembly of intracellular organelles.

A.5.2. Retrieval and retention of ER and Golgi proteins

Compartments on the secretory pathway require a set of resident luminal or membrane-bound proteins which are not carried away by vesicular flow to exert their specific function. Two mechanisms are considered to achieve this objective [217]: "retention signals" would allow proteins to join bulk flow in the secretory pathway until they reach their target organelle, where the signal would retain the protein by restricting it from accessing budding vesicles. In contrast, "retrieval signals" would relocate proteins that have left their target organelle back to their original residence.

This involves the assumption of retrograde transport.

A.5.2.1. Retrieval of proteins

Genetic modification of luminal ER proteins has shown that the luminal ER proteins with the C-terminal amino acid sequence KDEL (Lys-Arg-Glu-Leu) are retrieved to the ER after having travelled to the CGN and early Golgi compartments [234]. This suggests that the KDEL sequence is not a retention signal *per se*, but that it enables a receptor-mediated retrieval of ER proteins [236]. Deletion of the KDEL sequence leads to the secretion of normally ER-resident proteins indicating that default delivery of proteins is to the plasma membrane [234]. In yeast the related sequences HDEL and DDEL are recognized as retrieval signals [236]. Experiments with Saccharomyces cereviseae demonstrated that the C-terminal HDEL sequence can also retrieve a membrane spanning protein [315].

The yeast erd2-gene product, a 26 kD protein with seven membrane spanning domains, has recently been identified as the HDEL-receptor [ref.in 236]. A 50% homologue of this protein serves as the human KDEL-receptor [167,168]. In its unbound form it is located at the *cis*-Golgi, whereas ligand binding induces redistribution of the receptor to the ER [168]. For ER resident membrane spanning proteins two other retrieval signals have been identified: type I proteins (luminal N-terminus) have a double Lys-motif at the -3 and -4 position of their C-terminal end (-KKXX, X any amino acid), whereas type II proteins (luminal C-terminus) have a double ARG (RR) within the first five amino acids of the N-terminus [217]. The cytoplasmic double Lys motif has been shown to interact with coatomer [50], but the molecular details of KK- and RR-dependent retrieval to the ER are still unknown. It is also unknown how the KDEL-receptor is retained in its *cis*-Golgi position.

Recently, the amino acid sequence -YQRL- (Tyr-Gln-Arg-Leu) has been found to act as a retrieval signal in the cytoplasmic domain of TGN38, a TGN resident protein that cycles between plasma membrane and TGN [125,217]. In addition TGN38 has been demonstrated to contain a TGN retention signal in its membrane spanning domain [245].

A.5.2.2. Retention of proteins

Extensive mutational analysis led Munro and Nilsson et al. to conclude that Golgi glycosylation proteins do not possess retrieval signals in their cytoplasmic domain, but that localization to the Golgi is mediated by the membrane spanning domain and the flanking regions thereof [214,385]. However, no specific amino acid sequence could be identified that mediated retention. Two models have been developed to rationalize this phenomenon:

I. *medial*-Golgi enzymes are capable of forming hetero-oligomers [216]. When the cytoplasmic domain of the Golgi enzyme N-acetylglucosamintransferase I (NAGT) was replaced by that of an ER resident protein containing a KKXX signal, not only the NAGT but mannosidase II and probably other Golgi enzymes, too, were restricted to the ER [216]. This was explained as a consequence of oligomerization of "kin proteins" which was regarded as "necessary but not sufficient" for retention. Oligomerization could be related to the observation that the polar residues of the α-helical transmembrane domain of Golgi proteins are lined up on one side of the helix [181].

II. The second model [33] is based on the concept of a cholesterol gradient in the secretory pathway and in the Golgi [224]. It has been shown that the transmembrane domain of sialyltransferase could be replaced by polyleucines without affecting retention in the Golgi [214]. Rather than being sequence dependent, retention appeared to be based on the length and physical properties of the membrane-spanning region [214]. The transmembrane domain of Golgi proteins is shorter than that of plasma membrane proteins [33]. Membranes enriched in cholesterol are supposed to be thicker than cholesterol-poor membranes, chiefly because cholesterol leads to tighter packing of the phospholipid acyl chains. It was suggested for thermodynamic reasons that Golgi enzymes with short transmembrane domains would be denied access to budding vesicles enriched in cholesterol thus selectively directing plasma membrane proteins to the cell surface. The co-existence of cholesterol-poor and cholesterol-rich domains within a bilayer was indicated as a possible mechanism to increase the cholesterol concentration on the secretory pathway [33]. Essentially, this concept presumes that budding of vesicles leads to an increased cholesterol

concentration of the vesicle or that budding is facilitated in cholesterol-rich microdomains.

A.5.3. Endocytosis and receptor-cycling

A.5.3.1. General

Internalization of material from the outside medium into cells occurs either by (facilitated) diffusion across the plasma membrane or by endocytosis [2]. Typically the cytoplasmic leaflet of membrane areas destined for invagination and subsequent budding of endocytic vesicles is covered by a protein coat which will eventually engulf the budded vesicles. Two endocytic systems that seem to require a protein coat and apparently operate independently from each other have been identified thus far.

The first uses 'coated pits', membrane invaginations covered with a protein coat made of clathrin for internalization of macromolecules which bind to receptors gathered in the pit [192]. The coat consists of a protein complex termed clathrin which forms a characteristic 'honeycomb'-like membrane coat [233]. The second endocytic system was discovered only very recently [264]. It employs membrane invaginations termed 'caveolae' which are coated by a filamentous network of proteins and appears to be for uptake of small molecules such as 5-methyltetrahydrofolate [40]. To differentiate the latter system from endocytosis via clathrin-coated pits it has been named 'potocytosis' [9]. In the following the term 'endocytosis' will be used to denote clathrin-dependent endocytosis, whereas the use of potocytosis will refer to internalization of molecules via caveolae.

A.5.3.2. Receptor-mediated endocytosis via clathrin-coated pits

The concept of receptor-mediated endocytosis was based on the pioneering observation by Brown and Goldstein (see [97] and references therein) that cholesterol uptake by cells depended upon binding of plasma low density lipoprotein (LDL) to cell surface receptors, internalization of receptor-bound LDL, release of LDL with

subsequent degradation in the lysosomes and recycling of the unoccupied receptor to the plasma membrane surface. Electron microscopical studies demonstrated that the LDL-receptors were concentrated in 'coated-pits' and that the membrane coat on the cytoplasmic side of the pits consisted of a protein complex termed clathrin [97]. Clathrin has the form of a triskelion consisting of three copies of a heavy, 180 kD chain coupled with three copies of a 30 kD clathrin light chain [38]. Clathrin-coated pits cover 1-2% of the cytoplasmic surface of the plasma membrane [105,192], but are also found on the surface of the TGN where they seem to be responsible for directing the 275 kD mannose-6-P-receptor (M6PR) to the lysosomes [362]. The clathrin-coat of pits or endocytic vesicles has a typical polygonal, cage-like structure reminiscent of a 'honeycomb' [97].

Several other receptors, e.g. the transferrin-, asialoglycoprotein-, EGF- and insulin-receptor, also use clathrin-coated pits as their entry into the cell [32,92,261]. These receptors can roughly be divided into two classes [192]:

Class I receptors such as the EGF- or insulin-receptor are involved in cell signalling. After internalization they are not recycled to the cell surface, but degraded within the cells, providing a potential mechanism for down-regulation of receptors. Class I receptors are localized to clathrin-coated pits only upon binding of their respective ligands.

Class II receptors including the LDL-, transferrin- and asialoglycoprotein receptors are normally clustered in the clathrin-coated pits. They continuously cycle through the endocytic pathway in the absence and presence of ligands.

Most receptors internalized via clathrin-coated pits are membrane spanning proteins with a short cytoplasmic tail [233]. Interaction between receptors and clathrin is not direct, but mediated by two specific adaptor complexes for plasma membrane and TGN-coated pits respectively [233]. It is as yet unclear how membrane specificity is imparted to the adaptors, but the existence of specific adaptor receptors that could mediate interaction between adaptors and receptors has been considered [261]. Both adaptors are heterotetramers which can be separated from each other by hydroxyapatite chromatography, hence their names HA-1 and HA-2 [233]. The plasma membrane HA-2 adaptor is composed of two 100-110 kD subunits called α- and ß-adaptin and two smaller proteins of 17 and 50 kD molecular

weight. The TGN HA-1 adaptor consists of a ß'- and γ-adaptin and two 47 and 20 kD subunits [261]. ß- and ß'-adaptin are closely related to each other, but homology was also found between α- and γ-adaptins [233]. As already pointed out above, ß-adaptin also shares homology with ß-COP [73]. Another essential component of clathrin coated vesicle formation, dynamin, was recently identified following experiments with the temperature-sensitive Drosophila shibire mutant [335,336]. At the non-permissive temperature endocytosis is blocked in these mutants. Dynamin has now been found to possess GTPase activity and to be phosphorylated by protein kinase C [261].

It has been shown that similarly to its effect on ß-COP, BFA causes dissociation of γ-adaptin from the TGN, an effect that could be prevented by pretreatment with AlF_4^- [262]. Not surprisingly, ARF1 was found to be necessary for recruitment of adaptins to the TGN [ref.in 261]. In contrast BFA does not affect plasma membrane association of α- and ß-adaptin [261,38]. Plasma membrane invagination is stimulated by GTP, but resistant to treatment with mastoparan, AlF_4^- and GTPγS which inhibit budding of clathrin coated vesicles [38]. This was interpreted to indicate involvement of multiple G-proteins, including heterotrimeric ones, in receptor-mediated endocytosis [38].

Studies on mutant receptors showed that although the length of the cytoplasmic tails of plasma membrane receptors varies considerably, most contain a crucial tyrosine (phenylalanine) residue whose substitution inhibits endocytosis of the mutant receptor [233]. However, a unique signal sequence for recognition by the HA-2 receptor could not be identified. It has been suggested that the three dimensional configuration of the tyrosine-containing part of the cytoplasmic receptor tail is of prime importance for binding to the HA-2 adaptor [233]. A different target sequence seems to be recognized by the HA-1 adaptor. The 47 kD subunit of this adaptor has a kinase activity which phosphorylates two serine residues on the M6PR [362]. Deletion of the amino acid sequence containing these residues leads to a loss of the sorting function of the M6PR [362].

Clathrin-coated vesicles budded from the plasma membrane have a short half-life ($\leq$ 1 min [10]). *In vitro* studies demonstrated stripping of their clathrin coat by a 70 kD "uncoating" ATPase, hsc70, a member of the heat shock protein family

[233]. It is as yet unclear how the HA-2 adaptor which supposedly carries the targeting information is released from the vesicles [233]. Uncoating is a condition for fusion of incoming vesicles with the early endosomes and this process requires a NEM-sensitive protein [60,196,302]: in cell-free assays fusions can be blocked by treatment with NEM and replacement of NEM-treated cytosol with fresh cytosol restores the fusion capacity.

A.5.3.3. The fate of internalized receptors

Endosomal compartments have been defined morphologically and by functional methods measuring the appearance of internalized markers in isolated endosomal fractions from broken cells [105]. Generally, three different stations on the endocytic pathway are recognised (early endosomes, late endosomes and lysosomes), but a variety of different terms have been used to describe these compartments (e.g. sorting endosomes, recycling compartment). Therefore it is often very difficult to relate the results of different authors. Thus, the statement made by Hopkins in 1986 [120], that "it seems to be too early to adopt a rigid classification system" has not lost its validity.

Vesicles which have budded from a clathrin-coated pit are targeted to the early endosomes, an acidic (pH = 6.0-6.2) compartment which is normally described as a vesiculo-tubular structure at the cell periphery [105]. Functionally, the early endosomes are at an important crossing on the endocytic pathway. From here class I receptors (e.g.EGF-receptor) are directed on a degradative pathway to the late endosomes (a prelysosomal compartment, pH = 5.5) and thence to the lysosomes (pH = 4.5), whereas class II receptors (e.g.asialoglycoprotein receptor, LDL-receptor) are uncoupled from their ligands and recycle back to the surface [97]. The transferrin-receptor represents a somewhat different case as only the iron dissociates in the early endosomes while the receptor bound transferrin recycles back to the cell surface where it is released from the receptor in its iron-free form [97]. It has been suggested [105] that the early endosomes correspond to the CURL (compartment of uncoupling of receptor and ligand) isolated by Geuze et al. from rat liver extracts [92]. Kinetic studies indicate that recycling of receptors to the cell surface is quick,

occurring with a half-time of around 5 minutes [105].

Transport from early to late endosomes occurs via endosomal carrier vesicles and requires an intact microtubule system [103,105,145]. Disruption of the microtubules with nocodazole inhibits the appearance of internalized markers in the late endosomes [10]. By contrast, recycling of the transferrin receptor appears to be independent of microtubules [145,379]. Recycling fluorescent transferrin receptor is often used as a kind of definitive standard marker for the 'recycling pathway' [48,145,209,372]. While fluorescent transferrin certainly marks supposedly endosomal structures at the cell periphery, it also labels a mildly acidic (pH 6.4 $\pm$ 0.2) juxtanuclear region consisting of tubules and vesicles in the vicinity of the Golgi [372]. In contrast, fluorescent α_2-macroglobulin appeared in more acidic (pH 5.4) endocytic vesicles after uncoupling from its receptor [372]. This implies a function for receptor recycling of the juxtanuclear compartment. The juxtanuclear compartment might possibly correspond to the multivesicular body around the nucleus described by Hopkins [120]. According to a recent study sorting of recycling components from lysosomally direct material occurs in peripherally located 'sorting' endosomes, but recycling components accumulated in the juxtanuclear compartment before returning to the cell surface [197].

So far this discussion has tacitly assumed that early endosomes, late endosomes and lysosomes are preexisting compartments [99]. Nevertheless, it must be said that an alternative to this perception has been presented in the "maturation model" [215]. This model regards the early endosomes as the product of the fusion of uncoated endocytic vesicles which mature into late endosomes and lysosomes by the receipt of specific lysosomal enzymes from the Golgi apparatus [215]. This would mean that early endosomes and late endosomes do not possess typical resident proteins. Independent support for the maturation model came recently from a study with a lipophilic fluorophore, TMA-DPH [126]: intensity of the dye in different membranes as well as the uptake kinetics were considered to be explained more easily by the maturation model. However. other investigators favour a model regarding endosomes and lysosomes as preexisting compartments [99]. This latter view has recently been strengthened by the observation that small GTP-binding proteins of the *rab*-subfamily are also involved in the regulation of vesicular flow

through the endocytic pathway [103,238]. In particular *rab*5 and *rab*7 could be assigned to early endosomes and late endosomes respectively. The rather complex morphology of the early and late endosomes which is difficult to envisage as the result of a dynamic maturation process [99,328] was also used to argue that endosomes and lysosomes are preexisting compartments.

A.5.4. Potocytosis

Caveolae are plasma membrane invaginations on the cell surface which are distinct from clathrin-coated pits. They are abundant in fibroblasts, endothelial and smooth-muscle cells, but seem to be present in most cells [264]. Although originally thought to be uncoated, specific electron microscopic preparations led to the discovery of a "striated coat" on the cytoplasmic face of the caveolae, consisting of a filamentous envelope of proteins wrapped around the caveolae [264]. A 22 kD subunit of the coat, termed *caveolin*, was identified by crossreactivity with antibodies directed against a substrate protein of $pp60^{src}$-tyrosine kinase in chicken embryo fibroblasts [264]. Palmitoylation of caveolin might be necessary for anchoring this subunit in the membrane caveolae. Furthermore a conspicuous enrichment of cholesterol and sphingomyelin in the caveolae has been described.

The physiological function of caveolae in a cell has not been established as yet. The invaginate nature of their morphology has been interpreted as evidence for the ability of caveolae to pinch off the plasma membrane, thus forming endocytic vesicles for an alternative pathway for uptake of fluid and low molecular weight molecules [40,264]. However, several studies failed to identify endocytic vesicles originating from caveolae in endothelial cells, probably due to the lack of an appropriate marker [264].

Such a marker has now emerged in the folate receptor. Uptake of 5-methyltetrahydrofolate has been demonstrated to depend on a glycosylphosphatidylinositol (GPI)-anchored receptor which is clustered in caveolae [40]. Cholesterol depletion of the plasma membrane by filipin or nystatin causes unclustering of the receptor and the disappearance of caveolae, whereas the clathrin-coated pits are left intact [40]. These effects are accompanied by a marked reduction

of folate uptake.

It appears possible that in contrast to membrane-spanning receptors, GPI-linked receptors generally accumulate in caveolae. Support for this idea comes from the fact that a GPI-linked chimer of the CD4 receptor is internalized by a pathway which involves "uncoated" invaginations very reminiscent of caveolae [137]. By contrast the natural CD4 receptor uses clathrin-coated pits for internalization [137]. Secondly the GPI-linked Thy-1 antigen is absent from clathrin-coated pits, but assembles in membrane domains which are enriched in cholesterol and sphingomyelin. There it becomes resistant to detergents, a feature which it shares with caveolin which cannot be removed from membranes either by salt washing or Triton X-100 treatment [264]. Additional molecules concentrated in caveolae are ATP- and IP_3-sensitive Ca^{++}-channels, heterotrimeric G-proteins and 5'-nucleotidase, another GPI-anchored protein [45,300]. A recent study demonstrated that activators of protein kinase C inhibit potocytosis by inhibiting the formation of caveolae [300], thus indicating a possible role of these structures in cell signalling.

A.5.5. Are there additional endocytic pathways?

Watts et al.[118] have identified a new class of endocytic vesicles, that can be clearly distinguished from clathrin-coated vesicles. These 'macropinosomes' are transient, tubulo-vesicular structures of up to 0.5 to 2.5 μm diameter. Their formation is induced by EGF and seems to occur in regions of membrane ruffling that are subject to regulation by small GTP binding proteins of the *ras*-related *rho* subfamily [257]. In contrast to another new class of vesicles that are 95 nm in diameter and deliver their contents to early endosomes [109] macropinosomes do not fuse with endosomes nor is their morphology affected by brefeldin A [118].

A.6. Transport of sphingomyelin in eukaryotic cells

A.6.1. Lipid transport in eukaryotic cells

Since vesicular transport of proteins follows the established routes of the secretory pathway it seems inevitable that some lipids must go this way as well. However, there is convincing evidence available that certain lipids are able to bypass the Golgi on their way to the plasma membrane. Kaplan and Simoni reported in two different studies [133,134] that transport of phosphatidylcholine and cholesterol to the cell surface was not affected by inhibitors of vesicular transport through the Golgi like monensin [251]. Furthermore, the half-time at 25°C of only 2 minutes for transport of phosphatidylcholine from the ER to the plasma membrane [134] suggested that the pathway of phosphatidylcholine transport differed from the one for cholesterol since transport of cholesterol had a higher half-time of 10 minutes (at 37°C) and (in contrast to transport of phosphatidycholine) was affected by metabolic inhibitors [133,298]. Van Meer [345], however, pointed out that the original data of Kaplan and Simoni [134] did suggest an additional slower component of phosphatidylcholine transport with a half-time of 30-60 minutes, probably representing the pool being transported concomitantly with proteins through the secretory pathway. The transport of both phosphatidylcholine and cholesterol to the plasma membrane is much quicker than vesicular transport of proteins through the secretory pathway which has a half-time of around 20-40 minutes [54,194 for ref] or that measured for appearance of sphingomyelin at the cell surface [250]. Later studies [333,353] essentially confirmed the above conclusions, with subcellular fractionation demonstrating that bulk transport of the glycerophospholipids phosphatidylcholine, -ethanolamine and -serine did not follow the secretory pathway [353].

Kobayashi and Pagano [143] observed that appearance at the plasma membrane of a fluorescent labelled phosphatidylethanolamine analogue was not inhibited in mitotic cells, indicating that this important aminophospholipid was not transported by a vesicular mechanism [364]. One *caveat*, however, generally applies to the interpretation of data gained in studies using fluorescent labelled or short chain

lipids: in contrast to physiological lipids these analogues are able to diffuse as monomers between membranes [26,131,263]. Thus, they might give misleading information on intracellular lipid traffic. Nevertheless, the inability of cytoskeletal disaggregating agents and metabolic inhibitors to disrupt transport of phosphatidylethanolamine supports the concept of non-vesicular transport of this phospholipid species [54].

A.6.2. A role for proteins in lipid transport?

It is not clear to what extent non-vesicular mechanisms are physiologically involved in transport of other glycerophospholipids and cholesterol [26,54]. Such a mechanism could be provided by the lipid transfer proteins first discovered by Wirtz some 25 years ago [370]. Three major classes of these proteins have been identified [370]: one highly specific for phosphatidylcholine, called PC-TP (phosphatidylcholine transfer protein), one with specificity for PI and PC, termed PI-TP (phosphatidylinositol transfer protein) since it has a higher affinity for PI than for PC, and a group of non-specific lipid transfer proteins (ns-LTP, also called "sterol carrier protein 2").

Although originally considered to mediate exchange of lipids between membranes, a role for these proteins in lipid bulk transport has recently been advocated [358]. Voelker [358] suggested that an energy-independent cytosolic carrier protein is responsible for the quick transport of phosphatidylcholine to the ER to the plasma membrane. Consistent with this view is the correlation between the rate of phosphatidylcholine synthesis and PC-TP levels, which has been observed repeatedly [138,323].

Mutations of the SEC14 gene encoding the PI-TP in S.cerevisiae are lethal which suggests a crucial role of the protein in this organism [16]. The SEC14 gene product seems to control the relative content of PI and PC in Golgi-membranes [381], which might be important for flow through the secretory pathway [177,198]. However, in other yeasts, e.g.Yarrowia lipolytica, SEC14p is required for differentiation to the mycelial form [177]. *In vitro* studies demonstrated enhancement of phosphatidylserine transport from microsomes to mitochondria by nsLTP [359],

but experiments in CHO mutants devoid of the nsLTP clearly showed that phosphatidylserine transport to mitochondria was not dependent on this protein in intact cells [342]. Addition of nsLTP to crude rat microsome fractions increases esterification of cholesterol up to 2-fold implying a capacity of nsLTP to facilitate exchange of cholesterol between different organelle membranes [246,329]. Another study demonstrated that nsLTP is not a cytosolic protein, but located in the peroxisomes; peroxisome-deficient cells showed increased cholesterol synthesis [343]. The confinement of nsLTP to the peroxisomes may lend support to the prevailing concept that intracellular transport of cholesterol is vesicular [133,333,345]. This concept relies on the observation that (a) cholesterol transport appears to depend on metabolic energy and (b) newly synthesized cholesterol appears to accumulate in a vesicular fraction which can be separated from microsomes by density gradient centrifugation [162,333]. However, cholesterol makes its way to the plasma membrane with kinetics very dissimilar to those of vesicular transport and is clearly able to bypass the normal routes of vesicular protein transport through the exocytic pathway [133,333]. Hence, it was postulated that cholesterol transport to the plasma membrane follows a pathway distinct from the secretory pathway [133].

In conclusion, the studies discussed here demonstrate that lipid transfer proteins may well have a yet unclarified role in lipid metabolism and probably transport, but that their function in different cell types/organisms is diverse and probably not confined to mere participation in lipid metabolism.

A.6.3. The arguments for vesicular transport of sphingomyelin

Whereas transfer proteins for glycerophospholipids and cholesterol appear to be part of the standard equipment of cells [370], transfer proteins for sphingomyelin have only been found in either fetal [76] or transformed cell lines [75,334]. This could point to an exclusively vesicular mode of transport for sphingomyelin in normal cells. In fact theoretical arguments exist to support this view.

As pointed out in a foregoing section (A.3.3.) sphingomyelin is located in the outer leaflet of the plasma membrane and probably in the luminal bilayer leaflet of some unidentified intracellular compartment [4]. Present orthodoxy regards the

luminal leaflet of the *cis*- or *medial*-Golgi as the predominant site of sphingomyelin synthesis [90,129]. Since sphingomyelin does not undergo transbilayer movement [375], it was postulated to be delivered to the plasma membrane by vesicular transport along the established routes of the secretory pathway [345]. This model would explain the exclusive location of sphingomyelin on the outer leaflet of the plasma membrane as a consequence of fusion events that finally turn the luminal leaflet of a vesicle into the surface leaflet of the plasma membrane. By analogy, one would guess that transport of sphingomyelin by vesicles is also responsible for delivery of sphingomyelin to its intracellular location. The following experimental observations seem to support this view:

1. Plasma membrane sphingomyelin is restricted to the outer leaflet of the bilayer [8] and is supposed to be synthesized on the luminal site of an early Golgi-compartment [89,129]. Since sphingomyelin is not known to undergo transbilayer movement [345,375], there are few possibilities to explain the location of plasma membrane sphingomyelin other than by assuming vesicular transport.
2. In mitotic cells all vesicular transport is interrupted [364]. Addition to mitotic cells of labelled ceramide which carries a C_6-NBD-fluorescent group in the N-position of the sphingosine backbone to mitotic cells results in the synthesis of fluorescent-labelled NBD-sphingomyelin at a visibly intracellular site [143]. However, the newly synthesized NBD-sphingomyelin is not delivered to the plasma membrane surface, whereas newly synthesized PE is transported to the cell surface with kinetics indistinguishable from those in interphase cells. These findings are in accord with vesicular transport of sphingomyelin.
3. Lipsky and Pagano added NBD-labelled ceramide to cells treated with monensin, a known inhibitor of vesicular transport from the *medial*- to the *trans*-Golgi. NBD-ceramide is converted into NBD-sphingomyelin, but by comparison to control cells the amount of sphingomyelin removable from cells by back exchange with extracellular lipid vesicles is reduced by 50%. This was interpreted to reflect inhibition of sphingomyelin transport through the Golgi [175].

4. Several studies, mainly performed in rat liver hepatocytes, have found an increasing concentration of sphingomyelin and cholesterol in the constituents of the secretory pathway from ER to Golgi and plasma membrane [ref.in 33,153,345]. By analogy to the sorting of proteins on the secretory pathway van Meer [345] pointed out that the higher concentration of cholesterol and sphingomyelin in the plasma membrane demanded some kind of sorting mechanism. It was suggested that the ability of sphingolipids to aggregate by hydrogen-bonding could result in budding of sphingolipid enriched vesicles to the plasma membrane. Insertion of cholesterol into sphingomyelin enriched microdomains was also indicated as a means for sorting of cholesterol in the Golgi [345].

The use of fluorescent-labelled lipid analogues has certainly made possible a deeper insight into the mysteries of the location and trafficking of sphingomyelin in mammalian cells. However, these analogues suffer from the drawback that although they appear to be able to give a true image of metabolic pathways within cells their biophysical properties differ quite markedly from those of the natural molecules. Because of its relatively short fluorescent chain, NBD-ceramide can easily diffuse between membranes [226], a phenomenon that has not been demonstrated for natural ceramide to my knowledge. The capacity to undergo transbilayer movement and to diffuse between membranes creates a problem for the analysis of vesicular lipid traffic since fluorescent compounds such as NBD-ceramide might gain access to sites in the cell its natural counterpart cannot reach. This problem is illustrated by the fact that recycling of NBD-sphingomyelin and NBD-glucosylceramide through the endocytic pathway is largely conservative [144,151,209] whereas cycling of NBD-ceramide is not, as demonstrated by the appearance of fluorescent label in ER and Golgi membranes [174]. The difference between the NBD-labelled sphingolipid analogues is probably due to the hydrophilic headgroups of sphingomyelin and glucosylceramide which should prevent transbilayer movement and access of these lipids to the cytoplasm [284]. An added complication is the recent observation [193] that C5-DMB-GluCer, in contrast to general belief (Ph.Devaux, personal communication), might be able to undergo such transbilayer movement. Pagano &

Lipski [175] have reported that appearance at the cell surface of sphingomyelin synthesized from NBD-ceramide is inhibited by addition of monensin, an ionophore supposed to block vesicular protein transport between *medial-* and late Golgi compartments [251]. However, the effect of monensin on protein transport seemed to be substantially exceed the effect on sphingomyelin transport, a result difficult to reconcile with the conclusion that sphingomyelin transport follows vesicular traffic through the secretory pathway. For these inconsistencies it does not seem satisfactory to regard evidence obtained by the use of fluorescent-labelled sphingolipid analogues as proof for vesicular transport of sphingomyelin.

Newer studies also appear to question the concept introduced above that a sphingomyelin and cholesterol concentration gradient exists on the secretory pathway. In cells infected with vesicular stomatitis virus (VSV) the viral G-protein is transported to the plasma membrane through the secretory pathway [333]. The fungal metabolite brefeldin A (BFA) which is known to interfere with vesicular transport in the Golgi-apparatus blocks delivery of the VSV-G-protein, but not of cholesterol to the cell surface [333]. The kinetics of cholesterol transport to the cell surface ($t_{1/2} \approx$ 10 minutes) [133] are much quicker than those for transport of proteins from the ER to the plasma membrane ($t_{1/2} \approx$ 20-30 minutes) [367]. Accumulation of cholesterol in an unidentified class of vesicles also suggested that transport might occur by a vesicular pathway different from the secretory pathway [133].

Similarly, Shiao & Vance observed that treatment with monensin or BFA did inhibit protein transport to the cell surface, but not delivery of sphingomyelin [281]. In contrast to these authors Warnock et al.[363] found inhibition of sphingomyelin transport to the plasma membrane in BFA treated cells. Thus, the available (functional) data suggest that cholesterol does not follow the secretory pathway to the plasma membrane, whereas the situation is less clear for sphingomyelin. If both molecules are able to bypass the Golgi, why should there be a gradient for cholesterol or sphingomyelin in the Golgi?

Some doubt might be warranted as to the validity of the cell fractionation data concerning the relative content of cholesterol and sphingomyelin in distal Golgi-compartment, since the lipoprotein content of rat liver Golgi and endosome fractions

might considerably have affected these figures. In fact, a study by Taylor et al.[321] that managed to largely avoid this pitfall did not observe any enrichment of cholesterol and sphingomyelin in *trans*-Golgi fractions (purified 400-fold! with respect to galactosyltransferase) over ER membranes. However, when using a conventional fractionation protocol which gave a much lower specific activity of galactosyltransferase they found a higher content of cholesterol and sphingomyelin in the *trans*-Golgi. Therefore the often quoted concentration gradient on the secretory pathway for cholesterol and sphingomyelin might simply be a consequence of impure *trans*-Golgi preparations.

One might therefore ask whether the *cis*-Golgi site of sphingomyelin synthesis is truly the site for synthesis of plasma membrane sphingomyelin. An accumulation of fluorescent labelled ceramide in the *trans*-Golgi as observed by Pagano and coworkers [227,228] would require retrograde transport of ceramide through the Golgi-apparatus for the production of sphingomyelin. Although such retrograde movement might exist [273], it has as yet not been demonstrated unequivocally [266]. An additional sphingomyelin synthesis site distal to the *trans*-Golgi would circumvent this difficulty and avoid the complicated sorting mechanism considered by van Meer [345]. Some justification for this idea comes from the observation by Quinn and Allan [250] that the kinetics of labelling with ^{3}H-choline of the intracellular and plasma membrane pool of sphingomyelin are so different that it is almost impossible to explain their results with the assumption of only one sphingomyelin synthesis site in BHK-cells. Merrill has pointed out [202] that the kinetics of the resynthesis of plasma membrane sphingomyelin after breakdown by an extracellular sphingomyelinase [4,294] indicated a possible involvement of endocytosis in this step, an idea that would also require the assumption of two sites of sphingomyelin synthesis.

A.7. Involvement of sphingomyelin in cell signalling

In the last five years evidence has emerged [for reviews see 147,208] that plasma membrane sphingomyelin and its metabolites may act as a signal transduction mechanism analogous to that based on the hydrolysis of

phosphatidylinositolbisphosphate (PIP_2) to DAG and inositoltrisphosphate (IP_3) [23]. This theory originated from the observation in GH_3 rat pituitary neoplasm cells that DAG stimulated hydrolysis of sphingomyelin to ceramide and subsequent deacylation to sphingosine by a protein kinase C (PKC)- independent reaction [380]. Since sphingosine at μM concentration reversibly inhibits PKC this was considered to constitute the basis of a possible negative feedback control mechanism of PKC activation [203]. Subsequently, it was demonstrated that hydrolysis of sphingomyelin was not due to the acid lysosomal, but to a neutral, Mg^{++}-activated sphingomyelinase residing in the plasma membrane [291]. The analogy with the PIP_2 pathway was driven further by the discovery of a mammalian Ca^{++}-independent ceramide kinase distinct from the DAG kinase [15,149].

Fresh life was given to the hypothesis of a sphingolipid-dependent second messenger system when Davis and colleagues found that sphingosine stimulated phosphorylation of the epidermal growth factor receptor (EGRF) independently from its inhibitory effect on PKC [82]. However, consequent studies showed that the effect was most likely due to ceramide produced by rapid acylation of sphingosine [95]. The ceramide-dependent kinase appears to be a serine/threonine kinase, requiring the minimal recognition sequence X-Ser/Thr-Pro-X [147]. More recently, activation of a cytosolic serine/threonine protein phosphatase by ceramide but not phospholipids was described [63].

Vitamin D_3, tumour necrosis factor α (TNFα), interferon γ and interleukin-1ß act as stimulators of monocytic differentiation in HL-60 cells [107,147,208]. Recent studies [139,220,221] demonstrated that these agents also stimulate rapid hydrolysis of sphingomyelin and activation of a ceramide- dependent protein kinase. Their action can be mimicked by either application of bacterial sphingomyelinases or addition of a permeable ceramide analogue to the undifferentiated HL-60 cells. TNFα, bacterial sphingomyelinase and ceramide (but not other phospholipases (A_2,C,D) and sphingosine) were also able to stimulate ceramide-dependent protein kinase in HL-60 cell extracts [71]. This indicates a tight coupling of TNFα-receptor and the neutral plasma membrane sphingomyelinase. Two recent reports [128,219] have suggested that the induction of programmed cell death, apoptosis, is associated with the production of ceramide from sphingomyelin.

Whereas the present data suggest a role of ceramide as second messenger in cell differentiation or apoptosis, other sphingomyelin derivatives appear to be involved in cell proliferation. Thus, Olivera and Spiegel found recently, that sphingosine-1-phosphate mediates growth stimulation induced by PDGF and FCS mitogens [382].

Clearly, the possibility of a second messenger system acting via hydrolysis of sphingomyelin to ceramide is exciting, not the least because deacylation of ceramide could induce yet another second messenger system. Deacylation could be subject to regulation, too. However, before getting too excited it is probably wise to recall a remark by Michell and Wakeham [208] that kinetic data linking stimulated cellular sphingomyelin depletion to the appearance of its active metabolites are still lacking.

A.8. Clinical significance of sphingomyelin metabolism

In comparison to glycerophospholipids, the turnover of sphingolipids which occurs in the lysosomes is slow. However, perturbations of lysosomal sphingolipid degradation result in severe clinical disorders characterized by hepatosplenomegaly and neurological dysfunctions of a greater or lesser extent [150].

In Niemann-Pick disease, type A and B, the capacity of cells to hydrolyse sphingomyelin is greatly reduced, resulting in intracellular accumulation of sphingomyelin [166]. This is due to a deficient activity of the acid lysosomal sphingomyelinase [30], whereas the neutral membrane-resident sphingomyelinase shows normal activity [91,165]. Analysis of the cDNA clones now available for the acid lysosomal sphingomyelinase suggested that there are at least two isoforms of the enzyme [252]. More than 90% of the cDNA encodes for only one type of the isoenzymes. It has been suggested that a mutation in a region of the gene common to both enzymes is responsible for the more dramatic type A of Niemann-Pick disease, whereas abolition of the activity of the more abundant isoenzyme is the cause for the chronic and benign type B.

A third type of Niemann-Pick disease, NP-C, is less well understood than type A and B. Although increased levels of sphingomyelin are found in cells from patients with NP-C [30], this is not due to a defect of acid or neutral sphingomyelinase [24]. A defect in the saposins, a class of heat stable glycoproteins acting as activators of

lysosomal sphingolipid hydrolases [272], might be involved in the pathogenesis of this disease [153]. NP-C cells also show a conspicuous inability to esterify LDL-derived cholesterol whilst the esterification of endogenous cholesterol after application of bacterial sphingomyelinase is not affected [295].

A strong connection between cholesterol and sphingomyelin metabolism is also evident in atherosclerosis, although the available evidence is of a somewhat phenomenological nature. Atherosclerotic plaques are enriched in sphingomyelin [41,269] and it has been claimed that sphingomyelin contains an increased level of unsaturated fatty acid moieties in atherosclerosis [110]. In line with the parallel accumulation of cholesterol and sphingomyelin in atherosclerotic lesions is the enhanced activity of sphingomyelin synthesizing enzymes in hepatocytes of cholesterol-fed rats [110] which is accompanied by a downregulation of neutral sphingomyelinase activity. It has also been observed that the sphingomyelin levels of a variety of different organs including brain increase with rising age [148]. Apparently, this rise occurs at the expense of phosphatidylcholine levels and seems to be ubiquitous in vertebrates [91]. Although it has been speculated that this will increase the "microviscosity" of cell membranes, the clinical significance of the phenomenon is entirely obscure. Also, numerous authors have pointed out recently that the term "microviscosity of membranes" has no exactly defined physical basis and should therefore be avoided [25,56,341].

A.9. Effects of the fungal metabolite brefeldin A (BFA) on intracellular transport of vesicles

A.9.1. Brefeldin A inhibits protein transport from ER to Golgi

The fungal antiviral antibiotic brefeldin A (Figure A-4) has offered a plethora of new information on the pathways of vesicular transport in mammalian cells [see [142,235] for recent reviews]. The mechanisms of its action will be considered here, since the present study makes extensive use of the drug.

Interest in BFA arose originally because of its ability to almost completely inhibit transport of newly synthesized proteins, including membrane, viral and secretory proteins, from the ER to the Golgi cisternae [172,183,210,218,316]. Interestingly, the proteins retained in the ER as a result of BFA treatment acquire the complex glycosylation pattern of proteins which have been processed by *cis-/medial*-Golgi enzymes (N-acetylglucosaminyltransferase and mannosidase II) [64,172]. Surprisingly this was true even for ER resident proteins [172,332].

A.9.2. Brefeldin A merges ER and Golgi

Immunofluorescence and electron microscopy demonstrated that the Golgi stacks lose their distinctive perinuclear location and acquire a punctate appearance closely associated with the ER [172]. In fact, tubular and tubulovesicular processes appeared to link "Golgi-vesicles" and ER [172,274]. The inference that this indicated fusion of Golgi and ER membranes was corroborated by the observation that not only resident *cis-/trans*-Golgi-enzymes relocated into the ER [172], but also the alleged Golgi-membrane marker NBD-ceramide [171,174]. At first the *trans*-Golgi cisterna was considered to be unaffected by BFA. Later biochemical and morphological studies, however, demonstrated that *trans*-Golgi markers like galactosyltransferase also colocalize with ER markers in BFA-treated cells [3,29,42,270], whereas the TGN-enzyme sialyltransferase does not redistribute [42].

Thus the apparent paradox that proteins retained in the ER are modified by Golgi-enzymes appears to be the result of a fusion of Golgi and ER membranes

Brefeldin A

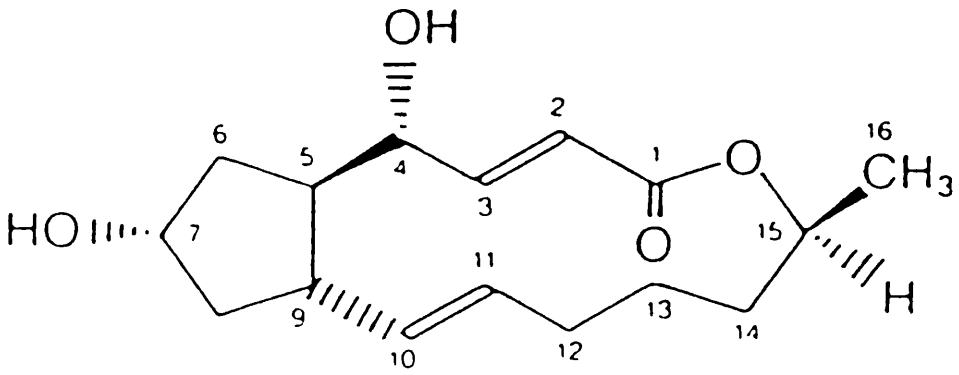


Figure A-4: Structure of brefeldin A. Brefeldin A is a cyclic lactone with 16 carbon atoms derived from palmitate. Derivatisation or changes in the chirality of the carbon atoms in position 4 or 7 respectively, abolish the effect of BFA on morphology and transport of cells [142].

which allows Golgi enzymes access to ER proteins. Effectively Brefeldin A blocks exit from this merged compartment to the TGN as shown by the accumulation of proalbumin in brefeldin A treated rat liver cells [210]. This explains why proteins which are normally secreted and processed by the sialyltransferase are resistant to neuraminidase in BFA-treated cells [170].

A.9.3. Molecular mechanisms of BFA action on Golgi and ER

Relocation of Golgi-enzymes to the ER is a quick process (5-15 minutes) [172], but the earliest detectable effect of BFA (after 30 seconds) is the dissociation of a 110 kD protein from the Golgi-cisternae [70]. Serafini and coworkers [280] were able to identify this protein as the ß-subunit of the cytosolic coatomer (COP)-complex. The membrane-bound form of this complex, which is in a dynamic equilibrium with the cytosolic coatomer, constitutes the non-clathrin protein coat of Golgi-derived vesicles [365,366]. In addition to its effect on the ß-COP brefeldin A prevents binding of the ARF, a small GTP-binding protein belonging to the *ras*-superfamily, to Golgi-membranes [67]. Pretreatment of permeabilized cells with GTPγS enhances binding of ß-COP and ARF to the Golgi-membranes and blocks the effect of brefeldin A on ß-COP and ARF, whereas treatment with GTPγS and BFA in the reverse order releases ß-COP and ARF from the Golgi-membranes [67,69]. AlF_4^- mimics the effect of GTPγS on binding of the ß-COP, but not on binding of ARF. Given that AlF_4^- only affects heterotrimeric, and not small GTP-binding proteins [93,132], this was interpreted to suggest the involvement of a heterotrimeric GTP-binding protein in the binding of ß-COPs. In accord with this view, binding of ß-COP to Golgi-membranes can also be stimulated by mastoparan, an activator of heterotrimeric G-proteins [156]. Finazzi et al.[84] could demonstrate that AlF_4^- protects a small proportion of ARF•GTP from hydrolysis thus enabling binding of ß-COP. Inhibition of the mastoparan effect by pretreatment with pertussis toxin might indicate that this GTP-binding protein belongs to the G_i/G_0 subfamily [156,93]. It is as yet unclear how trimeric G-proteins are involved in binding of coatomer [265].

Recently, two different groups [66,116] reported that a protease-sensitive Golgi

membrane enzyme is responsible for catalysing binding of guanine nucleotides to ARF. BFA inhibits the nucleotide exchange reaction [66,116] and thus blocks binding of the myristoylated ARF to Golgi membranes [66]. Membrane binding of the ARF, however, is conditional for association of ß-COP and coatomer with membranes [65]. It has been suggested to regard binding of ARF as the first committed step in the assembly of vesicle protein coats and that a putative family of compartment-specific, BFA-sensitive guanine nucleotide-exchanging proteins could thus control budding and fusion events in membrane traffic [116].

A.9.4. Unmasking by BFA of retrograde membrane flow from Golgi to ER

Inhibition of the assembly of the coatomer on Golgi cisternae by brefeldin A in a cell-free system also inhibits anterograde vesicular membrane traffic through the Golgi [172]. Whereas transport by coated vesicles is thus inhibited, BFA provokes the generation of uncoated tubular membrane structures between Golgi-cisternae even in the *in vitro* system, eventually leading to the fusion of Golgi compartments with concomitant mixing of their contents [172]. Similar tubulovesicular extensions have been observed *in vivo* [170,173] and have been regarded as the basis of the retrograde membrane flow from the Golgi to the ER [142,170]. The formation of these tubules depends on an intact cellular microtubule system [170] and is hindered by metabolic poisons and low temperature. Forskolin, which reverses dissociation of the ß-COP by BFA in a cAMP-independent way, also reverses the functional and morphological alterations induced by BFA [171].

The capacity of BFA to block anterograde vesicular transport through the secretory pathway unmasks retrograde tubulovesicular transport between Golgi and ER. This suggests that physiologically anterograde and retrograde membrane flow between Golgi and ER are tightly balanced. Support for this concept comes from the recent observation by Klausner and colleagues [123] that in human RD-4 cells overexpression of the human equivalent of the erd2-gene or a related gene, ELP-1, leads to a BFA-like phenotype with the dissociation of the ß-COP from the Golgi cisternae, blockage of secretion and fusion of ER and Golgi. According to Klausner and coworkers the similarity of the phenotypic changes caused by BFA and ELP-1

thus opens the interesting possibility that liganded products of erd2-related genes might inhibit binding of coatomer to the Golgi, thereby triggering budding of an uncoated vesicle which would then carry the ligand-receptor complex back to the ER.

A.9.5. Morphological effects of BFA on post-Golgi compartments

Although BFA blocks exit from the merged Golgi-ER compartment to the TGN it also exerts an effect on post-Golgi compartments. Analogously to the BFA-effect on ER and Golgi, extensive tubulation of TGN, endosomes and lysosomes has been reported as a consequence of BFA-treatment, leading to fusion of TGN and early endosomes after a lag time of around 10 minutes [173,371]. After prolonged incubation the merged TGN-early endosomes compartment may collapse into a perinuclear region around the microtubule organizing centre [173,254]. The extension of tubules is fully reversible upon removal of BFA, requires hydrolysis of GTP and an intact microtubule system [173,254,371]. Different opinions prevail with regard to the role of ATP in this process. In contrast to others [173] Reaves and Banting [254] could not demonstrate an ATP-requirement for the redistribution of the TGN. In some cell lines the TGN seems to preserve its morphological integrity in the presence of BFA, though the Golgi reveals the typical BFA-induced morphological changes [274]. Cells whose Golgi was disassembled by BFA-treatment were protected against ricin and a number of other toxins (including shiga toxin) [237,273,274]. By contrast, BFA did not affect the appearance of the Golgi in MDCK and PtK2 cells, two kidney epithelial cell lines. These cells were made more sensitive rather than protected against ricin by BFA-treatment [274]. Thus the toxic effect of ricin seemed to be negatively correlated with the BFA-effect on the Golgi structure. Entry into cells of diphtheria toxin which is supposed to occur via the endosomes [275] was not prevented by BFA [274].

A.9.6. BFA does not inhibit endocytosis and recycling of surface receptors

In spite of the dramatic effects of BFA on TGN and endosomes, endocytosis is not inhibited by BFA [173,209,210,274,371]. In particular the early endosomes retain their capacity to take up and recycle cell surface receptors (e.g. the transferrin receptor) even if they are part of a merged TGN/early endosome compartment [173,209,371]. Some discrepancy, however, exists as to how far further steps on the endocytic pathway are affected by BFA. Misumi et al.[210] did not find an inhibitory effect of BFA on the degradation of ^{125}I-asialofetuin in rat hepatocytes. Consistent with this observation Wood and colleagues [371] did not observe an inhibition of fluid phase transport from the early endosomes to the prelysosomal compartment (late endosomes), whilst Lippincott-Schwartz et al. noticed a clear repression of endosome-lysosome traffic in BFA-treated cells [173].

Recently Miller et al.[209] reported that BFA treatment of BHK cells inhibited release of VSV G-protein from the TGN to the cell surface, but caused no inhibition of endocytosis and recycling of the transferrin-receptor. Similarly, regulated secretion of sulfated secretogranin II in PC12 cells was completely blocked by BFA [209]. However, the target for BFA in this case is most probably not ß-COP since this component is associated with the Golgi apparatus. Instead, the BFA effect on constitutive and regulated secretion appears to be due to the BFA-dependent dissociation of γ-adaptin from the clathrin-coat of the TGN, but not the plasma membrane in BHK, VERO and MDCK cells [Miller & Moore, unpublished observation in 209,262]. Analogously to the recruitment of ß-COP to the Golgi apparatus, AlF_4^- and GTPγS increase binding of γ-adaptin to the TGN and block the effect of BFA [262]. Similarly BFA obstructs the transport of proteins from the TGN to the apical surface in MDCK-cells at concentrations where delivery to the basolateral surface is not influenced [178].

Lippincott-Schwartz et al.[173] and Miller et al.[209] attempted to conceptualize the effect of BFA on protein transport within different cell lines by assuming the existence of two "heterotypic" intracellular membrane systems in mammalian cells: an ER/*cis*-, *medial*-, *trans*-Golgi system on the one hand and a TGN/endosomes complex on the other hand. Heterotypic denotes compartments between which

transport is forbidden in the presence of BFA, homotypic organelles are those which exchange membranes in the presence of BFA. Nevertheless, the fact that BFA influences both systems might point to a common target for the drug. Ktistakis et al.[157] used the BFA-resistant cell line PtK_1 to show that dissociation of ß-COP from the Golgi depends on a nondiffusible factor, which may probably be involved in the regulation of γ-adaptin separation from the TGN-membrane, too.

A.9.7. Identification of ganglioside synthesis sites using BFA

Whereas much insight into the effects of BFA on protein transport and metabolism has been gained over the past few years, less attention was been directed towards the impact of BFA on lipid metabolism. The biosynthesis of gangliosides is supposed to be executed by membrane bound glycosyltransferases located at different stations of the secretory pathway [244,271,340]. This should make ganglioside biosynthesis susceptible to agents known to interfere with vesicular protein traffic through the secretory pathway. Indeed, two detailed studies [339,374] demonstrated that ganglioside synthesis beyond the formation of GM3 and GD3 (see nomenclature in 278) was inhibited by BFA. By contrast, lower sphingolipids (glucosylceramide, lactosylceramide, GM3, GD3) accumulated during treatment with BFA. In view of the more recent information that BFA blocks exit of vesicles from the *trans*-Golgi to the TGN [235] this suggests that the synthesis of higher gangliosides (GA2, GM2, GD2 and so forth) is a function of a distal part of the Golgi. This hypothesis supposes that access of simpler glycosphingolipids to these sites by vesicular transport is prevented by BFA.

However, in addition to the 20-fold increase in metabolic labelling of lower sphingolipids in primary cultures of mouse cerebellar neurons van Echten and coworkers [339] observed an approximately 60% reduction in the labelling of SM. This result is surprising since a single SM-synthesis site in the *cis-/medial*-Golgi (as proposed recently by several authors [90,129,175]) would not be expected to be affected by BFA. Also, it is difficult to see how the fusion of ER and Golgi membranes should suppress SM synthesis. Delivery of ceramide, which is most likely produced in the ER [185], to the phosphocholine transferase is considered to

be the rate limiting step of SM-synthesis [186], so if anything one would rather expect fusion of ER and Golgi to increase synthesis of SM.

A.10. Objectives of this study

The foregoing review of our present knowledge on sphingomyelin and its metabolism shows that there is clear relationship between the metabolism of sphingomyelin and cholesterol. Nevertheless, sphingomyelin and cholesterol appear to follow quite different pathways to the plasma membrane from their presumed sites of synthesis in the *cis*-Golgi and the ER respectively. Whereas little is known about the pathways of cholesterol transport, evidence based on sphingomyelin produced from fluorescent labelled ceramide suggested that sphingomyelin might follow the secretory pathway on its way to the cell surface. It has also been pointed out above that although present orthodoxy locates sphingomyelin synthesis to the *cis-/medial-*Golgi apparatus some experimental results obtained in the past point to the existence of a second sphingomyelin synthesis site in the cell. Therefore the following questions will be addressed in this study:

1. The effects of a new agent, brefeldin A, on the transport and metabolism of sphingomyelin will be analyzed.

2. The open question of one or two intracellular sphingomyelin synthesis sites will be addressed. Function and location of the presumed synthesis sites will be assessed.

3. The effects of inhibitors of sphingomyelin transport to the cell surface on cholesterol metabolism will be analyzed.

B. Materials and Methods

B.1. Materials

Radioactive ^{3}H-acetate and ^{3}H-choline were purchased from NEN Dupont (UK). Non-radioactive chemicals used in this study were:

ascorbic acid	BDH
brefeldin A	Sigma Chemicals
cytochalasin D	Sigma Chemicals
2-deoxy-D-glucose	Sigma Chemicals
forskolin	Sigma Chemicals
Minimal Essential Medium (Glasgow modification)	Gibco
lipid standards	Sigma Chemicals
monensin	Sigma Chemicals
nocodazole	Sigma Chemicals
penicillin	Imperial Chemicals
perchloric acid	BDH
Silica t.l.c. plates	Merck, Darmstadt, FRG
sodium cyanide	BDH
sodium molybdate	BDH
vinblastine	Sigma Chemicals
wheat germ cytosol	kind gift from Dr Paul Quinn, University College London

All organic solvents were of analytical quality and bought from BDH (UK). Ultima Gold (high flash point liquid scintillation cocktail) was obtained from Canberra Packard.

B.2. Media

Minimal Essential Medium (Glasgow modification, GMEM) was supplemented with bicarbonate (25 mM). The medium was sterilized by filtration of the freshly prepared solution through a Sartorius Sterilin filter (0.3 μm pore size) into sterile glass bottles. 10 ml aliquots were taken from each bottle and incubated for 24 hours at 37°C to check for bacterial contamination. The stock bottles were then stored at 4°C until further use.

Growth medium was completed by addition of 50 ml foetal calf serum (FCS), 50 ml tryptose phosphate broth and 10 ml of a penicillin-streptomycin-solution ([penicillin] = 5000 I.U./ml, [streptomycin] = 5000 uM) to 900 ml GMEM. For trypsinisation of cells a 0.25% solution of trypsin in Dulbecco's phosphate buffered saline containing 0.02% EDTA was used.

B.3. Methods

B.3.1. Culturing of BHK-21 cells

For the purposes of this study it was decided to use BHK-21 cells since this cell line is very well characterized in biochemical and stereological respects. A BHK-21 fibroblast clone was originally obtained from the EMBL (Heidelberg, Germany). Cell stocks were grown in Falcon flasks (75 cm^2) containing 15 ml growth medium (0.2 ml medium per cm^2) and routinely passaged after two days when the cells were just confluent. The supernatant was poured away and the cells which remained attached to the bottom of the flask were washed once with 3 ml medium A. Subsequently the cells were incubated with 2 ml of fresh medium A at room temperature until the cells visibly detached from the bottom of the flask. 5 ml growth medium were added to the flask taking the total volume to 7.5 ml (since a residual volume of about 0.5 ml could not be removed from the Falcon flask by decanting). The cell suspension (equivalent to 75 cm^2 of confluent cells) was sucked up and down in a sterile plastic pipette until there were no more cell clusters to be seen.

The cell suspension was then further diluted into growth medium (total volume equivalent to 0.2 ml medium per cm^2 petri dish) and seeded out into plastic Falcon petri dishes. Dilution was done on an area basis: if cells were needed on the following day a volume of the cell suspension equivalent to a fifth of the total area of the petri dishes had to be added to the growth medium, whereas a dilution factor of 15 was chosen for preparation of cells on the second day.

B.3.2. Radioactive labelling of cells

For short-term labelling experiments subconfluent cells were incubated for up to six hours in the presence of 20 μCi ^{3}H-acetate or 4 μCi ^{3}H-choline per dish. Cells were also labelled to equilibrium by incubating them for 48 hours with 5 μCi ^{3}H-acetate or 2 μCi ^{3}H-choline. During this time BHK-21 cells go through approximately four doublings, meaning that around 94% of the cell mass had been synthesized *de novo* during this time.

B.3.3. Extraction of lipids

Lipids were extracted routinely from BHK-21 cells by the method of Bligh & Dyer [27]. To this end the medium was removed quantitatively and 1.9 ml of a chloroform:methanol solution (1:2, v/v) were added to the dishes which were then stored for 30 minutes at -12°C to allow complete extraction of the lipids. Hydrophobically associated lipids are extracted by non-polar solvents as chloroform, whereas methanol is used here to disrupt hydrogen bonds and electrostatic interactions between membrane lipids and proteins. Thus there is no need to homogenize the cells since they are broken upon addition of the Bligh & Dyer solution [135]. The addition of Bligh & Dyer solution to plastic dishes is certainly unconventional. However, provided that there was no extracellular water present, the solution did not seem to dissolve the dishes. Equally, we did not observe an adverse effect of this technique on the lipid extraction by comparison with cells which had been scraped from the dishes.

The cell extract was then transferred quantitatively into glass test tubes. A

two phase system, chloroform and methanol-water, was generated by adding 0.6 ml chloroform and 1.1 ml isotonic NaCl to the cell extract. The phases were subsequently separated by centrifugation of the tubes for 5 minutes at 900 rpm. Under these conditions all water-soluble components (which in this case are cytoplasmic solutes) partition into the methanol-water phase, while the lipids are found in the bottom chloroform phase. After separation of the phases the chloroform phase was transferred into another set of glass test tubes with a glass Pasteur pipette. The chloroform phase was then evaporated to dryness *in vacuo*. In some cases a small volume of benzene (50 μl) was added prior to evaporation to facilitate removal of trace amounts of water. Subsequently the lipids were dissolved in 100 μl chloroform and transferred to silica plates for lipid analysis. Where required the samples were split into two 50 μl aliquots which were used for phospholipid and neutral lipid analysis respectively.

B.3.4. Lipid analysis

B.3.4.1. Separation of phospholipids

Phospholipids were routinely separated by thin layer chromatography (t.l.c.) using a modification of the solvent-system recommended by Skipski et al.[290] consisting of chloroform/methanol/acetic acid/water (75:25:12:2, by volume) and identified by comparison with standard lipids bought from Sigma. R_f values in this solvent were ~0.06 for sphingomyelin, ~0.13 for phosphatidylcholine, ~0.63 for phosphatidylethanolamine. Phosphatidylserine and phosphatidylinositol were not separated from one another, but ran as a common band between phosphatidylcholine and phosphatidylethanolamine (R_f value 0.39). Spots were visualized by staining with iodine vapour, scraped and transferred into Pico Prias counting vials (Canberra Packard). 0.2 ml of methanol/water/acetic acid (5:3:2, by volume) were added to the vials to elute the lipids from the silica gel. Finally 2 ml of Ultima Gold scintillation fluid were added and the samples counted for radioactivity in a Canberra Packard TR 2500 l.c.scintillation counter.

B.3.4.2. Separation of neutral lipids

Before loading the neutral lipid samples on t.l.c.plates 5 μl of a lipid carrier solution were added to the samples. The carrier solution contained ceramide, cholesterol, fatty acids, 1,2-diacylglycerol, triacylglycerol and cholesterol ester. For routine determination of neutral lipids a solvent consisting of benzene/diethylether/acetic acid (50:40:0.1, by volume) was used. This system offers a satisfactory separation of phospholipids, ceramide, cholesterol, diacylglycerol and triacylgylcerol/ cholesterol ester with R_f-values of ~0.15, ~0.36, ~0.51 and ~0.88. However, for reliable separation of triacylglycerol from cholesterol ester, t.l.c. plates were taken out of the tanks when the solvent was about 7 cm from the top, dried in a laminar flow hood and rerun in a system consisting of hexane/diethylether/acetic acid (80:20:1, by volume). R_f-values in this solvent were 0.92 for cholesterol ester, 0.75 for fatty acid methyl ester, 0.58 for triacylglycerol, 0.28 for fatty acid, 0.1 for cholesterol and 0.05 for diacylglycerol. Following staining with iodine vapour spots were then prepared for quantification of radioactivity as described above.

B.3.4.3. Distribution of radioactivity in cholesterol ester

In some experiments the distribution of radioactive label between the cholesterol and the acyl chain moiety of cholesterol was determined. Since staining with iodine vapour is destructive to cholesterol ester, the cholesterol ester spot had to be scraped from unstained plates. Cholesterol ester was then eluted from the silica with 1 ml 0.3 N methanolic sodium hydroxide (10 ml 3 N NaOH diluted to 100 ml with methanol) and hydrolysed for 2 hours at 60°C. After acidification with 0.1 ml 6 N hydrochloric acid the lipids were extracted into light petroleum (boiling point 30-60°C). The petroleum phase was evaporated in a desiccator and the dried residue separated in hexane/diethylether/acetic acid as above after addition of suitable carrier lipid. The spots corresponding to cholesterol, non-esterified fatty acid and fatty acid methyl ester were scraped and counted for radioactivity. More than 95% of the cholesterol ester radioactivity was recovered in cholesterol, non-esterified fatty acid

and fatty acyl methyl ester.

B.3.4.4. Analysis of sphingolipids

To analyze the distribution of radioactivity in sphingolipids total lipid extracts were subjected to mild alkaline methanolysis for deacylation of glycerophospholipids as described in [135]. 0.4 ml chloroform, 0.6 ml methanol and 1 ml freshly prepared 0.2 N methanolic sodium hydroxide (0.4 mg NaOH in 50 ml methanol) were added to the dried lipid extract and left for 20 minutes at room temperature. To generate a two phase system 0.4 ml methanol, 1.6 ml chloroform and 1.8 ml H_2O were added to the glass test tubes subsequently. The mixture was vortex-mixed and the phases separated by centrifugation (5 minutes at 900 rpm). The bottom phase containing sphingolipids and neutral lipids was evaporated and the residue dissolved in 50 μl chloroform. 5 μl of concentrated brain lipids, which are enriched in sphingolipids, were added as a carrier. The sphingolipids were then separated on t.l.c.plates in a solvent made up of chloroform/methanol/water (35:12:2, by volume). The spots were stained with iodine and identified by comparison with authentic lipids comprising sphingomyelin, glucosylceramide, lactosylceramide and ganglioside GM3. The radioactivity in individual spots was then determined as described previously.

B.3.4.5. Determination of phospholipid phosphorus

Phospholipids were extracted from plastic dishes (10 cm diameter) with 3.8 ml Bligh & Dyer solution as described above. After addition of 2.2 ml NaCl and 1.2 ml chloroform the samples were mixed and the phases separated by centrifugation. The chloroform (bottom) phase was evaporated in a vacuum dryer. Lipids were dissolved in 50 μl chloroform and separated on t.l.c. plates in a Skipski system. The individual spots were scraped and transferred into glass tubes for determination of phospholipid phosphorus according to a modified Bartlett procedure [18]. To some tubes an area of pure silica equivalent to the area covered by spots was added; these tubes were treated in exactly the same way as the ones containing phospholipids and

served as blanks.

To each of the glass tubes 0.2 ml perchloric acid (70%) were added and the tubes were heated for one hour at 180°C in a fume hood. Subsequently 1.5 ml H_2O, 0.1 ml sodium molybdate (5%) and 0.2 ml ascorbic acid were pipetted into the tubes which were then heated for 20 minutes at 60°C. After centrifugation of the tubes for 5 minutes at 2000 rpm the absorption of the supernatant was read at 830 nm and corrected for the absorption of blanks.

B.3.5. Treatment of cells with BFA

BFA was kept as a stock solution of 5 mg/ml in DMSO at 4°C. Generally cells were pretreated for 15 minutes with BFA before addition of radioactive lipid precursors. An equivalent amount of solvent was added to control cells to ensure strict comparability of the experimental conditions and this was also done where reagents other than BFA were used.

B.3.6. Treatment of cells with forskolin

Forskolin was stored at 4°C as a 100 mM solution in DMSO. To test whether forskolin would reverse or prevent effects of BFA on lipid metabolism cells were pretreated in the following way before addition of radioactive label: either with nothing or with BFA (0.25 or 1 μg/ml) or forskolin (100 μM) alone for 30 minutes. Other samples were pretreated first for 30 minutes with BFA (0.25 or 1 μg/ml) alone and then for another 30 minutes with BFA and forskolin (100 μM), or vice versa with 100 μM forskolin for 30 minutes first and then for a second 30 minutes period with forskolin and BFA. 10 μCi ^{3}H-acetate were added at the end of the preincubation period to each of the dishes and the cells further incubated for four hours. Treatment was finished by washing the cells three times with cold isotonic NaCl and subsequent extraction of the lipids with 1.9 ml Bligh & Dyer solution.

B.3.7. Measurement of LDH-activity

The extent of permeabilization of BHK-21 cells by digitonin was determined by the release of cellular lactate dehydrogenase (M_r = 140 kD) activity into the supernatant. Lactate dehydrogenase (LDH) catalyses the reduction of pyruvate by NADH:

$$Pyruvate + NADH + H^+ \rightleftharpoons L\text{-}Lactate + NAD^+ \quad (2)$$

The reaction can be followed by measuring the decrease in absorption of NADH at 340 nm. If reactants are available in excess the decrease in NADH absorption is a measure of LDH concentration. The routine LDH assay comprised a total volume of 200 μl: 20 μl supernatant were added to 145 μl PBS and 25 μl pyruvate (40 mM), the reaction was started by addition of 10 μl freshly prepared NADH (1 mM). The absorption of NADH was followed for 3 minutes in intervals of 15 seconds. LDH activity in the supernatant of digitonin-treated cells was compared to the activity of cells treated with 0.1% Triton X-100, which released all their intracellular LDH.

B.3.8. Preparation of mitotic cells

A modified version of the method of [83] was used for preparation of mitotic cells. BHK-21 cells were grown to subconfluence in 75 cm^2 Falcon flasks. Cells of one flask were seeded out into 850 cm^2 Corning roller bottles containing 50 ml growth medium plus 500 μCi ^{3}H-acetate. The bottles were incubated under constant rotation for two days at 37°C. To synchronize the cells in interphase thymidine was added to the bottles to a final concentration of 5 mM at the end of day one. Following incubation overnight for 10-12 hours at 37°C the bottles were rotated for 5 minutes at 200 rpm to remove loosely attached cells and cell debris. The medium was removed and the cells washed twice with cold growth medium. Subsequently the cells were reincubated for 3 hours in 50 ml radioactive (10 μCi ^{3}H-acetate/ ml) growth medium plus 40 ng/ml nocodazole. This treatment blocks the cells in metaphase after passing G2. Mitotic cells (which are less strongly attached to the bottle wall) were harvested by rotating the roller bottles for 15 minutes at 200 rpm.

The cells were then concentrated by centrifugation at 1000 rpm for 5 minutes and resuspended in 20 ml serum-free growth medium. Around $1.5\text{-}3 \cdot 10^7$ cells were thus obtained from each roller bottle equivalent to a mitotic yield of around 15-30%. The viability of cells was routinely checked with trypan blue which was normally excluded from more than 95% of cells.

B.3.9. Study of SM resynthesis in mitotic cells

Three 0.5 ml aliquots were taken from the resuspended mitotic cells for lipid determination before and after treatment of the cells with B.cereus sphingomyelinase (0.1 units/ml) for 20 minutes at 37°C. The remainder of the sample was divided into two portions and washed twice with growth medium with or without nocodazole (40 ng/ml). The cells were suspended to a volume of 12 ml in growth medium with and without nocodazole and seeded out into Falcon plastic dishes. Lipids were extracted from the dishes after one, two and four hours with 1.9 ml Bligh & Dyer solution.

B.3.10. Energy-depletion of BHK-21 cells

Cells were labelled for two days with ^{3}H-acetate in growth medium. To study the effect of energy depletion on sphingomyelin resynthesis 50 mM 2-deoxy-D-glucose and 50 μM sodium cyanide were added to the labelling medium 2 hours before the end of the labelling period. The radioactive medium was removed and the sphingomyelin resynthesis assay performed as described above in serum-free GMEM containing 50 mM 2-deoxy-D-glucose and 50 μM sodium cyanide.

B.3.11. Aluminium Fluoride effect on SM resynthesis

Cells labelled to equilibrium were treated with B.cereus sphingomyelinase. To investigate the effect of AlF_4^- on sphingomyelin resynthesis cells were incubated in growth medium supplemented with 30 mM sodium fluoride and 62.5 μM $AlCl_3$. As described by Melancon et al.[199] this leads to the formation of intracellular AlF_4^-.

B.3.12. Block of transport through the Golgi with monensin

Monensin was kept as a 10 mM stock solution in ethanol. Monensin was added to cells 15 minutes prior to addition of radioactivity to a maximum concentration of 10 μM and control cells received the same amount of pure ethanol (max.1 μl/ml).

B.3.13. Permeabilization of cells with digitonin

Digitonin was kept as a 10 mM stock solution in DMSO. The drug permeabilizes cell membranes by complexation of cholesterol. Cells labelled to equilibrium were treated with bacterial sphingomyelinase. After removal of the enzyme by washing, cells were incubated in 1 ml of ice-cold serum-free medium (which does not contain cholesterol) supplemented with 50 μM digitonin. The cells were then washed two times with 1 ml of medium enriched with FCS to remove any excess digitonin. The cells were then reincubated at 37°C for up to 3 hours in 1 ml of serum free medium or an intracellular replacement buffer or an intracellular replacement buffer supplemented with 20% wheat germ cytoplasm. Intracellular replacement buffer was prepared following a method developed by Prof.Jack Judah, University College London, and consisted of K-glutamate (100 mM), $MgCl_2$ (1 mM), NaCl (10 mM), $CaCl_2$ (0.1 μM), EGTA 3 mM, ATP (1 mM), creatine phosphate (5 mM), creatinephosphokinase (15 μg/ml), GTP (0.1 mM), DTT (500 μM) and PIPES buffer 25 mM. The pH of the buffer was adjusted to 6.8.

B.3.14. Disruption of the cytoskeleton with nocodazole or cytochalasin D

Nocodazole and cytochalasin D were made up as 10 mM stock solutions in ethanol. Cells labelled to equilibrium were preincubated in 1 ml GMEM containing 10 μM of the respective drug. Sphingomyelin breakdown and resynthesis was carried out in the presence of the drugs.

B.3.15. Treatment of cells with NEM

A 100 mM stock solution of NEM in distilled water was prepared freshly before use. Cells labelled to equilibrium were treated with exogenous sphingomyelinase as described above. After washing away the sphingomyelinase cells were briefly incubated for 5 minutes at 37°C in prewarmed GMEM containing 100 μM NEM. Cells were then washed two times with serum-free medium and further incubated in GMEM at 37°C to study resynthesis of sphingomyelin. Alternatively, 100 μM NEM were added after degradation of sphingomyelin and kept present during the whole resynthesis period. In a third mode cells were preincubated with 100 μM NEM at 37°C for 30 minutes in GMEM and all further steps (washing, degradation and resynthesis of sphingomyelin) were done in the presence of NEM.

C. Results

C.I. Effects of brefeldin A on lipid synthesis and sphingomyelin transport in BHK21

C.I.1. Motivation for the use of BFA in this study

In the foregoing section the effects of BFA on vesicular transport and the compartmental organization of a cell have been outlined. If SM is indeed only transported by vesicles as suggested by van Meer [345] and Kobayashi and Pagano [143] then BFA as a potent inhibitor of vesicular transport on the secretory pathway should prevent transport of SM from its synthesis site in the *cis*-Golgi to the plasma membrane surface. Secondly, a putative block of SM-transport to the cell surface by BFA could have repercussions on cholesterol esterification similar to those induced by breakdown of surface SM by exogenous sphingomyelinases [293,296]. Finally it appeared interesting to investigate what effect fusion of ER and Golgi would have on SM synthesis in a non-specialized cell line like BHK fibroblasts.

C.I.2. Experimental findings

C.I.2.1. Incorporation of ^{3}H-acetate into BHK21-cells

To monitor *de novo* synthesis of lipids in BHK cells ^{3}H-acetate was chosen as a radioactive label since it is readily taken up by cells and after metabolism to ^{3}H-acetyl-CoA will be incorporated into all lipids approximately in proportion to their mass (compare Table 2 and 3). Therefore, it was assumed that incorporation of acetate label into lipids gave a fair indication of relative lipid synthesis. The cells were incubated with ^{3}H-acetate (20 μCi per dish) for up to four hours in growth medium. Lipids were extracted, separated and analyzed for radioactivity in individual classes as described in the Materials and Methods section (B.3.3./B.3.4.). Figure I-1 shows the labelling with ^{3}H-acetate of SM, PC, cholesterol and triacylglycerol. At

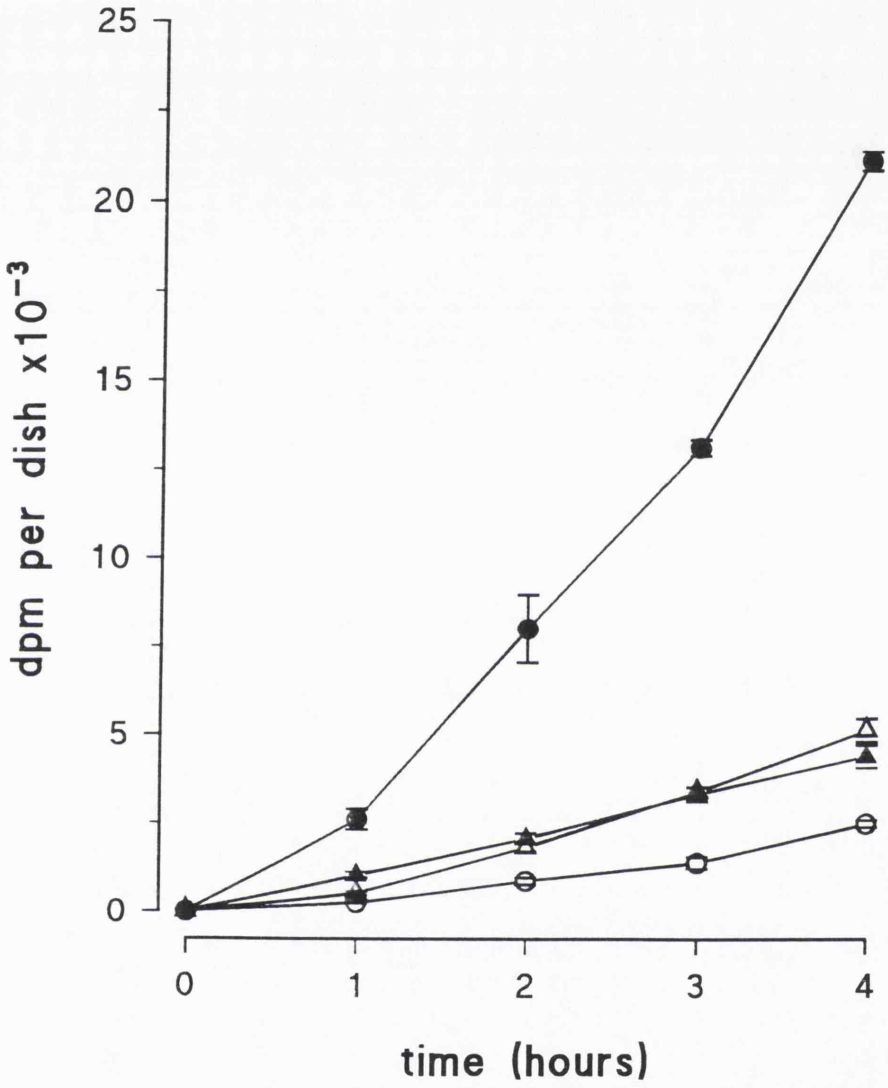


Figure I-1: Incorporation of ^{3}H-acetate into phosphatidylcholine, sphingomyelin, cholesterol and triacylglycerol. Cells were incubated for up to 4h with ^{3}H-acetate and lipids were extracted and analysed as described under Methods. Values shown are means and standard deviations for triplicate samples in one experiment which was repeated on two further occasions with substantially the same results. ● phosphatidylcholine; ○ sphingomyelin; ▲ triacylglycerol; △ cholesterol.

least 2000 d.p.m. were incorporated into each of these lipid classes after four hours.

C.I.2.2. Effects of BFA on incorporation of ^{3}H-acetate into lipids

In order to investigate the effect of a fusion of Golgi- and ER membranes on lipid synthesis BHK cells were labelled with ^{3}H-acetate for four hours in the presence of BFA. The addition of brefeldin A (0 - 5 μg/ml) to the growth medium caused dramatic alterations of the incorporation of ^{3}H-acetate into newly synthesised lipids (Figure I-2). After four hours radioactivity in sphingomyelin and cholesterol ester was increased 3 - 4fold over control cells, whereas corresponding decreases of ^{3}H-acetate labelling were observed in cholesterol (by 30 - 40%) and triacylglycerol (by 35 - 50%). A reduction of radioactive label was also measured in phosphatidylcholine (up to 15%), but no significant changes were observed in the other glycerophospholipids, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine (Table 1). Concentrations of BFA as low as 0.1 - 0.25 μg/ml (0.36 - 0.9 μM) were sufficient to trigger the above changes in labelling of the various lipids.

In addition to the effect on phospholipids and neutral lipids, distinct effects of BFA on ^{3}H-acetate labelling were noticed in two more sphingolipids, ceramide

Table 1: Incorporation of ^{3}H acetate into BHK cells exposed to BFA and/or sphingomyelinase. Lipids shown in this table were measured in the same experiment that is shown in Figure I-4 (unit = % of total lipid radioactivity).

	CONTROL	+SMase	+BFA	+BFA+SMase
PS/PI	3.6 ± 0.1	4.2 ± 0.4	4.4 ± 0.4	4.5 ± 0.7
PE	9.1 ± 0.6	9.0 ± 0.1	9.1 ± 0.5	9.1 ± 0.3
TAG	7.3 ± 1.3	8.6 ± 0.5	5.7 ± 1.2	6.1 ± 1.3
CHOL.EST.	0.6 ± 0.1	ND	2.4 ± 0.3	ND
total d.p.m.	34645 ± 3883	43191 ± 8022	33007 ± 2751	33998 ± 3308

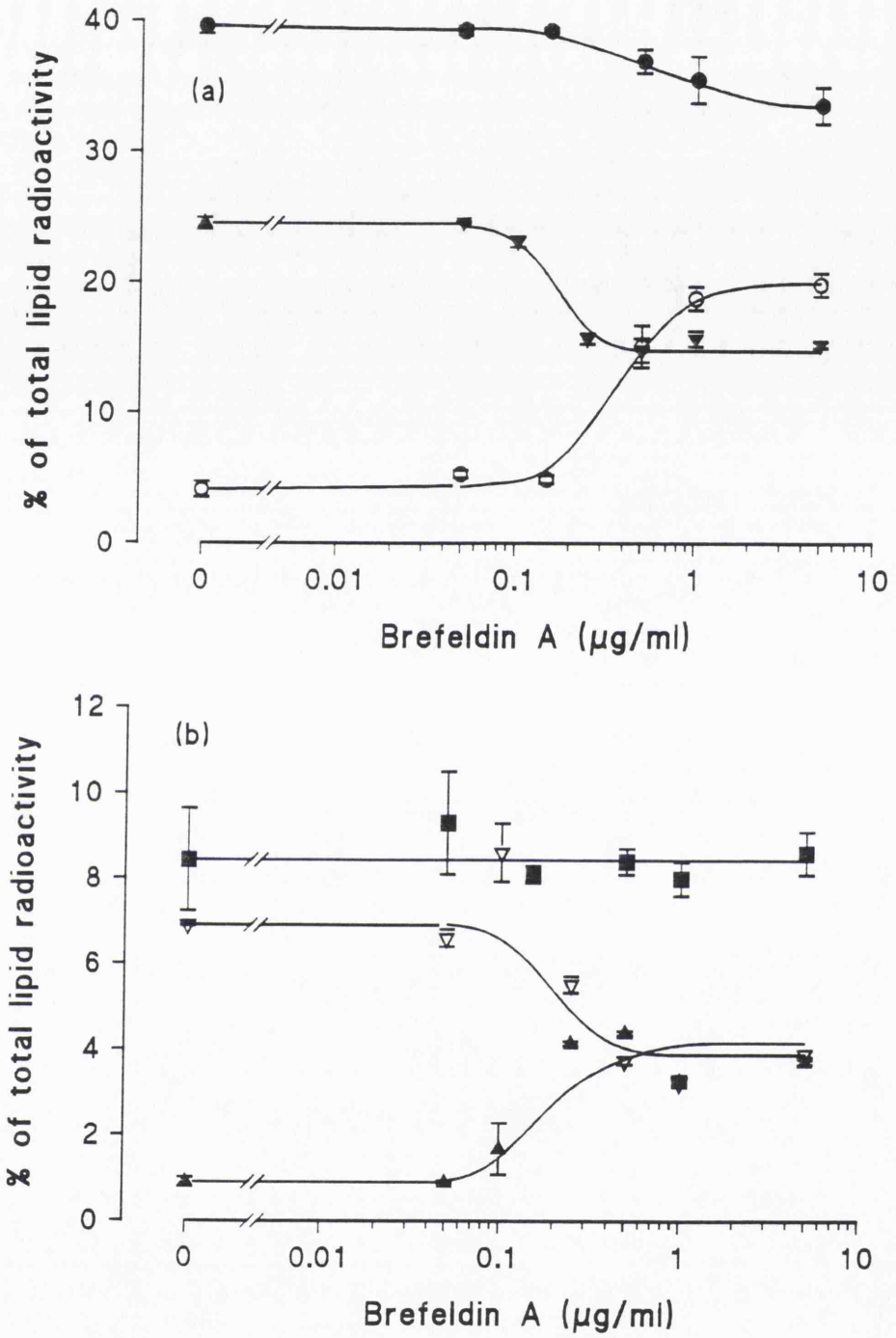


Figure I-2: Effect of BFA concentration on incorporation of ^{3}H acetate into lipids of BHK cells. Cells were incubated for 4h with ^{3}H acetate in the presence of different concentrations of BFA. Lipids were extracted and analysed as described under Methods. Values shown represent means and standard deviations for triplicate samples, virtually the same results were obtained in two further experiments. **(a)** ●, phosphatidylcholine; ○, sphingomyelin; ▾, cholesterol; **(b)** ▿, triacylglycerol; ▲, cholesterol ester; ■, phosphatidylethanolamine.

and monoglucosylceramide (Figure I-4, b and e respectively). Incorporation of label into monoglucosylceramide rose 3-4 fold, while there was a statistically highly significant reduction of radioactivity in ceramide by around 40% ($p < 0.005$, 6 degrees of freedom), which was consistently observed in all experiments. However, even at the highest concentration, 5 μg/ml BFA did not cause a net increase of ^{3}H-acetate uptake into BHK21 cells (Table 1). That is, the increment of ^{3}H-acetate label in sphingomyelin, cholesterol ester and monoglucosylceramide was roughly matched by a commensurate decrease of radioactivity in phosphatidylcholine, triacylglycerol and cholesterol. Thus fusion of ER and Golgi by BFA treatment directs utilization of ^{3}H-acetate towards synthesis of sphingolipids and away from phosphatidylcholine, cholesterol and triacylglycerol.

C.I.2.3. BFA increases net synthesis of sphingomyelin

Although the analysis of the incorporation of ^{3}H-acetate into lipids of BFA-treated cells indicated that BFA effectively increased synthesis of sphingomyelin, the possibility remained that this effect reflected changes of specific activity in the fatty acyl moiety of the molecule, e.g. by increased turnover of the N-linked fatty acyl chain, rather than net synthesis of sphingomyelin. Therefore it was decided to also label BHK cells with ^{3}H-choline. Choline marks the headgroup portion of sphingomyelin and phosphatidylcholine and is incorporated into these molecules on a one to one basis. Hence, an increased incorporation of choline label into either sphingomyelin or phosphatidylcholine will reflect a net increase of synthesis of these molecules.

BHK cells were incubated with ^{3}H-choline for up to 6 hours in the absence and presence of BFA (5 μg/ml). No changes were found in the labelling of phosphatidylcholine in control and BFA-treated cells (Figure I-3a). The water-soluble fraction, representing free choline and the choline metabolites glycero-3-phosphocholine, phosphocholine and CDP-choline, was equally unaffected by BFA. Around 95% of the water-soluble label chromatographed as phosphocholine and no significant difference could be detected between control and BFA-treated cells. However, in cells exposed to BFA a 3 - 4 fold rise in the radioactivity of

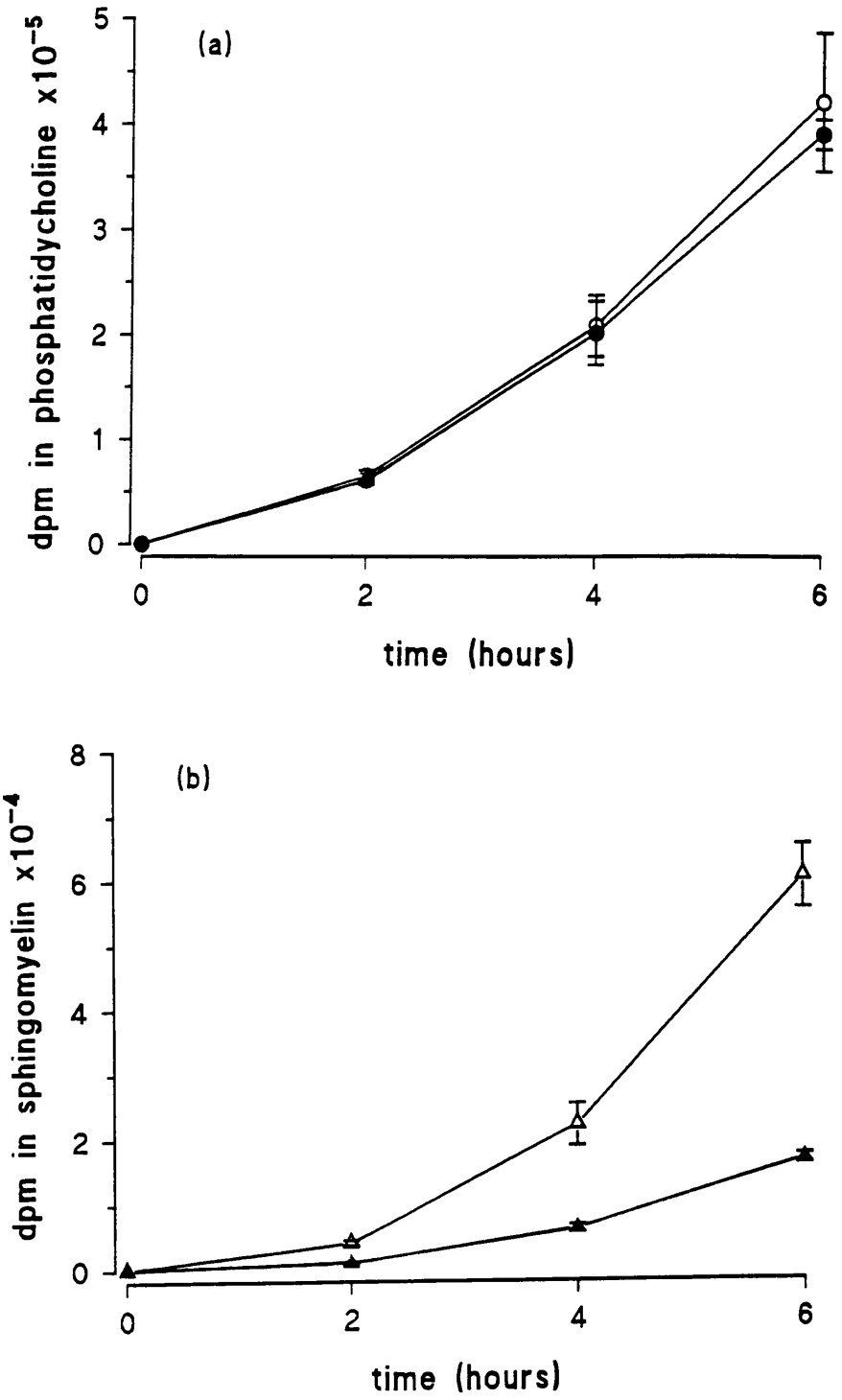


Figure I-3: Incorporation of ^{3}H-choline by BHK-21 cells in the presence or absence of BFA. Cells were incubated with ^{3}H choline for up to 6h in the **absence** (●,▲) or **presence** (○,△) of BFA (5μg/ml) and lipids were extracted and analysed as described under Methods. Values shown represent means and standard deviations for triplicate samples in one experiment which was repeated on two further occasions with virtually the same results. **(a)** phosphatidylcholine; **(b)** sphingomyelin.

sphingomyelin was observed, similar to that seen in ^{3}H-acetate labelled cells. In the experiment shown in Figure I-3, total incorporation of radioactivity after six hours was 457437 ± 16259 d.p.m. in controls and 522896 ± 20152 d.p.m. in BFA-treated. A t-test for unpaired and uncombined samples demonstrated that this difference was statistically significant ($p < 0.01$, 4 degrees of freedom).

Thus the increase of sphingomyelin labelling with ^{3}H-choline in BFA-treated cells appeared to represent a net increase of ^{3}H-choline uptake into the cells, in contrast to the situation in ^{3}H-acetate labelled cells, where increased incorporation of label into sphingolipids and cholesterol ester led to a redistribution, but not increased uptake of acetate in the cells. Although the increased radioactivity in sphingomyelin in ^{3}H-choline labelled cells could principally be due to increased turnover of the choline headgroup, a similar increase in ^{3}H-acetate labelled cells makes this possibility less likely.

C.I.2.4. BFA effect on intracellular transport of newly-synthesized sphingomyelin

Previous work by Quinn and Allan [250] had convincingly demonstrated the existence of two sphingomyelin pools in BHK cells, with the plasma membrane pool comprising roughly 60%-70% of total sphingomyelin. Although BFA clearly increased the synthesis of sphingomyelin in BHK cells the experiments described in the foregoing paragraphs left unclear where in the cells the newly synthesized sphingomyelin resided. To clarify this question cells were therefore labelled with ^{3}H-acetate for four hours in the presence and absence of BFA. The radioactive growth medium was then exchanged for nonradioactive, serum-free medium and the cells were treated with bacterial sphingomyelinase (B.cereus or S.aureus, 0.1 unit/ml, 20 minutes, 37°C). Treatment with sphingomyelinase was stopped by washing the cells twice with 1 ml of cold isotonic NaCl and lipids extracted and analyzed as described in the Materials and Methods section (B.3.4.).

In the experiment shown in figure I-4 (see also Table 1) around 40% of the newly synthesized sphingomyelin was accessible to degradation on the cell surface by exogenous sphingomyelinase ($p < 0.001$, 6 degrees of freedom). Breakdown of

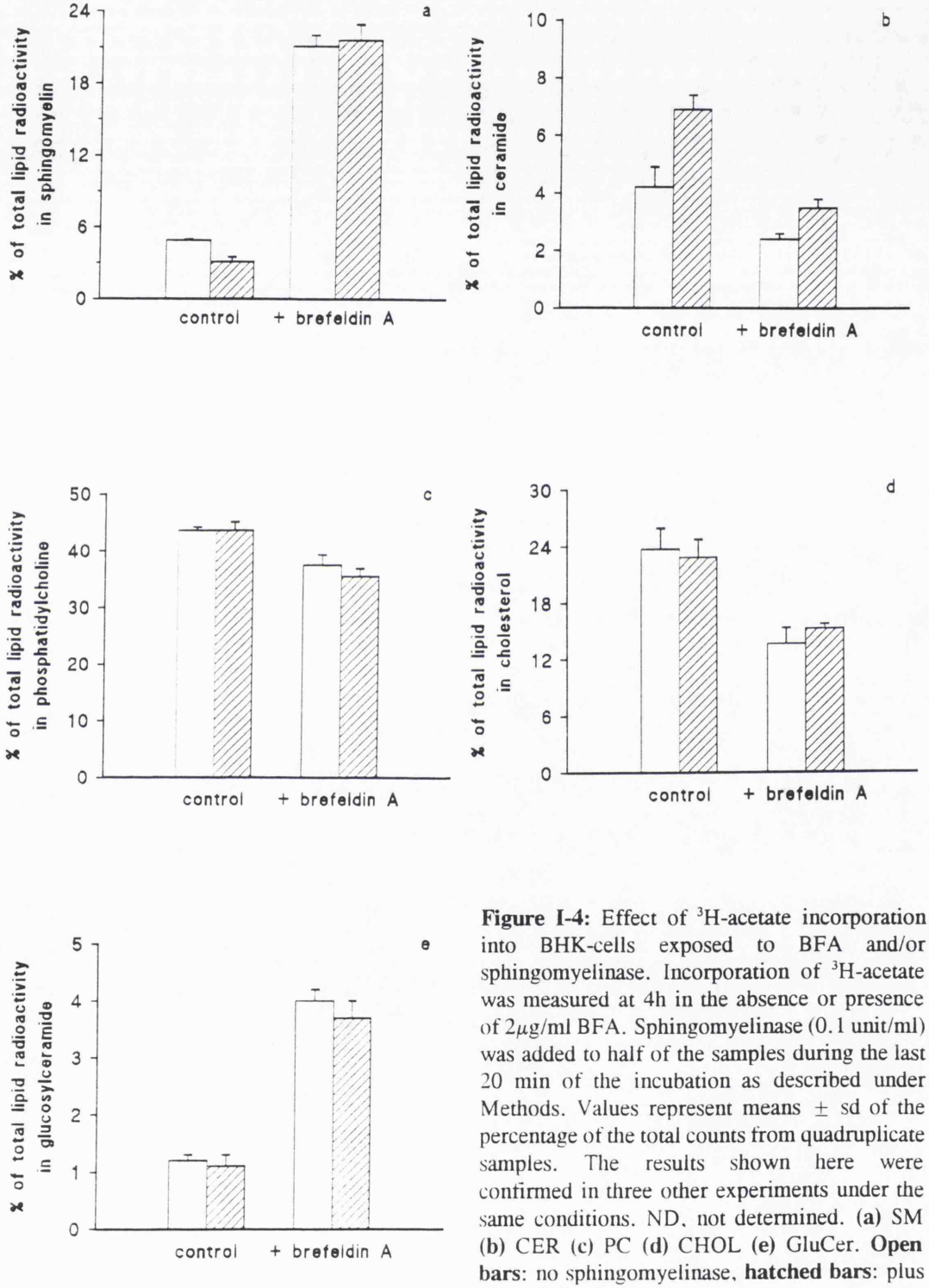


Figure I-4: Effect of ^{3}H-acetate incorporation into BHK-cells exposed to BFA and/or sphingomyelinase. Incorporation of ^{3}H-acetate was measured at 4h in the absence or presence of 2μg/ml BFA. Sphingomyelinase (0.1 unit/ml) was added to half of the samples during the last 20 min of the incubation as described under Methods. Values represent means $\pm$ sd 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. **(a)** SM **(b)** CER **(c)** PC **(d)** CHOL **(e)** GluCer. **Open bars**: no sphingomyelinase, **hatched bars**: plus sphingomyelinase.

sphingomyelin was accompanied by a concomitant rise in ceramide (Figure I-4b). In spite of the hugely increased levels of sphingomyelin labelling in BFA-treated cells, no surface breakdown of sphingomyelin was detected in treated cells. A modest, though statistically significant rise was found in ceramide, which was, however, much smaller than the equivalent rise in control cells. Thus it appeared that most of the newly synthesized sphingomyelin was intracellular, implying that BFA inhibited transport of the bulk of sphingomyelin to the plasma membrane.

Sphingomyelinase treatment did not have an effect on phospholipids other than sphingomyelin. Neutral lipids with the exception of cholesterol ester (see C.III.) were also unaffected by sphingomyelinase. This is consistent with earlier observations of Slotte et al.[293,296,297].

C.I.2.5. Effect of BFA on mass distribution of sphingomyelin in BHK cells

Since the experiments in the foregoing section left the possibility that some sphingomyelin reached the cell surface attempts were made to determine the quantity of sphingomyelin getting to the plasma membrane. Cells were therefore grown in petri dishes of 10 cm diameter in order to obtain a measure of mass changes in sphingomyelin by phospholipid phosphorus determinations as described earlier (B.3.4.4.).

Sphingomyelin accounted for 7.5% of lipid phosphate, equivalent to 13.9 nmol/dish, in unlabelled control cells (Table 2). This proportion did not change after an incubation period of five hours, although total cell phosphate increased by 40% over this period consistent with a doubling time of the cell line of around 12 hours (Figure I-5). Treatment with exogenous sphingomyelinase showed that around 65% of cellular sphingomyelin was found on the cell surface in control cells before and after the five hours incubation period (Table 2, Figure I-5a). Addition of BFA to the incubation medium in parallel samples did not significantly alter the amount of total phosphate per dish (around 250 nmol in both, control and BFA-treated cells).

BFA-treated cells revealed a drastic alteration of the distribution of cellular sphingomyelin. Whereas the intracellular sphingomyelin pool increased by 40% in control cells (Table 2, Figure I-5b), the increment in BFA-treated cells was 100%.

Table 2. Effects of BFA and sphingomyelinase on sphingomyelin content of BHK cells. Cells approaching confluence in 10cm dishes were incubated for 5h with or without BFA in complete growth medium. After replacement of the medium with 5ml of serum-free Glasgow MEM, 0.5 units of sphingomyelinase was added to half of the dishes and lipids were extracted and analysed as described under Methods. Values represent **A)** means ± sd of mole percent of total phospholipids from triplicate samples; **B)** nmoles 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 (0h) refers to samples which were extracted immediately before the 5h incubation period. PL is the total phospholipid in the samples (nmoles phosphorus). SM_t is total sphingomyelin in cells; SM_s is surface sphingomyelin calculated as SM_t minus sphingomyelin remaining after treatment with sphingomyelinase SM_i. Significantly different from sphingomyelinase-treated control $p < 0.01$.

	CONTROL (0 h)	CONTROL (5 h)	+SMase (5 h)	+BFA (5 h)	+BFA SMase (5 h)
(a)	percentage of total lipid				
SM	7.5 ± 0.1	7.5 ± 0.3	2.8 ± 0.2	7.9 ± 0.1	4.3 ± 0.2
PC	48.0 ± 1.0	48.1 ± 0.6	52.1 ± 0.3	44.9 ± 0.3	47.8 ± 0.6
PS/PI	13.1 ± 0.8	13.4 ± 0.2	14.1 ± 0.2	14.5 ± 0.2	15.4 ± 0.2
PE	26.5 ± 0.2	27.1 ± 0.9	27.1 ± 0.2	28.4 ± 0.3	27.9 ± 0.9
CL	4.8 ± 0.2	3.9 ± 0.1	3.9 ± 0.2	4.7 ± 0.3	4.4 ± 0.2
(b)	nmol of phospholipid				
PL	185 ± 7	258 ± 10	256 ± 12	246 ± 11	240 ± 15
SM_T	13.9	19.3	7.2	19.4	10.3
SM_I	5.1	7.2		10.3	
SM_S	8.8	12.1		9.1	

Concomitantly, much less sphingomyelin was accessible to attack by outside sphingomyelinase in BFA-treated cells (48%) than in control cells (65%). This means that the amount of sphingomyelin on the cell surface in BFA cells (9.1 nmol)

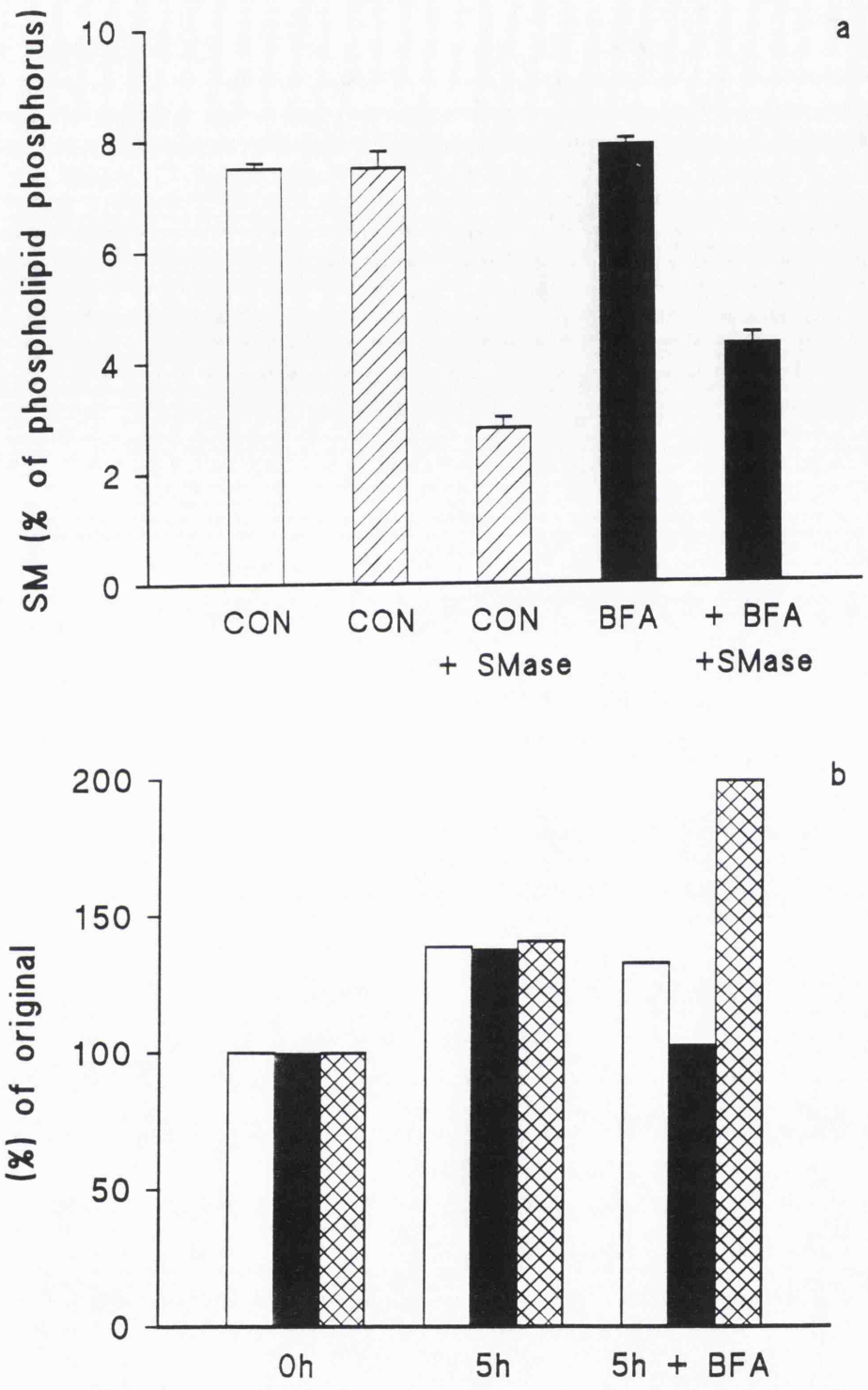


Figure I-5: Influence of BFA treatment on the mass of internal and external sphingomyelin pool. The details of the experiment are described in the legend to Table 2, the original values are also given in this table. **(a) open bars:** control cells before the incubation period, **solid bars:** control cells after the 5h incubation period, **solid bars:** cells treated with BFA for 5h **(b)** Values are related to the absolute amounts (nmols) of intracellular sphingomyelin, surface sphingomyelin and total phospholipids in controls cells before the start of the incubation period. **Open bars**: total phospholipids; **solid bars**: surface sphingomyelin (SM_s); **crossed bars**: intracellular sphingomyelin (SM_i).

was practically unchanged from that in control cells before the incubation (Table 2) while control cells had 12.1 nmol sphingomyelin on the surface. Hence, these results confirmed the conclusion that BFA inhibits arrival of newly synthesized sphingomyelin at the plasma membrane.

C.I.2.6. Does BFA cause breakdown of sphingomyelin in BHK cells?

In some cell lines BFA seems to stimulate a plasma membrane resident neutral sphingomyelinase as recently reported by Hannun and colleagues [169]. To

Table 3: Distribution of radioactivity in BHK cells labelled to equilibrium with ^{3}H acetate and exposed to BFA in non-radioactive medium. Cells were incubated for 48h with ^{3}H acetate, washed with non-radioactive medium and treated with 2μg/ml BFA for 4h as described under Methods. Values represent **(a)** means ± sd of percentages of total lipid radioactivity from quadruplicate samples and **(b)** the percentage of total phospholipid label present in the individual phospholipids. The total dpm per dish ± s.d. is quoted. The results shown here were confirmed in three other experiments under the same conditions. Significantly different from controls: * $p < 0.01$.

	Control		+ BFA	
	(a)	(b)	(a)	(b)
(a) phospholipids				
SM	6.3 ± 0.3	9.6	6.6 ± 0.2	10.1
PC	32.5 ± 1.1	49.6	32.4 ± 1.1	49.8
PS/PI	7.8 ± 0.6	11.9	8.2 ± 0.3	12.6
PE	15.5 ± 0.2	23.7	14.8 ± 0.5	22.7
CL	3.4 ± 0.3	5.2	3.1 ± 0.4	4.8
(b) neutral lipids				
CHOL.EST.	1.1 ± 0.1		3.0 ± 0.3	
CHOL	22.4 ± 0.8		20.4 ± 0.7	
CER	0.38 ± 0.07		0.26 ± 0.03	
NEFA	0.25 ± 0.03		0.24 ± 0.02	
DAG	0.8+0.1		1.0 ± 0.1	
TAG	6.1 ± 0.4		7.7 ± 0.2	
CELL AQU.	2.7 ± 0.2		2.6 ± 0.2	
Total d.p.m.	50205 ± 3341		50236 ± 4258	

exclude the possibility that such an activity caused degradation of plasma membrane sphingomyelin in BFA-treated cells, which might be confused with inhibition of sphingomyelin transport to the plasma membrane, BHK cells were labelled to equilibrium with ^{3}H-acetate or ^{3}H-choline for 48 hours, washed and incubated for another four hours in non-radioactive medium. Table 3 (column b) shows that the distribution of radioactive acetate label between the phospholipids very much resembled that obtained by measurements of phospholipid phosphorus (see Table 2). It was assumed that neutral lipids would show a similar relationship between mass and radioactivity as phospholipids, although the mass of neutral lipids was not determined directly.

Addition of BFA to the incubation medium did not change total lipid radioactivity by comparison to control cells, nor were there any significant changes in the distribution of label among the major lipids (Table 3 and 4). BFA exerted a clear effect on the levels of ceramide label, where a 50% drop was observed, and also on cholesterol ester radioactivity which rose 3-fold from 1.1% to 3% of total radioactivity. The additional radioactivity in cholesterol ester appeared to be derived from cholesterol as indicated by the concomitant, statistically significant ($p < 0.01$) drop in cholesterol label from 22.4% to 20.4% of total radioactivity. However, this decrease in cholesterol was not significant in all experiments and due to the experimental error it was not possible to decide unequivocally whether the extra radioactivity in cholesterol ester stemmed exclusively from cholesterol or whether part of it was derived from the added acyl-chain.

C.I.2.7. Analysis of ^{3}H-acetate distribution in cholesterol ester

In separate experiments the origin of the radioactivity in cholesterol ester in equilibrium-labelled control and BFA-treated cells was clarified. As in the experiments described above cells were incubated in non-radioactive medium in the presence and absence of BFA for two or four hours. After separation of the neutral lipids on t.l.c.-plates cholesterol ester spots were scraped, eluted with methanolic NaOH and subjected to alkaline methanolysis for two hours at 60°C as described in Materials and Methods. Subsequently the breakdown products of cholesterol ester,

Table 4: Distribution of radioactivity in BHK cells labelled to equilibrium with ^{3}H choline and exposed to BFA in non-radioactive medium. BHK-21 cells were labelled with 2 μCi of ^{3}H-choline per dish for 48 hours and then treated as described in the legend to Table 3. For the choline-labelled cells, the radioactivity in the medium and in the intracellular aqueous fractions were also measured.

	control	+ BFA
choline label in		
SM	8.8 ± 0.5	9.4 ± 0.5
PC	39.8 ± 1.7	40.3 ± 1.0
Cell aqueous phase	38.5 ± 1.9	40.0 ± 0.8
Medium	12.9 ± 0.6	11.3 ± 0.8

fatty acid methyl ester (FAME), non-esterified fatty acids (NEFA) and cholesterol, were separated and counted for radioactivity.

Table 5 demonstrates the results of this procedure. Close to 95% of the radioactivity in cholesterol ester was recovered in breakdown products. On average 36.2 ± 0.5% of the total radioactivity resided in the combined NEFA and FAME-fractions, whereas 61.9 ± 1% were recovered in cholesterol, the remainder being in cholesterol ester. Thus the ratio between the radioactivity of the cholesterol and the acyl moiety of cholesterol ester was 1.7. This was virtually the same ratio as that between the number of carbon atoms in the cholesterol (n = 27) and acyl portion (n = 16) of the molecule if one assumes that the bulk of the cholesterol ester acyl chains is derived from palmitoyl-CoA. This meant that even after a cold chase which greatly diminished the specific activity of NEFA the cholesterol ester acyl chain had the same specific activity as the cholesterol ester cholesterol. Moreover, the increase in radioactivity of the fatty acid portion of cholesterol ester was too large to be derived from either the non-esterified fatty acid pool or the aqueous fraction of the cell. Therefore it was unlikely that these lipids acted as donors of the cholesterol ester fatty acyl chain. Alternatively, the fatty acyl chain could have been derived from preformed lipids, suggesting the activity of a transacylase. However, it was not possible to identify the origin of the fatty acyl chain from the experiments presented here.

C.I.2.8. Reversibility of the BFA-effect on lipid metabolism by forskolin

Klausner and colleagues recently reported that *forskolin*, a well established activator of adenyl cyclase, could prevent the morphological changes induced by BFA in several mammalian cells [171]. Furthermore, forskolin was also able to reverse the BFA effect on protein secretion and causes reassociation of the *β-COP* with the Golgi membrane. However, studies with forskolin analogues and phosphodiesterase inhibitors disclosed that forskolin exercised its BFA antagonizing effect independently from its impact on the intracellular levels of cAMP [171]. It was suggested that BFA and forskolin interacted in a competitive way. Here, the consequences of the combined BFA and forskolin actions on cellular lipid metabolism are reported.

Table 5: The distribution of radioactivity in the cholesterol and fatty acyl moieties of cholesterol ester of cells incubated with or without BFA. Cholesterol ester isolated by tlc from ^{3}H acetate-labelled cell samples treated for 2 or 4h with or without BFA in the same experiment as that shown in Table 3 and was subjected to alkaline methanolysis as described under Methods. The counts in the original samples before methanolysis were compared with counts recovered in cholesterol, cholesterol ester, free fatty acid and fatty acyl methyl esters after methanolysis and separation by tlc. Results are means of dpm from duplicate determinations which varied by less than 5%. The proportion of counts found in the fatty acid moiety of cholesterol ester was calculated as counts in NEFA+FAME as a percentage of counts in NEFA+FAME+cholesterol. In three similar experiments, the mean value was found to be 35.7 ± 0.6%

	incubation period (h)				
	0 h	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
recovery	96.1%	95.1%	93.8%	96%	95.2%
(NEFA+FAME)%	35.8%	36.2%	36.7%	36.7%	35.7%

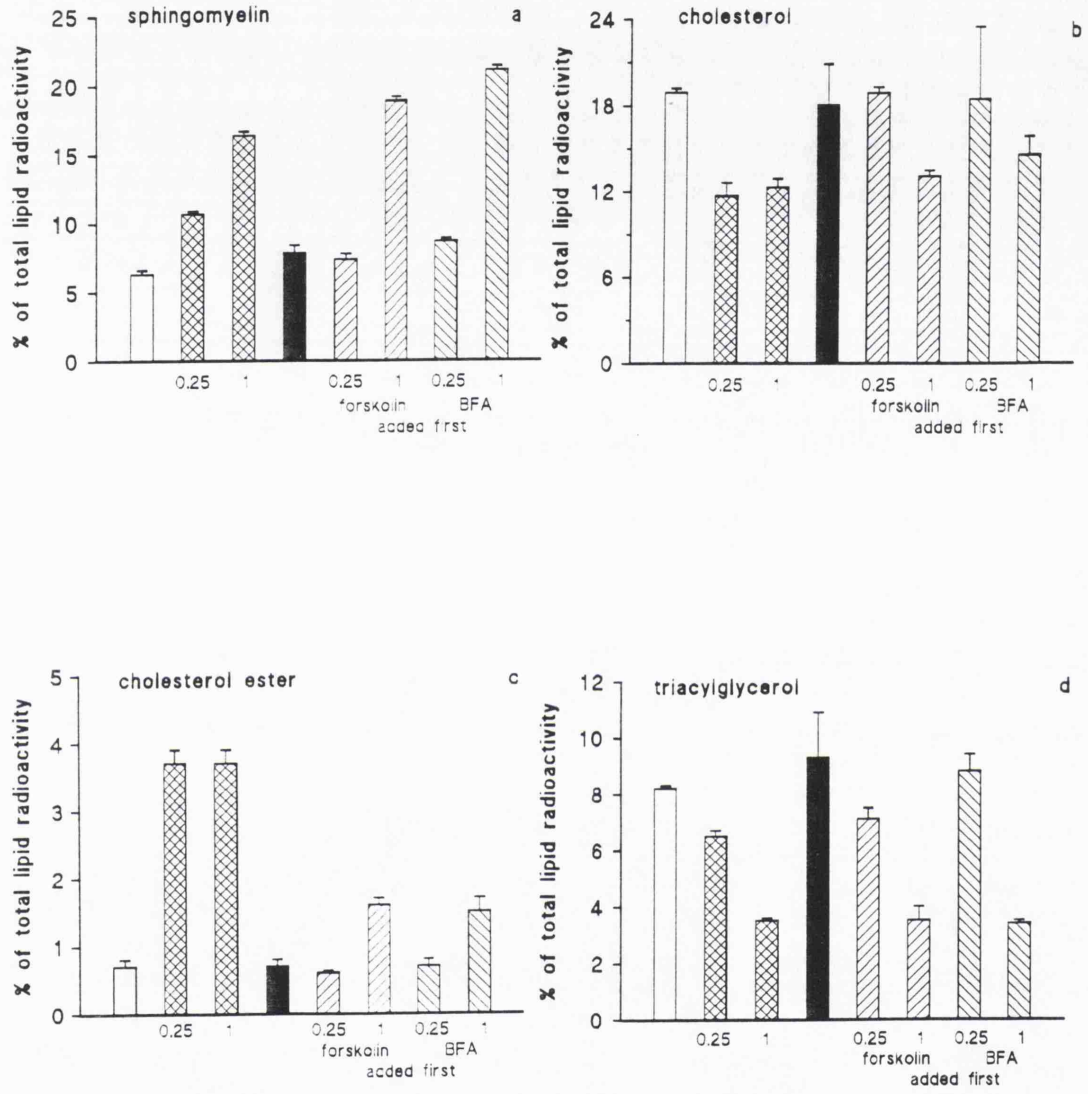


Figure I-6: Forskolin can reverse and prevent BFA induced changes in the incorporation of ^{3}H-acetate into BHK-21 cells cells. Cells were incubated for 4h in 3.5cm dishes in the presence of ^{3}H acetate and BFA and/or forskolin as described under Methods. Results are given as percentages of the total lipid radioactivity and are expressed as means ± SD from triplicate determinations in one experiment which was repeated twice with substantially the same results. Numbers on the abscissa denote the concentration of BFA (μg/ml). **Open bars**: control, **cross-hatched bars**: BFA alone, **solid bars**: forskolin (100 μM) alone, **hatched (/) bars**: pretreatment with forskolin, **hatched (\) bars**: pretreatment with BFA.

BHK cells were pretreated with no addition to the growth medium, with added forskolin, added BFA or a combination of BFA and forskolin as described in Materials and Methods (B.3.7.). 20 μCi ^{3}H-acetate were then added to each dish and the lipids extracted after incubation for four hours. The results of the lipid analysis are summarized in Figure I-6. Labelling of sphingomyelin increased by 70% or 250% in cells treated with 0.25 or 1.0 μg/ml BFA, whereas incorporation of ^{3}H-acetate was scarcely affected in cells treated with forskolin alone. The effect of the lower BFA concentration (0.25 μg/ml) on sphingomyelin labelling was completely abolished by forskolin (Figure I-6a), but forskolin had no effect on cells treated with 1 μg/ml BFA. The adverse effect of forskolin on the BFA-enhanced sphingomyelin labelling was observed regardless whether forskolin was added before or after BFA.

The analysis of other lipids (Figure 6d, Table 6), whose labelling was known to be influenced by BFA confirmed that forskolin could overcome the effects of low-dose BFA, but not of high-dose BFA: the BFA-induced reductions in the labelling of phosphatidylcholine, ceramide, cholesterol and triacylglycerol were reversed by forskolin at low BFA-concentrations, while a higher dose of BFA abrogated the protective effect of forskolin. By contrast, cholesterol ester showed a slight deviation from this pattern. Forskolin was able to considerably reduce the stimulating effect of BFA on labelling of cholesterol ester even at the higher concentration of BFA. This behaviour was constantly observed in all experiments, but the reasons for this peculiarity are not obvious. However, the effects of forskolin on changes in lipid metabolism caused by BFA are consistent with the model put forward by Lippincott-Schwartz et al.[225] which regards forskolin as a competitive inhibitor of the BFA action.

C.I.3. Discussion

C.I.3.1. Scope of the discussion

Treatment of BHK21 cells with BFA had a twofold effect on cellular lipids: the fungal metabolite nearly completely inhibited transport of sphingomyelin to the cell surface and caused striking changes in the labelling of other BHK cell lipids with

Table 6: Forskolin can reverse and prevent BFA-induced changes in the labelling of lipids with ^{3}H-acetate. Values for glycerophospholipids and ceramide were taken from the same experiments that is described in Figure I-6. * Forskolin was added before brefeldin A.

	Pretreatment of BHK cells before addition of ^{3}H-acetate							
BFA [μg/ml]...	control	0.25	1	0	0.25	1	0.25*	1*
Forskolin [μM]...	control	0	0	100	100	100	100*	100*
PC	35.6 ± 0.9	35.1 ± 0.8	33.3 ± 0.4	36.3 ± 0.5	36.4 ± 0.6	33.4 ± 0.6	38.2 ± 0.6	31.5 ± 0.6
PS/PI	8.5 ± 0.3	9.6 ± 0.3	8.6 ± 0.2	7.4 ± 0.5	7.9 ± 0.1	8.8 ± 0.3	8.3 ± 0.1	7.9 ± 0.1
PE	9.5 ± 0.3	10.5 ± 0.4	9.7 ± 1.0	9.2 ± 0.3	10.4 ± 0.8	8.3 ± 0.1	10.2 ± 0.5	10.1 ± 0.1
CER	1.5 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	2.0 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	1.4 ± 0.1	1.0 ± 0.1
Total (d.p.m.)	78219 ± 1195	62230 ± 7869	67925 ± 7054	63557 ± 5493	45192 ± 1001	57994 ± 3481	35438 ± 2384	58847 ± 1542

^{3}H-acetate (Figure 4,5; Table 1,2). The changes in labelling were most conspicuous in sphingolipids and steroids, while glycerophospholipids were affected not at all (PE,PS,PI) or to an only minor extent (PC). The BFA effect was maximal at concentrations between 1 and 5 μg/ml, which are commonly used to study the effects of BFA on organelles and protein translocation in cells [69,70,142,171]. However, in the present work effects on incorporation of ^{3}H-acetate into lipids were already observed at much lower concentrations (0.1 - 0.25 μg/ml). The relation of the above summarized BFA effects on BHK lipids to each other and the alterations of the compartmental organization of the cell induced by BFA are discussed below.

C.I.3.2. Intracellular transport of sphingomyelin is vesicular

Degradation of plasma membrane sphingomyelin with exogenous, bacterial sphingomyelinases demonstrated that BFA largely inhibited the appearance on the cell surface of newly synthesized radioactive sphingomyelin (Figure 4). Determinations of phospholipid phosphorus in unlabelled cells treated with BFA for five hours confirmed this conclusion (Figure 5, Table 2). Apparently BFA did not change the total amount of sphingomyelin in the cells, but whereas in control cells the surface pool of sphingomyelin amounted to 12.1 nmol after the incubation, it was only 9.1 nmol in BFA-treated cells, not significantly different from the surface pool in control cells before the incubation (8.8 nmol).

The inhibition of sphingomyelin transport to the plasma membrane in BFA-treated cells is most readily explained by van Meer's concept [345]. This proposes that sphingomyelin can only be transported by vesicles in cells, since it is located on the luminal side of intracellular organelles and does not undergo rapid transbilayer movement (see A.6.3.). This interpretation would place the experimental results presented herein line with several other studies [333] which reported that BFA inhibits vesicle-mediated protein transport through to the cell surface. Previous experimental support for van Meer's hypothesis relied on sphingomyelin that was synthesized from water-soluble ceramide precursors [34,143]. However, this is the first study to show that transport of endogenous, authentic sphingomyelin depends on a vesicular mechanism. If sphingomyelin transport was dependent on cytosolic

lipid transfer proteins [370] it is difficult to see how BFA should inhibit its arrival on the cell surface. In fact, it has been demonstrated that BFA does not hinder transport to the plasma membrane of phosphatidylethanolamine [354] whose intracellular transport occurs most likely by a non-vesicular transport mechanism [143].

C.I.3.3. Increased labelling of sphingomyelin as a consequence of the fusion of ER and Golgi apparatus

BFA did not influence the total incorporation of ^{3}H-acetate into lipids (see Table 1), but caused a 3-4 fold enhancement of labelling of sphingomyelin, monoglucosylceramide and cholesterol ester at the expense of triacylglycerol, phosphatidylcholine and cholesterol (Figure 2). Thus BFA exerted a profound effect on the balance between different pathways of lipid synthesis. Since incorporation of ^{3}H-choline into sphingomyelin was enhanced to a similar extent as that of ^{3}H-acetate in BFA-treated cells, the higher radioactivity of sphingomyelin in BFA-treated cells did seem to represent *de novo* synthesis of the whole molecule and not just the ceramide backbone or the N-acyl-linked fatty acyl chain. The increased labelling of sphingomyelin in BFA-treated cells found in the experiment presented above is in excellent agreement with data of other authors in other cell lines [34,114] who also observed a distinct increase of sphingomyelin as a consequence of BFA treatment using a short chain ceramide [34] or radioactive serine or choline [114] as sphingomyelin precursors. Brüning et al.[34] concluded further that the increased synthesis of sphingomyelin is not due to a direct effect of BFA on the phosphocholine transferase. However, these authors did not detect an increase of glucosylceramide synthesis.

C.I.3.4. Potential mechanism of the BFA effect on sphingomyelin labelling

BFA is well known to inhibit egress of transport vesicles from the merged ER-Golgi compartment to more distal compartments [142]. The data presented in Figure 4 and 5 (see also C.I.3.2.) clearly demonstrate that BFA also inhibits the

transport of sphingomyelin to the plasma membrane where it normally resides in the external leaflet [5]. However, a mere diversion of sphingomyelin destined for the plasma membrane into an intracellular pool would not be sufficient to explain the huge increase of sphingomyelin labelling found in BFA-treated cells. The increased intracellular sphingomyelin synthesis therefore appears as the consequence of the fusion of ER and Golgi-membranes induced by BFA.

Synthesis of the sphingomyelin precursor ceramide has long been assumed to be localized in the ER [352], but experimental support for this hypothesis has been provided only recently [185]. Thus the ceramide synthesis site in the ER is spatially separated from the sphingomyelin synthesis site attributed to the *cis*-Golgi [90,129]. Fusion of ER and Golgi membranes would join the enzymes responsible for ceramide synthesis in the same compartment. Since biophysical evidence [186] suggests that the provision of ceramide to the phosphocholine transferase is the limiting step of cellular sphingomyelin synthesis utilization of ceramide for sphingomyelin synthesis should increase. Indeed, the experimental results presented here support this notion, as BFA treatment significantly reduced the levels of ceramide mass (Table 3) and also of ceramide labelling (Figure 4b). This in turn could release negative feedback-inhibition of the serine-palmitoyl-CoA ligase, the rate limiting enzyme of sphingolipid synthesis [352]. Consequently palmitoyl-CoA utilization for synthesis of sphingolipids would be increased and this could explain increased labelling of glucosylceramide as well as the decreased labelling of phosphatidylcholine and triacylglycerol in BFA labelled cells (Figure 2) since both these lipids contain palmitate as a major fatty acyl chain component (David Allan, personal communication). The reversibility of the BFA effect on lipid metabolism by forskolin apparently supports this conclusion since forskolin is also known to reverse the BFA induced changes of the compartmental organization of a cell [171]. Brüning et al.[34] reported that pretreatment of cells with nocodazole and AlF_4^- which inhibit the BFA action in cells also inhibit the effect of BFA on cellular lipid metabolism [34].

The model outlined above makes several important predictions that are open to experimental tests:

1. A transport step would be rate-limiting for the synthesis of the important plasma membrane lipid sphingomyelin and also other sphingolipids. The prevailing view is that ceramide transport from the ER to the *cis*-Golgi occurs by vesicles [345]. As described in the introduction heterotrimeric and small GTP-binding proteins are involved in the regulation of transport through the secretory pathway [37,67,103]. Together with the results reported here this could indicate that the proteins coordinating intracellular traffic exert indirect control of metabolic processes, too.

2. Utilization of ceramide for synthesis of sphingomyelin or glucosylceramide and its derivatives may be regulated dynamically, i.e. by the relative activities of the enzymes catalyzing the respective reactions. This forecast is based on the fact that the ratio between sphingomyelin and monoglucosylceramide does not seem to be greatly affected by BFA, it is ~ 4 in control and ~ 5 in BFA-treated cells (Figure 4).

Although Brüning et al.[34] and Hatch & Vance [114] also concluded that the BFA-effect on SM synthesis is due to fusion of ER and Golgi, other recent studies suggest that this model might be too simplistic. An analysis of sphingolipid synthesis in mitotic cells [46] found that synthesis of higher glycosphingolipids (GA2) was reduced by more than 95%, glucosylceramide synthesis was not affected and that of sphingomyelin reduced by 40%. This could suggest non-vesicular transport of ceramide from the ER to the Golgi or differential distribution of the relevant enzymes to mitotic intracellular vesicles. A differential redistribution of sphingolipid synthesizing enzymes in BFA treated HepG2-cells has been reported recently [350], BFA caused redistribution of the glucosyltransferase, but not of the PCCP to the ER.

C.I.3.5. The connection between sphingomyelin and cholesterol metabolism

In addition to its conspicuous effect on sphingomyelin metabolism BFA causes a distinct change in the synthesis of neutral lipid: most prominent is the 4 fold rise in the labelling of cholesterol ester, whereas labelling of cholesterol and

triacylglycerol is reduced in the presence of BFA (Figure 2 and 6). A similar stimulating effect of BFA on the synthesis of cholesterol ester has recently been reported by Stein and coworkers [306] in $HepG_2$ and Caco-2cells at BFA concentrations as low as 30 ng/ml, whereas incorporation of label into triacylglycerol remained unaffected. These authors also reported that in peritoneal macrophages triacylglycerol was much more strongly affected by BFA than cholesterol ester, but this effect was only noticed at the higher concentrations of BFA comparable to those used in the present study.

In the present work BFA significantly inhibited incorporation of ^{3}H-acetate into cholesterol (Figure 2). Only part of this drop can be accounted for by utilization of cholesterol for production of cholesterol ester, so that the difference is most likely due to an inhibition of cholesterol synthesis in BFA-treated cells. Cells labelled to equilibrium with ^{3}H-acetate and treated with BFA revealed a 3-fold increase of cholesterol ester radioactivity from 1% to 3% of total lipid counts (Table 3) which very likely represents a corresponding change in the proportion of cholesterol ester mass. A commensurate decrease was observed in the relative mass of cholesterol, but the decline in cholesterol radioactivity was not clearly significant in all experiments.

Alkaline methanolysis of the cholesterol ester, however, demonstrated that the radioactivity in cholesterol ester originated from cholesterol and the acyl chain in proportion to the number of carbon atoms in the respective molecules (Table 4) suggesting that both molecules had the same specific activity even after cold chase. Experiments by Stein et al.[306] showed that inhibition of the acyl-CoA:cholesterol acyltransferase by the Sandoz component 58-035 prevented esterification of cholesterol. However, under the chase conditions applied in the present study only very little radioactivity was recovered in non-esterified fatty acids, which was not surprising since the prolonged chase period should have decreased the specific radioactivity of fatty acids and fatty acyl-CoA. Thus, the cholesterol and fatty acid portions of cholesterol ester would only have the same specific radioactivity if both molecules were derived from preexisting pools of these molecules. Hence, it appears more likely that phosphatidylcholine and triacylglycerol, both relatively losing radioactivity in the presence of BFA (Figure 2), act as donors of the fatty acid

moiety in a reaction analogous to the lecithin:cholesterol acyl transfer [312]. This could possibly explain why Slotte and coworkers did not find an inhibition of cholesterol esterification in ATP-depleted cells despite the ATP-dependency of the ACAT-reaction [298].

C.I.3.6. Esterification of cholesterol - a general response to depletion of the plasma membrane of sphingomyelin?

Previous work by other authors has demonstrated that degradation of plasma membrane sphingomyelin by exogenous sphingomyelinases results in increased esterification of plasma membrane cholesterol [293] and down-regulation of the key enzyme of cholesterol synthesis, HMG-CoA reductase [106]. Furthermore, the breakdown of cell surface sphingomyelin entails redistribution of cholesterol from a plasma membrane pool, accessible to external cholesterol oxidase, to a pool which is oxidase resistant [296]. Even if HDL_3 is present in the medium the flow of cholesterol is specifically directed towards the cell interior [293,297].

In the present work the plasma membrane was depleted of sphingomyelin by addition of BFA to the cell culture (Figures 4 and 5, C.I.3.2). From Tables 2 and 4 it can be calculated that sphingomyelin equivalent to around 0.8% of total lipid was prevented from reaching the cell surface by treatment of cells with BFA, while the drug caused a rise in cholesterol ester equal to 2% of total lipids. Assuming molecular weights of 770 and 690 for sphingomyelin and cholesterol ester respectively, it infers that around 3 mols of cholesterol are esterified per mol of sphingomyelin that does not reach the cell surface. This ratio matches the normal ratio of sphingomyelin and cholesterol in the plasma membrane [58] which supports the conclusion that the esterified cholesterol is actually derived from the plasma membrane.

Thus it appears that the cells tightly regulate the ratio in the plasma membrane of cholesterol and sphingomyelin and that esterification is a mechanism for cells to scavenge excess cholesterol. In fact the results presented in Figure 6 and Table 6 suggest that labelling of cholesterol ester is an extremely sensitive indicator of any disturbances of this balance, since there are already remarkable changes in the

radioactivity of cholesterol ester at BFA concentrations where sphingomyelin and cholesterol labelling are affected to a much lesser extent. In addition to esterification of excess cholesterol, cells react to a depletion of sphingomyelin in the plasma membrane by reducing cholesterol synthesis. It is, however, unknown how the cells normally coordinate vesicular transport of sphingomyelin and transport of cholesterol which appear to follow a different route [333]. Also, it is not known how a relative decline in plasma membrane sphingomyelin could elicit the above responses in cholesterol metabolism. Work by Gupta and Rudney [106] indicates that a minute proportion of excess plasma membrane cholesterol is converted into oxysterols which appear to have a regulatory effect on the HMG-CoA reductase activity.

C.II. Two sites of sphingomyelin synthesis in BHK21 cells

C.II.1. Effect of monensin on vesicular transport through the Golgi apparatus

Monensin is a lipophilic, cationic ionophore which acts as a diffusing carrier mediating the exchange of Na^+, K^+ and H^+ [249]. Within minutes after addition of monensin to mammalian cells the Golgi cisternae undergo a conspicuous morphological change from flattened cisternae to dilated balloons while the endoplasmic reticulum remains apparently unaffected [100,319]. The functional equivalent of these morphological alterations is a remarkable slowing of vesicular transport of newly synthesized proteins through the secretory pathway [319].

Analysis of the posttranslational modifications of secreted proteins, e.g. Ig, or the spike proteins of Semliki Forest Virus, demonstrated that these proteins failed to acquire Endo-H-resistance in monensin-treated cells [100,251,320] which meant that these proteins did not gain access to a late Golgi compartment [319]. Immunohisto-chemical studies illustrated further that viral nucleocapsids accumulated in the *medial* part of the Golgi [251]. Together these observations lead to the conclusion that proteins traverse the Golgi apparatus from *cis* to *trans* and that monensin blocks protein transport between *medial* and *trans* Golgi [100].

The conspicuous swelling of Golgi cisternae caused by monensin [319] might be due to dissipation of monovalent cation gradients (Na^+, K^+, H^+) between intra- and extracisternal which together with the Golgi Cl^- transporter could cause influx of water into the Golgi cisternae. Nonetheless, it is unclear how these effects precisely relate to the functional consequences of monensin treatment. In view of the recent debate regarding the influence of membrane curvature on transport through the Golgi-apparatus [198] it seems conceivable that the geometry of swollen cisternae is simply unfavourable to budding of vesicles.

C.II.2. Objectives of the application of monensin in this study

The last decade has seen a remarkable shift of opinion concerning the intracellular location of sphingomyelin synthesis. Whereas earlier studies proposed

a synthesis site on the plasma membrane [61,186,361,334], a dominant *cis-/medial-*Golgi synthesis site of sphingomyelin is now generally accepted [90,129,176]. However, Futerman et al.[90] attributed a small proportion of total sphingomyelin synthesis to the plasma membrane. This possibility was reinforced by Malgat et al.[184] who found synthesis of sphingomyelin in microsome and plasma membrane fractions of rat liver and brain and concluded that two sites of sphingomyelin synthesis existed in these cells. Quinn and Allan [250] found that there were two metabolically different pools of sphingomyelin in BHK 21 fibroblasts, one intracellular and one plasma membrane pool, which did not mix with each other.

No attempt was made in the latter study to test whether the differential labelling of the two sphingomyelin pools also reflected two different sites of synthesis. Monensin appeared to be a suitable agent with which to address this question, since it stops transport through the Golgi, but in contrast to BFA does not cause a fusion of the Golgi with other compartments. It was postulated that monensin might be able to metabolically separate the two sites of sphingomyelin synthesis, depending on their localisation on the exocytic pathway.

C.II.3. Results

C.II.3.1. Effect of monensin on the incorporation of ^{3}H-choline into BHK 21 cells

BHK cells were labelled for four hours with ^{3}H-choline (2 μCi/ml) in the absence and presence of monensin (1 μM). After four hours the radioactive medium was removed and the cells were washed twice with 1 ml serum-free medium. Some samples of both control and monensin-treated cells were incubated for a further 20 minutes period with exogenous sphingomyelinase (B.cereus 0.1 unit/ml) in serum-free medium in order to determine the amount of radioactive sphingomyelin which had reached the plasma membrane. Around 30% of the sphingomyelin was degradable on the cell surface in control cells (Figure II-1). Significantly, in monensin-treated cells the incorporation of ^{3}H-choline into sphingomyelin was decreased by a similar extent. To test what proportion of newly synthesized

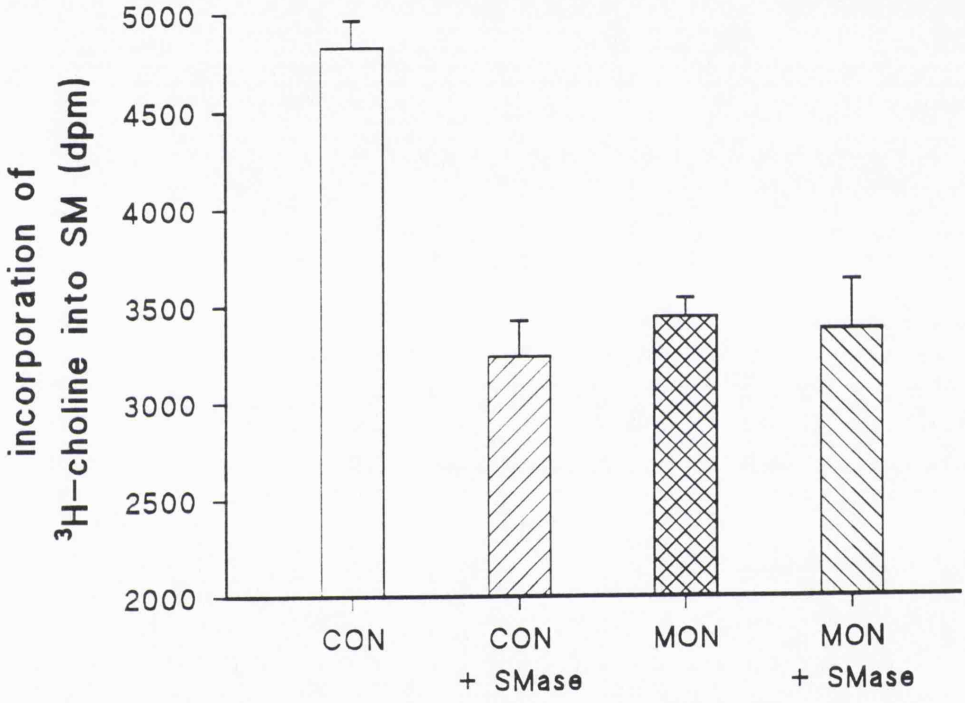


Figure II-1: Effect of sphingomyelinase and/or monensin on ^{3}H-choline labelling of sphingomyelin in BHK cells. Cells were labelled with ^{3}H-choline for four hours in the presence or absence of monensin as described under Methods. For the last 20min of the incubation, cells were treated with sphingomyelinase (0.1 units) before extraction and analysis of lipids. Results are expressed as means $\pm$ SD of triplicate determinations in one experiment which was repeated twice with similar results. CON, control; SMase, sphingomyelinase; MON, monensin.

sphingomyelin resided in the outer leaflet of the plasma membrane in monensin-treated cells, were exposed to sphingomyelinase after they had been incubated with monensin. The second treatment with sphingomyelinase did not cause a reduction of radioactivity in sphingomyelin beyond the 30-35 % decrease seen in cells treated with either sphingomyelinase or monensin alone. The radioactivity of the sphingomyelinase-insensitive pool of sphingomyelin which was assumed to be inside the cells was about the same in control and monensin-treated cells.

Thus monensin seemed to specifically inhibit synthesis of the plasma membrane pool of sphingomyelin, whereas it did not affect the intracellular pool of sphingomyelin. Alternatively, one could interpret the above results as indicating an equal reduction by monensin of the synthesis of internal and external sphingomyelin plus an additional inhibition of the delivery of sphingomyelin to the cell surface.

C.II.3.2. Incorporation of ^{3}H-acetate into BHK cell lipids in the presence of monensin

To investigate whether synthesis of lipids other than sphingomyelin, in particular the sphingomyelin precursor ceramide, was also influenced by monensin, BHK cells were labelled for four hours with ^{3}H-acetate following the protocol given above. Essentially, monensin affected incorporation of ^{3}H-acetate into sphingomyelin similarly to that of ^{3}H-choline. Treatment with monensin caused a reduction of the incorporation of ^{3}H-acetate into sphingomyelin by 40 % in comparison to control cells (Figure II-2a). Degradation of surface sphingomyelin by sphingomyelinase in control cells yielded a reduction in the radioactivity of sphingomyelin by up to 50%, whereas exposure of monensin-treated cells to sphingomyelinase decreased sphingomyelin radioactivity beyond the levels observed after monensin alone. Digestion of radioactive plasma membrane sphingomyelin by sphingomyelinase was accompanied by an equivalent rise in the radioactivity of ceramide (Figure II-2b, lanes 3 and 4) as expected from the hydrolysis of sphingomyelin to phosphorylcholine and ceramide. However, a clear rise of ceramide labelling by about 60% (statistically significant, $p < 0.05$) was also observed in monensin treated cells. Notwithstanding, the combined radioactivity of

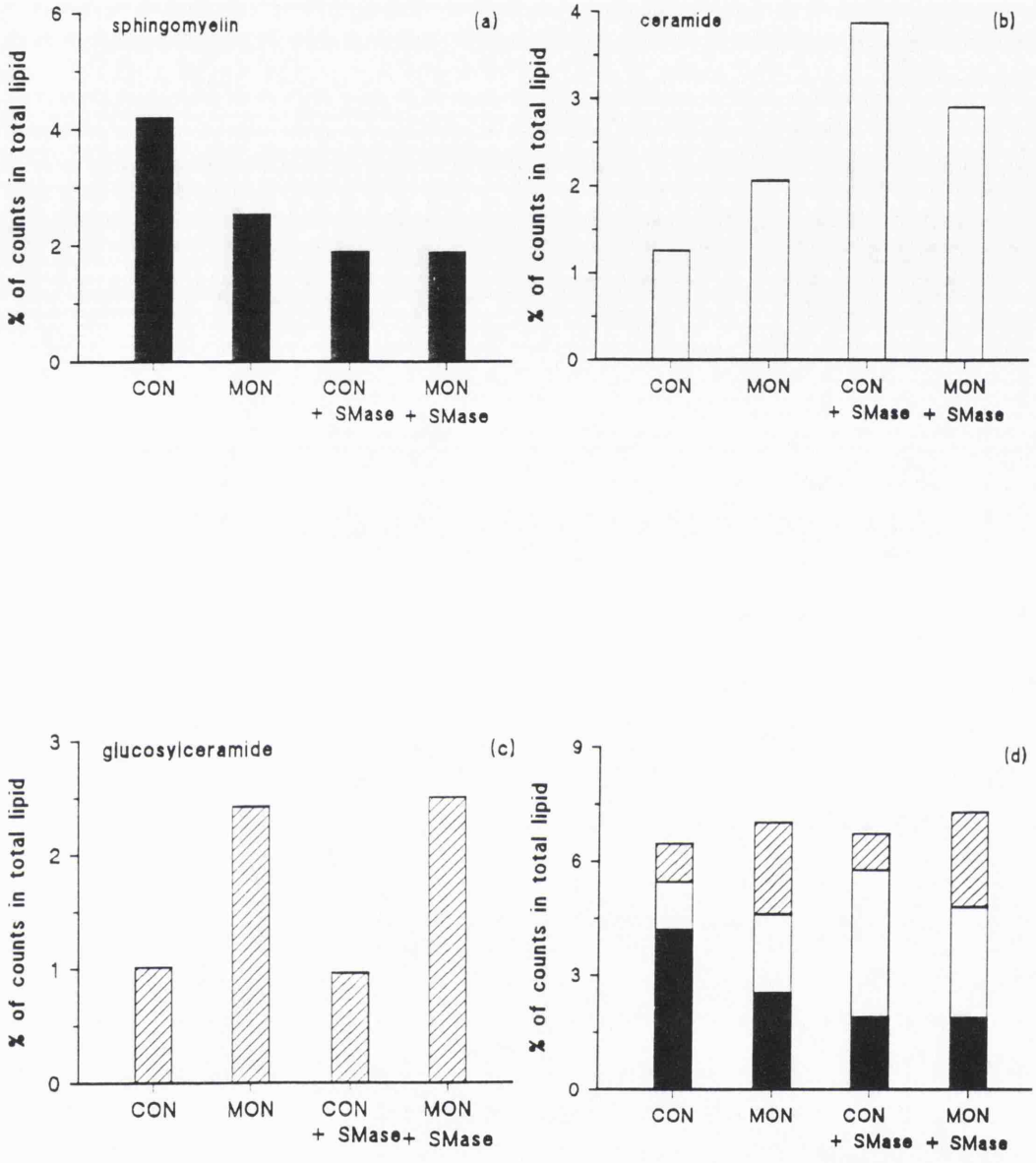


Figure II-2: Effects of sphingomyelinase and/or monensin on ^{3}H-acetate labelling of sphingomyelin, ceramide and glucosylceramide in BHK cells. The experiment was carried out as for Fig 1. except that ceramide and glucosylceramide were measured in addition to sphingomyelin. Results are expressed as the percentage of total lipid radioactivity present in sphingomyelin (solid bars), ceramide (open bars) and glucosylceramide (hatched bars). Values represent the means of four experiments which were each based on triplicate samples corresponding to the different conditions employed. Variation from the mean values shown was no more than ± 10%.

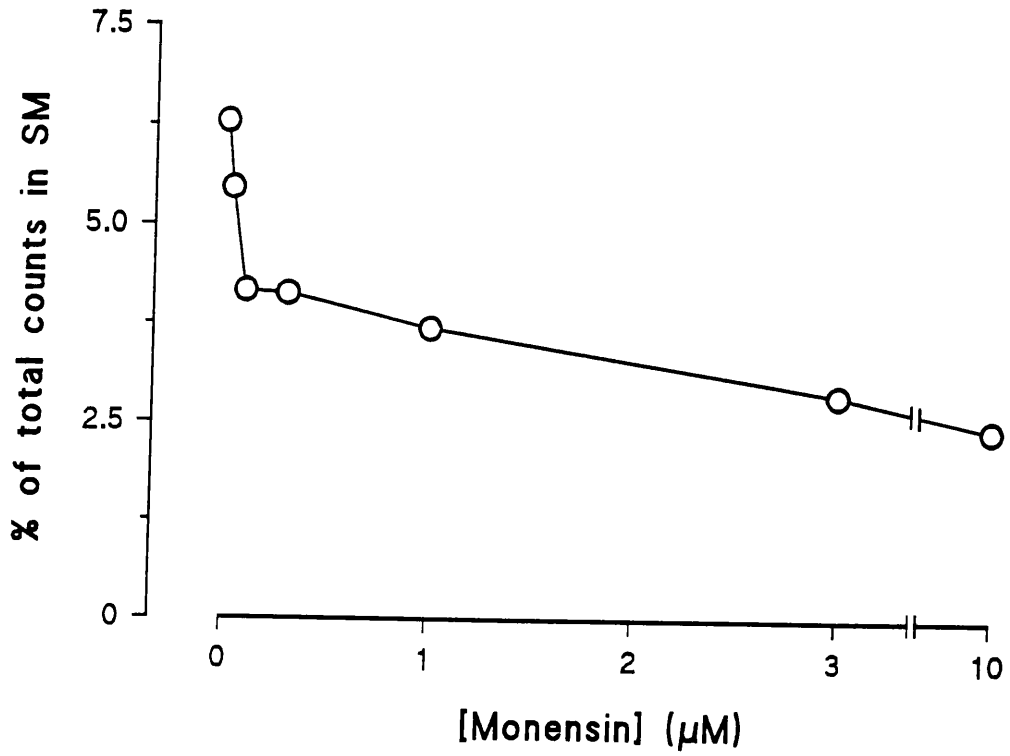


Figure II-3. Effect of different monensin concentrations on labelling of sphingomyelin. Cells were incubated as in the legend to Figure 2, but using different concentrations of monensin up to 10μM. Results are expressed as means $\pm$ SD of triplicate determinations in a single experiment which was repeated twice with similar results.

sphingomyelin and ceramide in monensin-treated cells was less than that in control cells. The missing radioactivity, however, was recovered in glucosylceramide which rose around 2-3 fold in monensin-treated cells (Figure II-2c). In contrast, sphingomyelinase treatment of control or monensin-treated cells did not affect the levels of glucosylceramide. As shown in Figure II-3, the effects of monensin on sphingomyelin labelling were maximal at around 3 μM and already half-maximal at a concentration as low as 0.1 μM.

The results obtained with ^{3}H-acetate again suggested that monensin largely inhibited synthesis of the sphingomyelin pool which in control cells was accessible to digestion by an exogenous sphingomyelinase at the plasma membrane surface. This inhibition resulted in an accumulation of ceramide which, however, did not match the decline in sphingomyelin radioactivity measured in monensin-treated cells. Instead, the remainder appeared in glycosphingolipids and here largely in glucosylceramide. Thus monensin did not change total incorporation of radioactivity into sphingolipids, but appeared to effectively redirect utilization of ceramide originally destined to become plasma membrane sphingomyelin, into glucosylceramide. Apparently, the ceramide accumulating in the presence of

Table II-1: Distribution of ^{3}H-acetate label among phospholipids of BHK cells. Radioactivity in individual lipids was measured after four hours incubation with ^{3}H-acetate with or without addition of monensin (1 μM) or sphingomyelinase as described under Methods. Values represent the percentage if total lipid radioactivity (means $\pm$ s.d. of triplicate determinations) and are derived from a single experiment which was repeated three times with substantially the same results. Monensin did not cause a significant change in total ^{3}H-acetate incorporation. * significantly different from control values ($p < 0.01$, degrees of freedom ≥ 6), NL: neutral lipids.

	CONTROL	MONENSIN	CONTROL + SPHINGOMYELINASE
SM	5.6 ± 0.2	3.4 ± 0.3*	3.2 ± 0.1*
PC	47.1 ± 1.8	46.4 ± 2.1	48.2 ± 2.3
PS/PI	10.2 ± 0.5	10.3 ± 0.3	10.6 ± 0.5
PE	12.5 ± 0.7	12.7 ± 0.8	12.2 ± 0.3
NL	25.5 ± 1.9	27.3 ± 2.5	26.7 ± 1.7

monensin was not used for synthesis of sphingomyelin for the internal pool, which did not significantly change in radioactivity.

C.II.3.3. Monensin effect on labelling of phospholipids and neutral lipids

In contrast to the distinct alterations of labelling of sphingolipids caused by monensin, no significant effect on phosphatidylcholine, phosphatidylethanolamine and the combined fraction of phosphatidylinositol and phosphatidylserine could be detected (Table II-1). Correspondingly, monensin did not cause a major change in the overall labelling of neutral lipids. However, this was not found to be true for cholesterol ester (Figure II-4). Here, monensin caused a 2-3 fold enhancement of the conversion of cholesterol to cholesterol ester, almost identical to the increased conversion induced by sphingomyelinase treatment. Although both monensin and sphingomyelinase treatment deplete the plasma membrane of sphingomyelin it has to be noted that in the latter case the plasma membrane is enriched in ceramide whereas this is not the case in monensin-treated cells which have less sphingomyelin on their surface. This implies that ceramide is unable to assume the role of a sphingomyelin substitute in a putative cholesterol:sphingomyelin complex and that the relative lack of sphingomyelin in the plasma membrane, but not the presence there of ceramide, is the most likely reason for the increased esterification of cholesterol in the presence of monensin.

C.II.3.4. Resynthesis of sphingomyelin in the presence of monensin

The experiments presented in C.II.3.2. suggest that a block of vesicular transport through the Golgi apparatus by monensin resulted in the inhibition of synthesis of plasma membrane sphingomyelin and a reciprocal accumulation of the sphingomyelin precursor ceramide. One explanation for this observation could be that monensin inhibits access of ceramide to a site where it would normally be turned into plasma membrane sphingomyelin. This site would have to be distal to the monensin block between *medial-* and *trans*-Golgi [100] and would be in addition to the known sphingomyelin synthesis site presumed to be in the *cis-/medial*-Golgi [90,129].

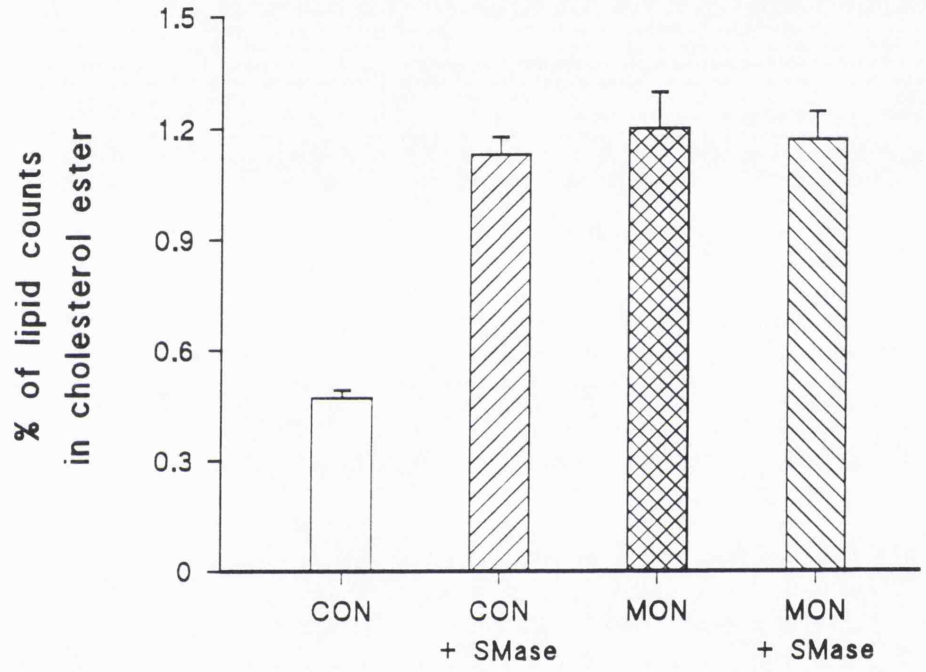


Figure II-4: Effect of monensin and/or sphingomyelinase on labelling of cholesterol ester. Cells were labelled with ^{3}H-acetate in the absence or presence of monensin (1 μM) and sphingomyelinase as described in the legend to table II-1. After separation of the cholesterol ester radioactivity was counted and expressed as a percentage of total radioactivity. Results represent means $\pm$ SD of triplicate determinations.

Whereas the evidence gained thus far pointed to the existence of such a site, proof for a functioning second sphingomyelin synthesis site was still lacking.

To address this problem an older observation by Allan and Quinn [4] was used. These authors reported that in cells labelled to equilibrium with ^{3}H-palmitate, sphingomyelin which had been degraded by exogenous sphingomyelinase was apparently completely resynthesized when the cells were further incubated after removal of the enzyme.

Figure II-5 illustrates the principle of this assay in BHK21 cells labelled to equilibrium with ^{3}H-acetate (5 μCi/ml) as described in Materials and Methods (B.3.2.). The supernatant was removed and the cells washed two times with 1 ml serum-free medium. Subsequently, the cells were treated for 20 minutes with sphingomyelinase (S.aureus or B.cereus, 0.1 units/ml) in serum-free medium to digest surface sphingomyelin. After removal of the sphingomyelinase and washing of the cells (two times 1 ml growth medium) the cells were allowed to recover for three hours. In accordance with the results of Allan and Quinn [4] sphingomyelin was almost completely resynthesized by the cells after this period. Breakdown and resynthesis of sphingomyelin were mirrored by reciprocal changes in the levels of ceramide, allowing the conclusion that the ceramide produced by degradation of sphingomyelin was also utilized for resynthesis of sphingomyelin. Resynthesis of sphingomyelin occurred with a half-time of around 64 minutes in this experiment, analysis of further six experiments for this parameter yielded a mean value of 64 $\pm$ 18 minutes. In an extension of the work of Allan and Quinn, however, Figure II-5 clearly demonstrates that the resynthesized sphingomyelin was at the plasma membrane surface where it was accessible to a second breakdown by an exogenous sphingomyelinase.

To establish whether resynthesis of plasma membrane sphingomyelin occurred before or after the monensin block the resynthesis of sphingomyelin was examined in the absence or presence of monensin (10 μM). If resynthesis of sphingomyelin was not affected by monensin and if the resynthesized sphingomyelin was degradable on the cell surface, this approach would provide direct evidence for a second sphingomyelin synthesis site distal to the *medial*-Golgi since monensin would block reappearance on the plasma membrane surface if resynthesis occurred in the *cis-/medial*-Golgi site

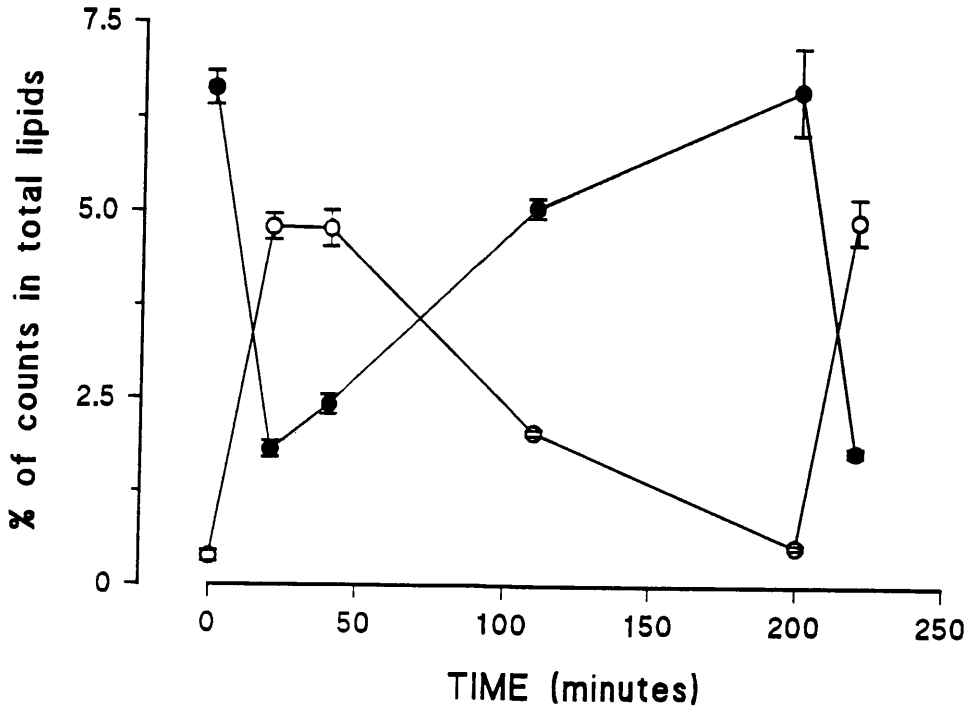


Figure II-5: Resynthesised sphingomyelin is at the cell surface. Cells were labelled to equilibrium with ^{3}H-acetate for 48 hours (see Methods). Exogenous, bacterial sphingomyelinase was added to degrade to break down surface sphingomyelin. After removal of the enzyme and washing of the cells, cells were allowed to resynthesise sphingomyelin for three hours. The recovery period was followed by a second treatment with sphingomyelinase to test whether resynthesised sphingomyelin was on the surface or not. Results represent means of triplicates ± sd of a single experiment. ●, sphingomyelin; ○, ceramide;

of sphingomyelin synthesis.

After degradation of surface sphingomyelin and subsequent washing of the cells, 10 μM monensin were therefore added to half of the samples. Figure II-6a shows the results of this experimental approach. In contrast to its effect on *de novo* synthesis monensin did not inhibit resynthesis of sphingomyelin. Determinations of phospholipid phosphorus gave essentially the same results (Figure III-6b). A second treatment with sphingomyelinase illustrated that the resynthesized sphingomyelin was on the cell surface both in control and monensin treated cells. Hence, resynthesis of sphingomyelin is not a function of the synthesis site proximal to the monensin block since resynthesized sphingomyelin would not be degradable by exogenous sphingomyelinase in this case. Furthermore, the experiment proved that monensin *per se* did not inhibit sphingomyelin synthesis as long as there is ample precursor ceramide in the appropriate place.

C.II.4. Discussion

C.II.4.1. Ceramide transport: vesicular or protein-mediated?

The experiments presented in the foregoing section show that in BHK 21 cells the ionophore monensin, a known inhibitor of vesicular protein transport through the Golgi-apparatus [100,251,319], largely suppresses synthesis of plasma membrane sphingomyelin while synthesis of the internal pool of sphingomyelin is not affected by the drug (Figures II-1 and 2). This observation becomes readily explicable if one assumes that synthesis of plasma membrane sphingomyelin depends on vesicular transport of ceramide through the Golgi. In this case one would predict an accumulation of ceramide in monensin-treated cells and/or a redirection of ceramide that cannot be used for synthesis of plasma membrane sphingomyelin into other sphingolipids or into intracellular sphingomyelin. As Figure II-2 reveals ceramide does show the behaviour expected from a vesicular transport modus for this common precursor of all sphingolipids. This finding appears difficult to reconcile with the assumption that transport of ceramide is mediated by a cytoplasmic carrier protein. It also rules out the possibility that ceramide can bypass the Golgi on the way to the

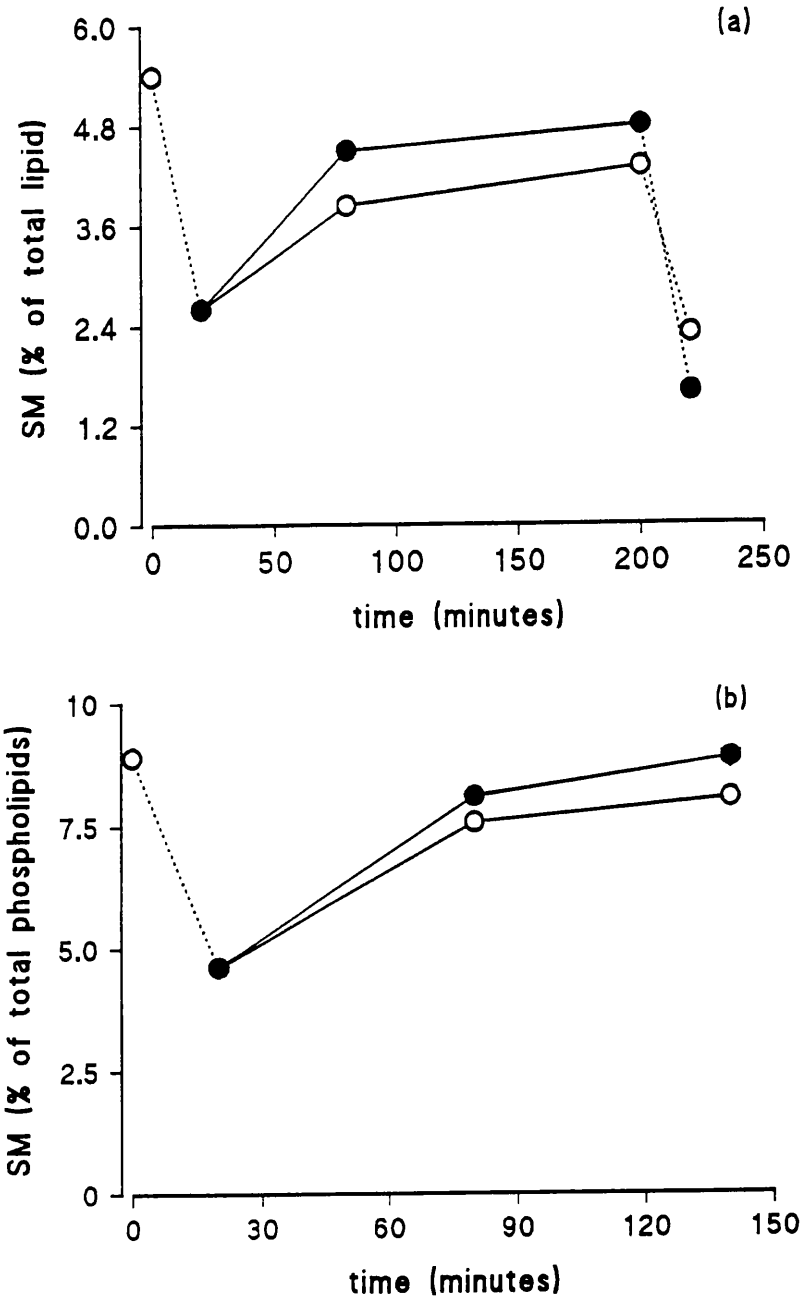


Figure II-6: Resynthesis and return to the surface of sphingomyelin degraded by exogenous sphingomyelinase is not affected by monensin (a). Cells were treated with sphingomyelinase as described previously. Resynthesis of sphingomyelin was followed in the presence or absence of 10μM monensin and succeeded by a second treatment with sphingomyelinase to determine the amount of surface sphingomyelin at the end of the incubation. The dotted lines indicate the periods when sphingomyelinase was present. Results represent the means of triplicate determinations in a single experiment which was repeated twice with the same result. **(b).** Cells were grown to subconfluence in plastic dishes ($\varnothing = 10$ cm) and treated as described under (a). Individual phospholipid classes were determined by phosphorus determinations as detailed in Materials and Methods. ○, control cells; ●, monensin-treated cells.

second site of sphingomyelin synthesis via a vesicular transport route different from the secretory pathway. A vesicular pathway independent of the Golgi was recently postulated to explain why inhibitors of vesicular transport failed to inhibit transport of newly synthesized cholesterol from the ER to the plasma membrane [133,333].

The results presented here are consistent with synthesis of the intracellular pool of sphingomyelin in the known *cis-/medial*-Golgi site [90,129]. In addition they suggest that in BHK cells, plasma membrane sphingomyelin is not made in the *cis-/medial*-Golgi, but at a later stage on the secretory pathway. The somewhat higher levels of labelled sphingomyelin remaining in monensin-treated cells in comparison to those treated with sphingomyelinase (Figure II-2) is readily explicable, since the monensin block was not complete at a concentration of 1 μM (Figure II-3), [319], i.e. some ceramide could transgress it and gain access to the second sphingomyelin synthesis site. If ceramide was primarily transported by cytoplasmic proteins then it should be able to reach this second site and then be transported as sphingomyelin to the cell surface. The fact, however, that there is virtually no labelled sphingomyelin on the surface of monensin-treated cells argues against this possibility.

The lack of a monensin effect on synthesis of the internal sphingomyelin pool involves no conceptual difficulty, since in BHK cells monensin specifically blocks vesicular transport between *medial-* and *trans*-Golgi [100,319] and would thus not be expected to hinder transport of ceramide from its site of synthesis in the endoplasmic reticulum [185] to the sphingomyelin synthesis site in the *cis-/medial*-Golgi [90,129].

Although the preceeding paragraphs have intrepreted the findings of this study largely in favour of vesicular transport of ceramide, a study by Collins & Warren [46] came to a somewhat different conclusion. These authors oserved that in mitotic cells synthesis of the immediate ceramide derivatives, glucosylceramide and sphingomyelin, was inhibited far less than that of higher gangliosides in late Golgi compartments. Since vesicular transport of proteins between ER and early Golgi compartments is inhibited during mitosis, this could indicate the existence of a protein mediating ceramide transport between ER and early Golgi. Thus it may be that ceramide is tranported by two modes: by a carrier protein between ER and Golgi and by vesicles within the Golgi. While the experiments discussed here cannot

elucidate the mode of ceramide transport between ER and Golgi, results which are presented later (C.III.2.6.) suggest that plasma membrane ceramide does not gain access to the glucosylceramide synthase located in the Golgi. This renders transport of ceramide between these structures unlikely regardless whether vesicular or protein-mediated transport is considered.

C.II.4.2. The metabolic fate of ceramide

In addition to its inhibitory effect on sphingomyelin synthesis, monensin causes a pronounced rise in the labelling of monoglucosylceramide (Figure II-2). This result is consistent with data published by other authors [271] who also observed a marked increase of labelling of glucosylceramide in the presence of monensin at concentrations similar to those used in the present study (Figure II-3). However, the monensin effect on sphingomyelin and ceramide synthesis may have escaped the attention of these authors, as ^{3}H-galactose was used as radioactive label in their experiments.

The total radioactivity incorporated into sphingolipids was about equal in control and monensin-treated cells (Figure II-2), suggesting that a significant proportion of the ceramide which could not be turned into plasma membrane sphingomyelin was instead utilized for synthesis of glucosylceramide. Several studies [51,89,130,327] have localized this reaction mainly to the *cis-/medial*-Golgi, although significant activity of the UDP-glucose:ceramide glucosyltransferase has also been found in other Golgi fractions, including distal [130] and pre-/early-Golgi fractions [89], but not in plasma membrane fractions [89]. Since monensin blocks vesicular transport between *medial-* and *trans*-Golgi, ceramide will most likely accumulate in the *medial*-Golgi. Thus more ceramide is available for conversion to glucosylceramide in this compartment and therefore the increased synthesis of glucosylceramide can be interpreted as a consequence of the inhibition of ceramide progress further through the Golgi apparatus. In addition to enhanced synthesis of glucosylceramide, the inhibition of synthesis of higher gangliosides [278] could also contribute to the accumulation of radioactivity in glucosylceramide in monensin-treated cells.

Surprisingly, none of the accumulating ceramide appeared to be turned into

sphingomyelin destined for the intracellular pool of this lipid. It is therefore unlikely that retrograde transport of ceramide occurred in monensin-treated cells, since in that case ceramide should gain access to the *cis-/medial*-Golgi site and be metabolized to internal sphingomyelin. In view of the enhanced synthesis of glucosylceramide this indicates a spatial separation of the UDP-glucose:ceramide glucosyltransferase and the PCCP, with the latter residing in a compartment proximal to the *medial*-Golgi. These conclusions raise the intriguing question of how the cell normally regulates the flow of ceramide into glucosylceramide, sphingomyelin destined for the intracellular pool and plasma membrane sphingomyelin.

C.II.4.3. Inconsistencies between results obtained using radioactive and fluorescent labelled precursors of sphingomyelin

The inhibitory effect of monensin on synthesis of plasma membrane sphingomyelin is a novel and unexpected finding. It is at variance with a previous study by Lipsky and Pagano who used NBD-ceramide to study the metabolism and transport of its sphingolipid metabolites [175]. In control cells around 80% of the added NBD-ceramide was turned equally into NBD-sphingomyelin and NBD-glucosylceramide within 30 minutes and 50% of each of these metabolites was extractable by back exchange from the plasma membrane. Addition of monensin to the cells reduced the proportion of extractable NBD-sphingomyelin to 25%, but also caused an increase in the proportion of total NBD-lipids recovered in NBD-sphingomyelin from 40 to 50%, largely at the expense of NBD-glucosylceramide. Thus, monensin seemed to inhibit transport, but not synthesis of sphingomyelin in this work [175].

The precise reasons for the discrepancy between the results of Lipsky and Pagano's study and the present work are unclear. The increase in the synthesis of sphingomyelin is, however, reminiscent of the somewhat higher rate of sphingomyelin resynthesis observed in some of our experiments in the presence of monensin (Figure II-6). If the resynthesis site distal to the Golgi is also the site normally responsible for synthesis of plasma membrane sphingomyelin, the addition of monensin would block delivery of ceramide to this site (Figure II-1 and 2), but

not delivery of ceramide produced at the plasma membrane by exogenous sphingomyelinase (Figure II-6). Likewise, NBD-ceramide inserted into the plasma membrane at 0°C should gain access to the resynthesis site after warming to 37°C and should be turned into sphingomyelin. In the presence of monensin the synthesis of sphingomyelin by the resynthesis site would lack the competition of unlabelled endogenous ceramide, probably explaining the somewhat quicker resynthesis of surface sphingomyelin (Figure II-6) and also the finding of Lipsky and Pagano [175] that monensin increased synthesis of NBD-sphingomyelin.

Nevertheless, if the above explanation held true for the increased synthesis observed by Pagano [175], one would expect more sphingomyelin to be available for back exchange with phospholipid vesicles at the cell surface. One reason for this discrepancy between prediction and experimental reality could be due to the use of fluorescent labelled ceramide by Lipsky and Pagano [175] which is known to be capable of monomeric diffusion between intracellular membranes [176]. Bishop and Bell [26] have already pointed out that a *caveat* has to be applied to results obtained by such an approach. Alternatively, one might suspect that resynthesis could potentially take place at an intracellular location. It is conceivable that during the rather short incubation period of 30 minutes applied by Lipsky and Pagano [175] only part of the NBD-sphingomyelin produced at the resynthesis site travelled back to the surface [151] and thus the bulk of the NBD-sphingomyelin produced at this site might not be amenable to back exchange. The next chapter will deal with the question of an intracellular location of the resynthesis site.

C.II.4.4. Cholesterol ester formation

A common theme occurring with monensin, BFA (see C.I.) and sphingomyelinase-treated cells [293,296] is the depletion of plasma membrane sphingomyelin which is associated with an enhancement of the conversion of cholesterol to cholesterol ester (Table 4,5; Figure II-4). Clearly, the cell attaches paramount importance to the maintenance of the sphingomyelin:cholesterol balance in the plasma membrane, since esterification of cholesterol is rapid and markedly increased after only 20 minutes (Figure II-4), [293]. The details of the mechanism

underlying this process are not known, although it is presumed to be mediated by the acyl-CoA:cholesterol acyltransferase (ACAT), [299,306], an enzyme usually found in a smooth microsome fraction, supposed to mainly consist of ER membranes [43,113,255]. A location of the ACAT in the ER poses a conceptual problem at first glance, as transport of plasma membrane cholesterol to the ER is very slow ($t_{1/2} \geq$ 46 h),[299,325] and it is thus difficult to see how the rapid esterification of cholesterol could be catalysed by an ER resident enzyme . However, cholesterol might normally be "tied down" in the plasma membrane by some unknown mechanism in which sphingomyelin plays a dominant role. This cholesterol is apparently "freed" by depletion of plasma membrane sphingomyelin in BFA, monensin or sphingomyelinase-treated cells. Apparently, the cell is able to rapidly transfer some of this cholesterol to the endoplasmic reticulum where it is turned into cholesterol ester. A yet unsolved problem is the question why it should be advantageous for the cells to store excess cholesterol in the form of cholesterol ester. Cholesterol ester is the most hydrophobic of the common lipids and is very unlikely to remain in a bilayer, rather it would form a separate phase, e.g.droplets.

C.II.4.5. Two sites of sphingomyelin synthesis sites?

The results presented here provide convincing evidence for the existence of a second sphingomyelin synthesis site in BHK cells. This confirms a suggestion by Malgat et al.[184] who obtained cell fractionation data which were consistent with two sites of sphingomyelin synthesis in rat liver and brain. It also seems conceivable that the two sites of sphingomyelin synthesis inferred from the present study are the cause of the different labelling of intracellular and plasma membrane sphingomyelin observed by Quinn and Allan in BHK cells [250]. In principle, it is possible that the distal, second sphingomyelin synthesis site which was detected by studying sphingomyelin resynthesis in the presence of monensin (Figure II-6) is not the site responsible for normal de novo synthesis of plasma membrane sphingomyelin, in which case one would have to assume three cellular sphingomyelin synthesis sites. This possibility cannot be rejected a priori on the basis of the experiments presented here and will have to be tested in the future. However, the principle of Ockham's

razor [85,247] suggests a minimum of two sites and this is the assumption which is made here and subsequently.

The experiments described above give no hint regarding the location of the second synthesis site other than placing it distal to the *medial*-Golgi. If this site was located on the plasma membrane as previously suggested [61,186,334,361], it seems somewhat bewildering that only one [175] of the more recent studies [129,348] found any PCCP activity in a plasma membrane fraction. The question of the location of the distal sphingomyelin synthesis site will therefore be addressed in the following chapter.

C.III. Resynthesis of plasma membrane sphingomyelin - an intracellular process?

C.III.1. Introduction

The results described in the preceding chapter clearly indicate the existence of two sites of sphingomyelin synthesis in BHK-21 cells: one prior to the monensin-block, most probably identical to the *cis-/medial*-Golgi site identified previously by other authors [90,129], and a second one distal to the monensin block between *medial-* and *trans*-Golgi [100] which is responsible for resynthesis of plasma membrane sphingomyelin after degradation by exogenous sphingomyelinase (see C.II.3.5.). This latter site could also be the site for *de novo* synthesis of plasma membrane sphingomyelin. Several possibilities exist as to the location of the second sphingomyelin synthesis site (referred to as the resynthesis site in the following):

1. The site could be located intracellularly in a more distal compartment of the secretory pathway, e.g. in the *trans*-Golgi cisterna or the *trans*-Golgi-network (TGN). In this case ceramide produced at the cell surface would have to be internalized to gain access to the resynthesis site. A membrane recycling pathway from the cell surface to the *trans*-Golgi has been proposed by Farquhar [81]. Some support for this hypothesis came from the observation that the desialylated transferrin receptor is resialylated after endocytosis [301], a process believed to occur in the *trans*-Golgi or TGN [94]. However, the kinetics of resialylation are much slower than those of receptor recycling. This could indicate that asialo-transferrin receptor follows a pathway distinct from that of transferrin receptor in iron-uptake [301]. A recent study clearly showed that "intact" transferrin receptor is not present in compartments of the secretory pathway [48].
   Nevertheless, a sphingomyelin resynthesis site in the *trans*-Golgi/TGN cannot be discounted *a priori*, in particular since the TGN has strongly been advocated by van Meer et al.[348,350,356] as the site responsible for sorting of sphingolipids in epithelial, polarized cells. A putative sphingomyelin synthesis site in the TGN could be imagined to participate in this sorting process.

2. Alternatively the second sphingomyelin synthesis site could simply be located on the plasma membrane as originally proposed by several groups [61,186,334,361]. Based on cell fractionation studies [90,129] and microscopic studies using fluorescent-labelled sphingomyelin precursors [175,357] this concept has not been substantiated. Only a minor proportion ($\sim$ 10%) of total cellular sphingomyelin synthesis was somewhat reluctantly allocated to the plasma membrane by one group [90]. However, it is difficult to find any obvious flaw in the earlier studies suggesting a plasma membrane site of sphingomyelin synthesis. Consequently, the plasma membrane cannot be excluded *a priori* as the site of sphingomyelin resynthesis.

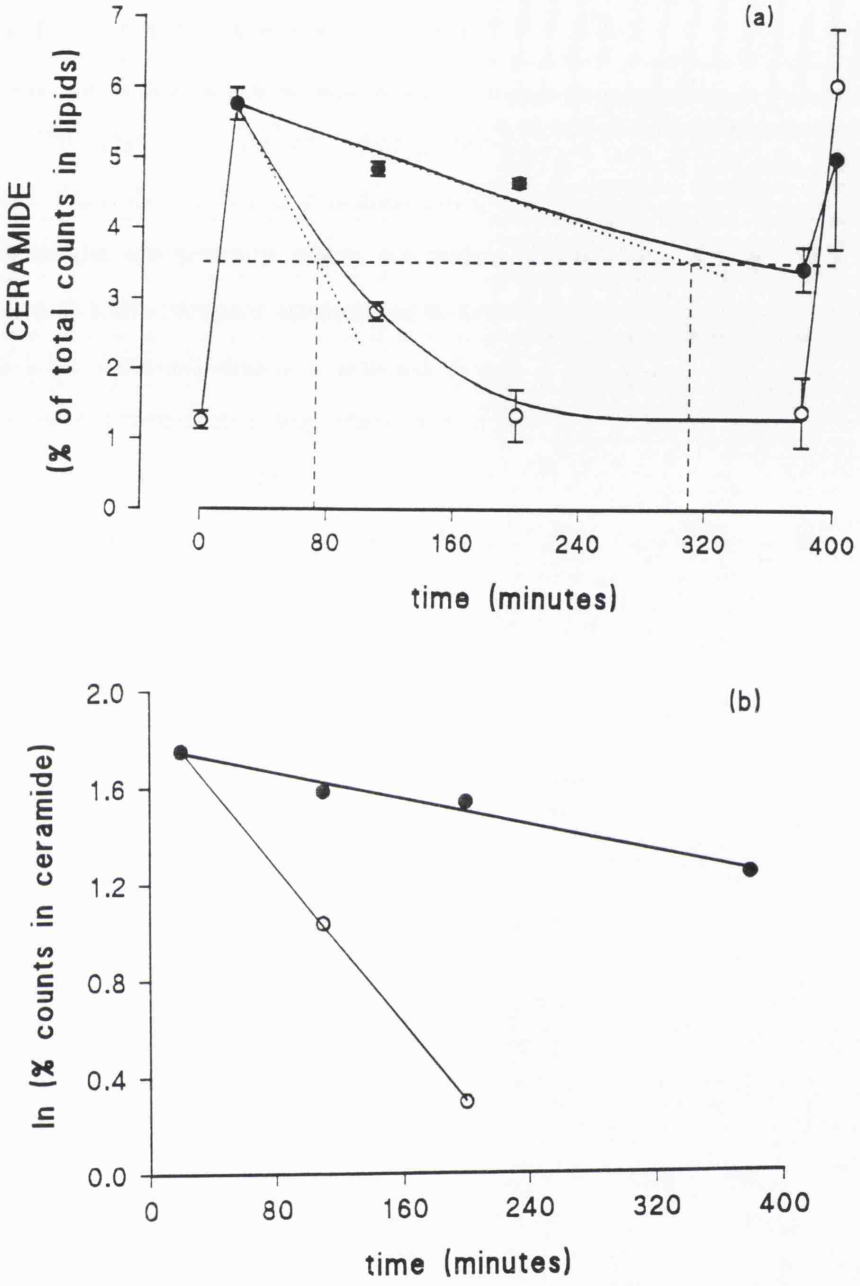
3. Finally, the hidden site of sphingomyelin resynthesis could reside somewhere on the endocytic pathway. Merrill and Jones [202] pointed out that the kinetics of sphingomyelin resynthesis [4] are consistent with an intracellular location of the resynthesis site on the endocytic pathway. Synthesis of NBD-sphingomyelin from exogenous NBD-ceramide has been demonstrated, but is believed to occur in the Golgi [175]. However, there is little reason to assume that endogenous ceramide is able to diffuse across aqueous phases and probably, sphingomyelin resynthesis from plasma membrane ceramide is on the recycling pathway revealed by exogenous fluorescent sphingomyelin or glucosylceramide [144,146,151].

Experimental approaches to distinguish between these possibilities are presented below.

C.III.2. Results

C.III.2.1. Temperature effect on sphingomyelin resynthesis

One approach to clarifying whether the sphingomyelin resynthesis site resides in the *trans*-Golgi/TGN is to block exit from these compartments to the plasma membrane. In this case sphingomyelin which had been degraded on the cell surface would probably be resynthesized by the presumed *trans*-Golgi/TGN site, but it would



III-1: Temperature effect on sphingomyelin resynthesis (a). Cells labelled to equilibrium were treated with SMase as outlined in Methods and resynthesis studied at low temperature (17°C). Since resynthesis of sphingomyelin from ceramide produced by exogenous SMase is largely conservative (see figure II-5), reutilization of ceramide was used to monitor sphingomyelin resynthesis. Values are means ± sd (n = 3) from a single experiment. **(b)** Linearization of a. Control: $x = -8.1 \cdot 10^{-3} + 1.92$, low temperature: $x = -1.38 \cdot 10^{-3} + 1.77$, regression coefficient > 0.99 for both regression lines. The line obtained by linearization depends critically on the correctness of the last value, in particular where equilibrium is reached (as in the controls) small changes can substantially alter the outcome of the analysis. (○) controls, (●) low temperature

not be transported to the plasma membrane subsequently and would consequently not be degradable by exogenous sphingomyelinase.

Lowering the temperature to below 20°C inhibits exit of VSV G-protein from the TGN to the plasma membrane, whereas temperatures below 15°C were alleged to inhibit exit from the CGN (intermediate compartment) [101,267]. Some confusion prevails as to the temperature effect on endocytosis: whereas uptake of Semliki Forest virus and LDL-receptor are stopped below 16°C, uptake of asialoglycoprotein and pinocytotic internalization is noticed down to temperatures of 10°C [74]. Resynthesis of sphingomyelin was therefore studied at 17° to block exit from the TGN, but to leave other transport processes intact.

Following treatment with B.cereus sphingomyelinase and removal of the enzymes by washing BHK-21 cells were incubated for six hours at 17°C and 37°C respectively. Figure III-1 shows the results of this approach. Resynthesis of sphingomyelin was monitored by measuring reutilisation of plasma membrane ceramide which had been produced by degradation of surface sphingomyelin. This is possible as resynthesis of sphingomyelin appears to be largely conservative (see C.II.3.4. and C.III.2.5.). As expected, much less ceramide was converted into sphingomyelin in cells kept at 17°C. The half-time for utilization of ceramide for resynthesis of sphingomyelin was 5.5 times higher at the lower temperature than the control value at 37°C ($t_{1/2} \approx 290$ minutes at 17°C, $t_{1/2} \approx 53$ minutes at 37°C) equivalent to a Q_{10} coefficient of 2.35. This value is midway between a Q_{10} of 2 and 2.7 for enzymatic reactions [312] and endocytosis [308] respectively. Linearization of the resynthesis data yielded a similar Q_{10} of 2.42. Hence, this approach did not seem to yield a decision whether the reduced rate of ceramide utilization was due to a simple temperature effect on a surface enzyme reaction or a consequence of a diminished rate of endocytosis.

A second treatment of BHK-21 cells with extracellular sphingomyelinase revealed an increase of ceramide in cells kept at either 37°C or 17°C throughout resynthesis. There was no statistically significant difference between control cells and "low temperature" cells which indicated that the resynthesized sphingomyelin was at the cell surface and suggested that *trans*-Golgi or TGN were probably not the sites of sphingomyelin resynthesis, since return of sphingomyelin to the cell surface

survived the < 20° block.

C.III.2.2. Brefeldin A and sphingomyelin resynthesis

The inability of low temperature to block appearance of resynthesized sphingomyelin in the outer leaflet of the plasma membrane could be regarded as an argument (but no proof) against a location of sphingomyelin resynthesis in either the *trans*-Golgi or the TGN. Nevertheless, it was clearly desirable to obtain independent evidence for this conclusion. Wood et al.[371] reported that BFA fused TGN and early endosomes in NRK-cells, but Miller et al.[209] demonstrated that TGN and early endosomes continued to exist as separate compartments in BHK-21 cells in the presence of BFA. The latter authors also showed that BFA inhibits exit of VSV G-protein from the TGN to the cell surface in BHK-21 cells [209]. In contrast, treatment with BFA does not affect plasma membrane recycling as monitored by NBD-SM [209] nor does BFA prevent budding of clathrin-coated endocytic vesicles from the plasma membrane [262].

Cells labelled to equilibrium were treated with sphingomyelinase and prepared for resynthesis as described above (C.II.3.4.). BFA (5 μg/ml) was added to half of the samples and resynthesis followed for three hours. As Figure III-2 shows BFA does not have a detectable effect on sphingomyelin resynthesis nor on ceramide reutilization. Similarly BFA does not prevent resynthesized sphingomyelin from appearing on the external plasma membrane leaflet. If sphingomyelin resynthesis took place in the *trans*-Golgi or the TGN, one would not expect the resynthesized sphingomyelin to be amenable to a second breakdown by exogenous sphingomyelinase in BFA-treated cells. Thus the absence of a BFA effect on sphingomyelin resynthesis provides further evidence against a location of resynthesis in either *trans*-Golgi or TGN and suggests that resynthesis of sphingomyelin occurs distal to the TGN, perhaps at the plasma membrane or in the endocytic system.

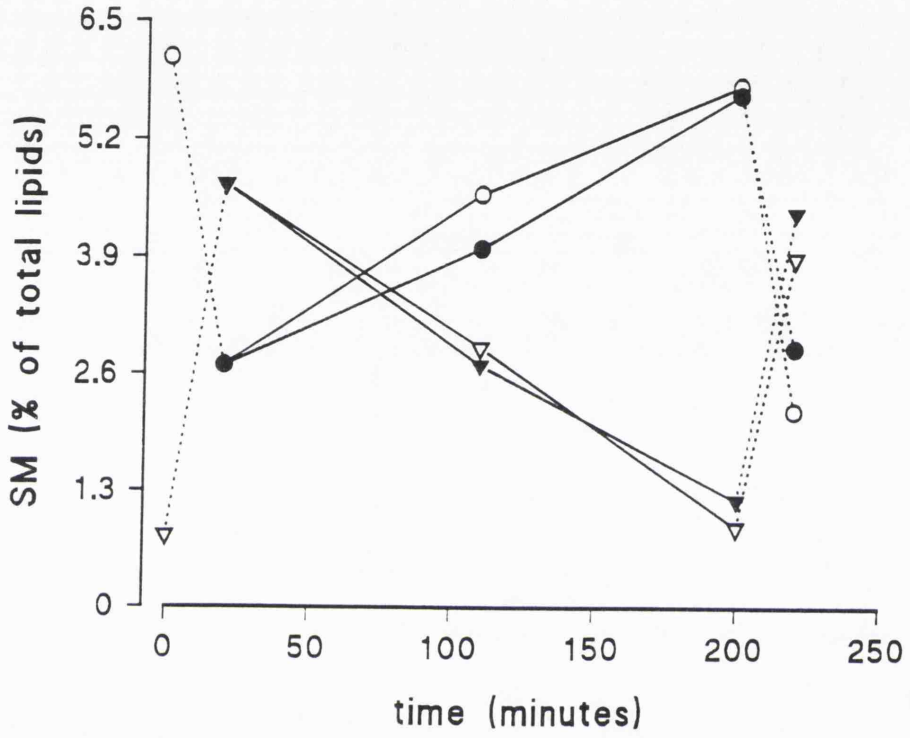


Figure III-2: Brefeldin A does not affect resynthesis of sphingomyelin and its return to the surface of BHK cells exposed temporarily to sphingomyelinase. Cells were labelled to equilibrium with ^{3}H acetate, treated with sphingomyelinase and then allowed to resynthesise sphingomyelin after removal of the enzyme, as described under Methods. After 3h, a further aliquot of sphingomyelinase was added to some samples in order to determine the proportion of surface sphingomyelin after resynthesis (dotted lines show where SMase was present). Parallel determinations were carried out with 5 μg/ml BFA present during resynthesis (solid symbols). Values are expressed as the percentage of total lipid radioactivity present as sphingomyelin (○,●) or ceramide (▽,▼) and represent the means of triplicate determinations (s.d. ≤ 10%) in one experiment which gave results representative of three similar experiments. Open symbols: control cells.

C.III.2.3. NEM and digitonin block resynthesis of sphingomyelin

The NEM-sensitive fusion protein is a mandatory component of the fusion machinery of the secretory pathway [267, see A.5.1.]. Its presence is also necessary for fusion of endocytic vesicles [105]. Mild treatment of cytosol with NEM (100 μM, 5 minutes) has been reported to inhibit vesicular transport through the secretory pathway [20,369] and the endocytic system [60,105] in permeabilized cells and replacement of NEM-treated cytosol with fresh cytosol restores the transport processes. These observations stimulated us to test the effect of NEM on the resynthesis of sphingomyelin in intact BHK-21 cells (Figure III-3).

After degradation of sphingomyelin in BHK-21 cells labelled to equilibrium with ^{3}H-acetate were treated with NEM (100 μM) either for 5 minutes, followed by washing and resynthesis for one hour (a) or for the whole of a two hour resynthesis period (b). In a third experiment cells were pretreated with NEM for 30 minutes prior to degradation and resynthesis (for 3 hours) of sphingomyelin in the presence of NEM. NEM showed a pronounced inhibitory effect on the resynthesis of sphingomyelin in all cases. Whereas the presence of the drug during resynthesis decreased resynthesis by around 80% compared with control cells, even the very short term treatment achieves a convincing reduction of resynthesis by 60%. The NEM effect certainly cannot prove an absolute requirement of endocytosis for the resynthesis of sphingomyelin since the effect could also be due to unspecific reactions with cellular proteins exposing free SH-groups, probably even the sphingomyelin synthase itself. In the latter case, however, a location of the enzyme in the outer leaflet of the plasma membrane would be somewhat surprising as cells by and large show little tendency to expose SH-groups to environments whose redox-potential is not under their own control [David Allan, personal communication].

As already mentioned, fusion of vesicles depends on an elaborate protein equipment consisting of both membrane-bound and cytosolic components [265]. The cytosolic components are likely to be lost by permeabilisation of the plasma membrane provided that the permeabilizing agent produces holes big enough to allow diffusion of proteins into the external buffer. As a consequence one can expect any process depending on vesicular transport, including endocytosis, to be inhibited in

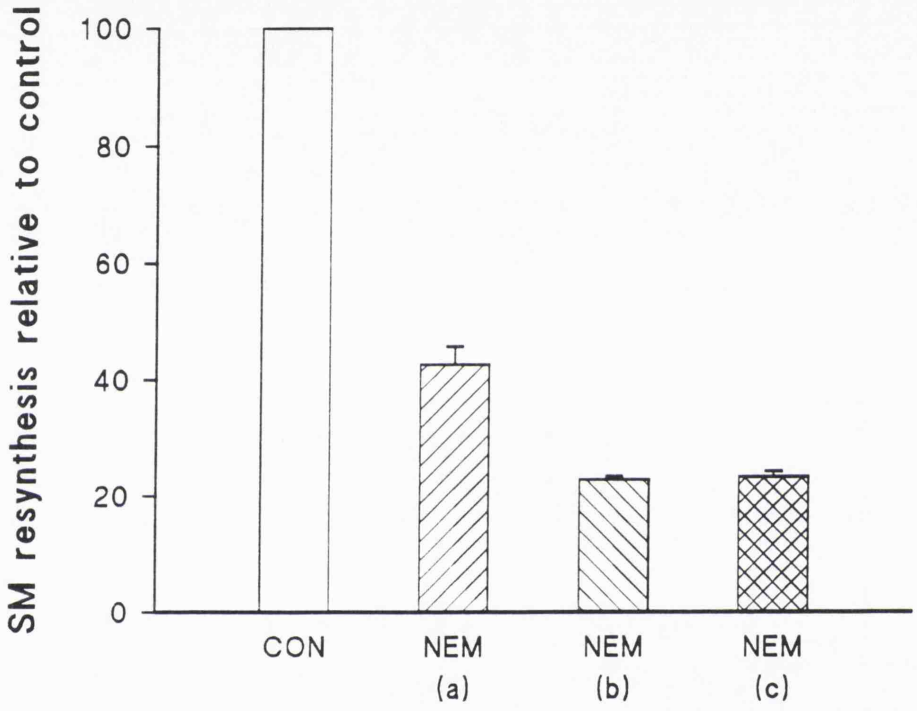


Figure III-3: NEM is a strong inhibitor of sphingomyelin resynthesis. Figures represent results of different experiments in which BHK-cells labelled to equilibrium with ^{3}H-acetate were treated in different ways by NEM as described in the text. **a:** (/)- **hatched bars:** cells were treated for 5 minutes with NEM after degradation of sphingomyelin, **b:** (\)**-hatched bars:** NEM was present during the whole resynthesis period, **c: cross-hatched bars:** cells were pretreated with NEM prior to degradation of sphingomyelin. Sphingomyelin breakdown was around 60% in all cases.

permeabilized cells. Accordingly, the effect of digitonin on the resynthesis of sphingomyelin was investigated.

Treatment of BHK-21 cells with 50 μM digitonin for 5 minutes at 0°C in serum-free medium results in a complete release of LDH, a protein with M_r=140 kD [324], as demonstrated by the identical activity of LDH in the supernatant of digitonin and Triton-X 100 (0.01%) treated cells (Figure III-4a). BHK-21 cells were therefore treated by the same digitonin protocol after degradation of sphingomyelin and removal of the sphingomyelinase. The resynthesis of sphingomyelin was then studied in unpermeabilized cells incubated in serum-free medium and in permeabilized cells incubated in serum-free medium or an intracellular replacement buffer containing 20% wheat germ cytoplasm (Figure III-4b). Sphingomyelin resynthesis was completely abolished in all permeabilized cells and in cells incubated in the cytoplasmic replacement buffer sphingomyelin levels even dropped slightly.

Again this result would be consistent with, but does not prove a requirement of endocytosis for resynthesis of sphingomyelin. On the other hand the effect of digitonin in complexing plasma membrane cholesterol might suppress the activity of the phosphocholine transferase in the plasma membrane, although it appears questionable whether this assumption could explain the radical abolition of sphingomyelin resynthesis in permeabilized cells.

C.III.2.4. ATP-depletion and treatment of cells with AlF_4^- inhibit resynthesis of sphingomyelin

Certainly the inhibition of sphingomyelin resynthesis by digitonin and NEM cannot be interpreted as anything like an unequivocal proof of a requirement of endocytosis for resynthesis of sphingomyelin. Nevertheless, they prompted further investigations into this direction. The combined treatment of cells with the metabolic inhibitors 2-deoxy-glucose (DOG, 50 mM) and sodium azide (NaN_3, 3 - 5 mM) has been reported on numerous occasions to inhibit endocytosis and, particularly relevant, internalization of fluorescent-labelled sphingomyelin and glucosylceramide [144,146,151,308]. In the present work, however, sodium azide was replaced by

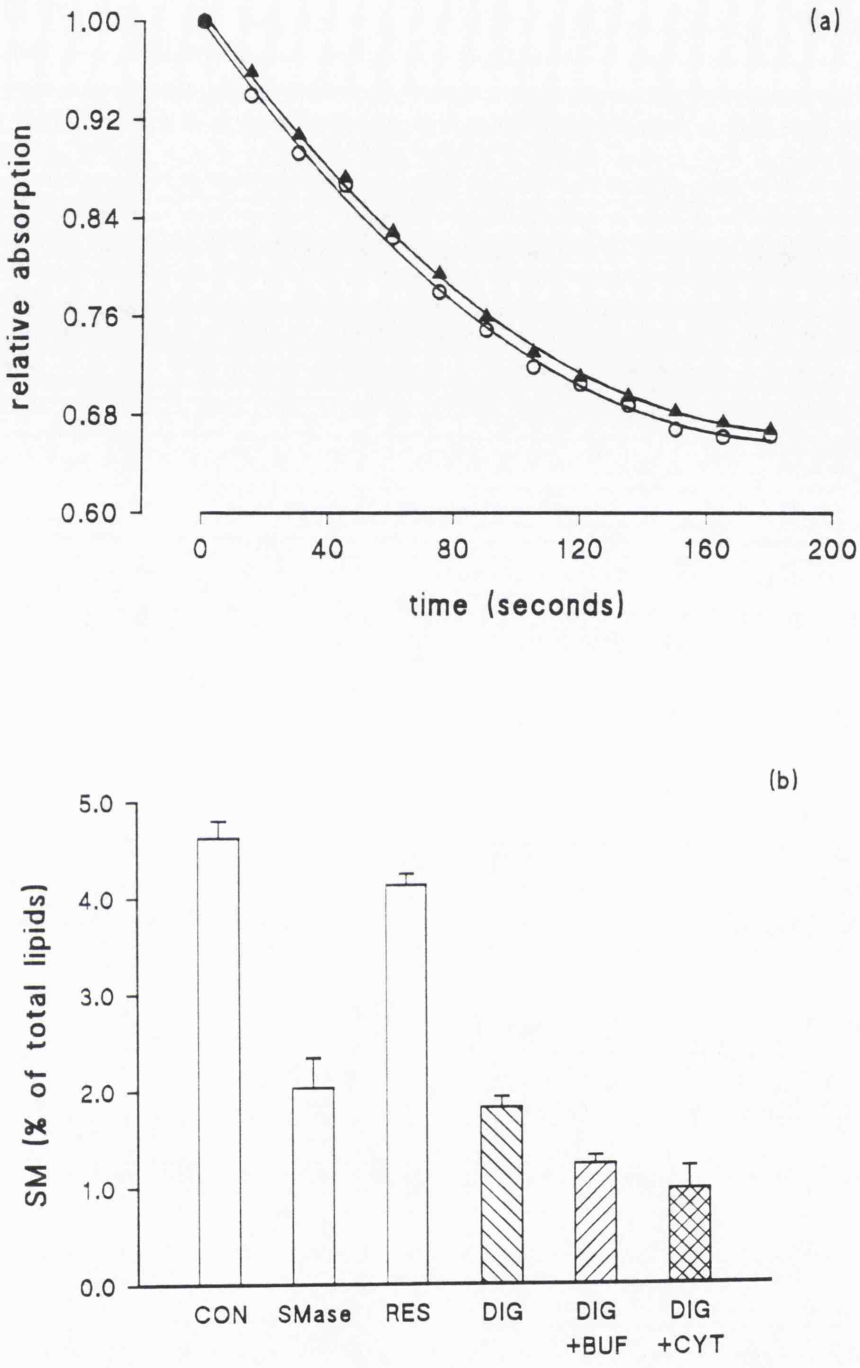


Figure III-4: Study of sphingomyelin resynthesis in permeabilized cells. (a) Release of LDH in digitonin treated cells.(○)cell lysed with triton X-100, ▲ cells lysed with digitonin. **(b)** Resynthesis of sphingomyelin in permeabilized cells. Cells were permeabilized with digitonin as described in Methods (B.13.3.).

sodium cyanide (NaCN, 100 μM) to lower ATP levels since the latter agent inhibits mitochondrial respiration at a much lower concentration (NaCN: ED_{50}=10 nM, NaN_3: ED_{50}=1 mM, [62]).

BHK-21 cells were pretreated for two hours with 50 mM DOG and 100 μM NaCN which were also present during all other steps of the experiment. Degradation and resynthesis of plasma membrane sphingomyelin were then assessed as described in C.II.3.4.. After one hour, resynthesis of sphingomyelin was reduced by 60% in the cells treated with DOG and KCN by comparison to control cells, but the difference was diminished to 20% after three hours (Figure III-5). The recovery of sphingomyelin resynthesis was somewhat unexpected, given the seemingly rigid ATP-depletion protocol. However, measurements of the cellular ATP levels under the above conditions showed that the ATP level dropped by only 40% after 60 minutes and remained at that level even when the incubation was prolonged to three hours (personal communication, Dr Jacqueline Whatmore, UCL).

Additionally the effect of AlF_4^- on the resynthesis of sphingomyelin was checked. AlF_4^- activates the α-subunit of heterotrimeric GTP-binding proteins but not small GTP-binding proteins. These experiments were originally instigated by the idea that stimulation of a heterotrimeric GTP-binding protein that was recently demonstrated to inhibit endosome fusion *in vitro* [47] might also decrease resynthesis of sphingomyelin. Subsequent to degradation of sphingomyelin, BHK-21 cells were therefore incubated with growth medium supplemented with 30 mM NaF and 62.5 μM $AlCl_3$, a protocol given with slight variations by several authors for activation of heterotrimeric GTP-binding proteins in intact cells [69,199]. As Figure III-5a shows treatment with AlF_4^- largely suppresses resynthesis of surface sphingomyelin after one and also after three hours.

It was clearly tempting to attribute this effect to an activation of the GTP-binding protein causing an inhibition of endocytosis. However, NaF by itself is a well-known metabolic inhibitor [308] with pronounced effects on the ATP-levels of BHK-21 cells: within 15 minutes after addition of NaF the ATP-level is reduced to about 13% of the original value and drops further to values of 5% and less than 1% after one and three hours respectively (personal communication, Jacqueline Whatmore, UCL). To test whether NaF alone was able to elicit the same inhibitory effect on

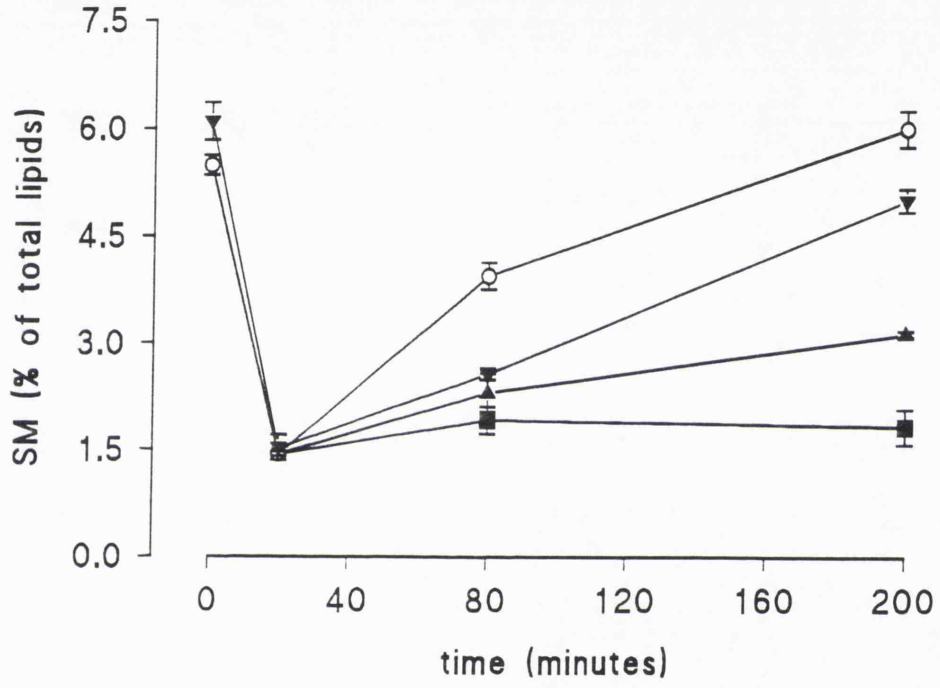


Figure III-5: Time course of sphingomyelin resynthesis in energy depleted cells. Cells labelled to equilibrium with ^{3}H-acetate were treated with sphingomyelinase and allowed to resynthesize sphingomyelin either alone (○), in the presence of 100 μM KCN plus 50 mM deoxyglucose (▼), in the presence of 30 mM NaF plus 62.5 μM $AlCl_3$ (▲) or 30 mM NaF alone (■).

sphingomyelin resynthesis as a combination of NaF and $AlCl_3$ BHK-21 cells were treated for 30 minutes with B.cereus sphingomyelinase in the presence and absence of 30 mM NaF and were then allowed to recover for 3 hours in growth medium with or without NaF. As shown this treatment inhibited sphingomyelin resynthesis to the same extent (or even slightly more) as the combination of NaF and $AlCl_3$.

C.III.2.5. Resynthesis of sphingomyelin is blocked in mitotic cells

It has been known for some years that practically all vesicular transport processes, including endocytosis, come to a halt during mitosis [364]. In determining whether a certain biological phenomenon critically depends on vesicular transport, one might regard the study of this phenomenon in mitotic cells as the 'gold standard' for this decision. Kobayashi and Pagano could thus demonstrate that after insertion into the plasma membrane of mitotic cells the fluorescent-labelled sphingolipid precursor NBD-ceramide is metabolised to sphingomyelin and glucosylceramide, but that transport of these metabolites to the cell surface is prevented [143]. Notwithstanding, NBD-ceramide is capable of monomeric diffusion between a variety of intracellular organelles [229] and consequently these experiments did not allow rejection of an endocytic site for resynthesis of sphingomyelin. However, natural ceramide is far more hydrophobic than phospholipids and its fluorescent-labelled analogues and therefore would be more likely to be transported in vesicles by cells, especially since a carrier protein for ceramide has not yet been identified. If resynthesis of sphingomyelin was indeed dependent on endocytosis in BHK-21 cells, one would postulate this reaction to be inhibited during mitosis.

Mitotic cells were therefore prepared according to Featherstone et al.[83] as described in Materials and Methods. The mitotic cells were suspended in serum-free growth medium and exposed to B.cereus sphingomyelinase (0.08 units/ml) for 20 minutes at 37°C. After being washed twice with growth medium in the absence and presence of nocodazole the cells were seeded out into Falcon plastic dishes to measure resynthesis of sphingomyelin. It should be made clear, however, that a major problem occurs in this washing step since treatment with sphingomyelinase renders the mitotic cells very sensitive to the shear stress of centrifugation. Lysis of

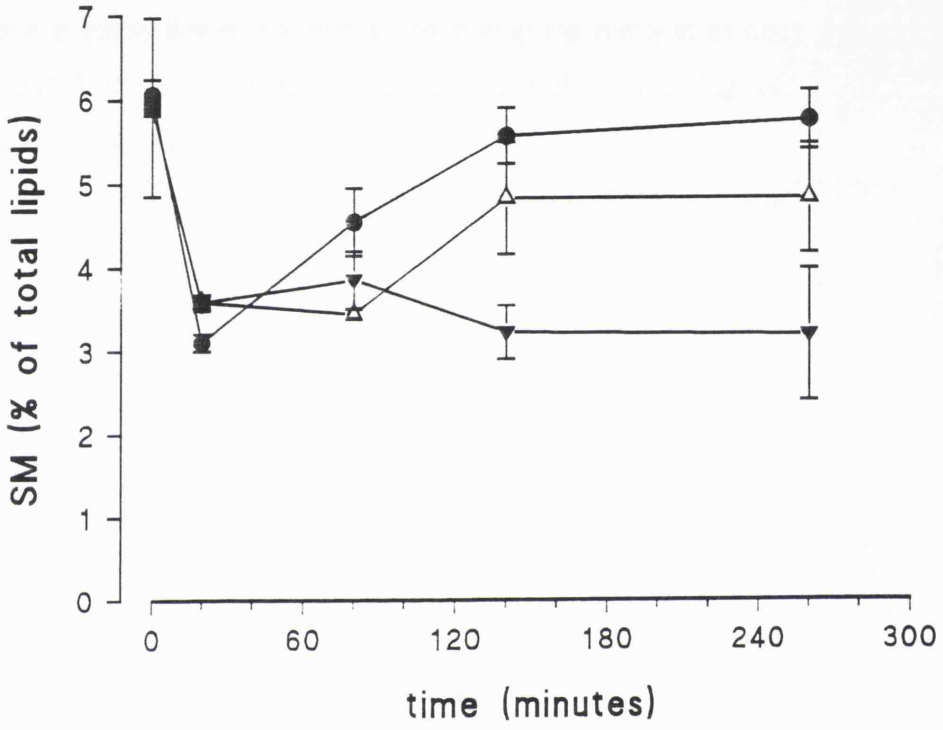


Figure III-6: Mitotic cells do not resynthesize sphingomyelin Labelled mitotic cells were prepared and incubated with sphingomyelinase as described under Methods. After removal of the enzyme, half of the cells were allowed to enter G_0 (▵) by washing out the nocodazole, whereas the remaining cells were further incubated in the presence of nocodazole and thus kept in mitosis (▾). The experiment shown is one of three which gave similar results. Values represent the means ± SD of triplicate determinations at each time point. (●) Interphase cells which had not been treated with nocodazole were also treated with sphingomyelinase and allowed to resynthesize sphingomyelin.

the mitotic cells during washing became particularly extensive when cold solutions were used for washing. Allan and Walklin [8] attributed a similar phenomenon in sphingomyelinase treated human erythrocytes to the formation of holes in the plasma membrane as a consequence of the phase separation of cholesterol set free by hydrolysis of sphingomyelin. Addition of 0.2% albumin to the medium containing the sphingomyelinase seemed to partly protect the cells against lysis.

Figure III-6 compares the resynthesis of sphingomyelin in cells kept in mitosis and control cells allowed to enter G_0 following the removal of nocodazole. Control cells started to resynthesize sphingomyelin with a time lag of one hour when compared to cells that had not been treated with nocodazole. The time lag before resynthesis started in control cells was related to the time necessary for the cells to emerge from mitosis. Resynthesis in control cells was around 60% of the degraded sphingomyelin while there was no detectable resynthesis of sphingomyelin in the mitotic cells. Thus resynthesis of sphingomyelin appeared to crucially depend on operative vesicular transport mechanisms.

C.III.2.6. How conservative is the "recycling pathway"?

The experiments presented in the foregoing paragraphs point to a location of the sphingomyelin resynthesis site in the endocytic system, possibly the early endosomes which have been associated with recycling of receptors generally [192]. Furthermore, resynthesis of sphingomyelin is largely conservative, i.e. virtually all ceramide produced by degradation of plasma membrane sphingomyelin is turned back into sphingomyelin (Figure II-6, III-2, [4]). This observation contrasts quite markedly with experimental studies of Pagano and coworkers using NBD-ceramide [143,175,176]. Within 30 minutes after incorporation into the plasma membrane this fluorescent-labelled ceramide analogue was almost completely converted into equal amounts of sphingomyelin and glucocerebroside [175,176]. NBD-ceramide was also found to be rapidly turned into sphingomyelin and glucosylceramide in mitotic cells [143]. This led to the postulate of a spontaneous movement of fluorescent ceramide from the plasma membrane to the sites of sphingomyelin and glucosylceramide synthesis in early Golgi compartments [151]. However, the physico-chemical

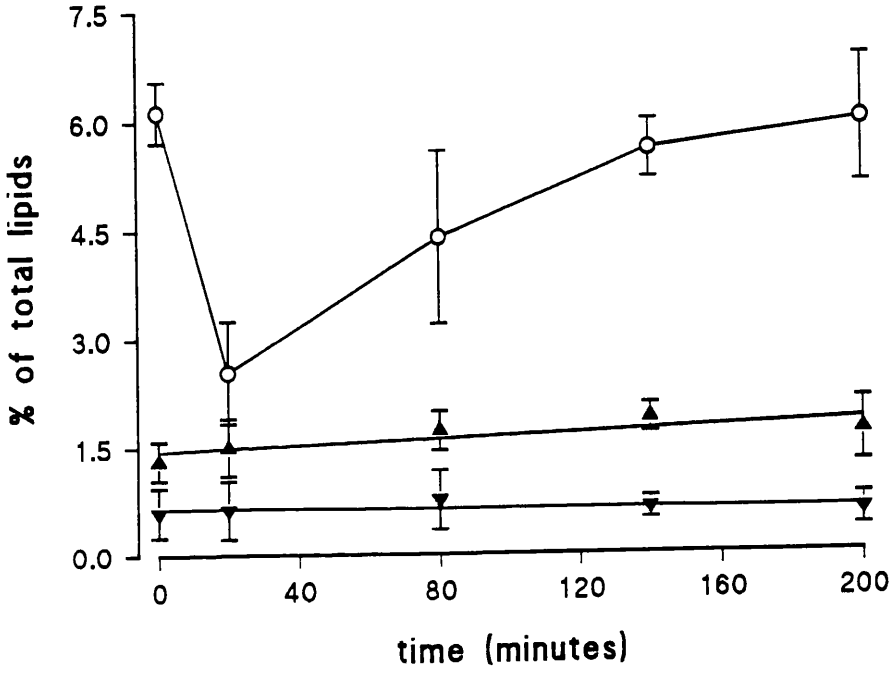


Fig III-7: Leakage of plasma membrane ceramide into the Golgi apparatus. BHK-21 cells were labelled to equilibrium with ^{3}H-acetate and after treated in the same way as described for Figure II-5. Sphingomyelin (○), glucosylceramide (▼) and ganglioside GM3 (▲) after alkaline methanolysis of total lipids (B.2.2.). Values represent the means ± SD of three experiments done in triplicate determinations.

properties of NBD-ceramide are undoubtedly very different from those of natural ceramide. This prompted us to examine whether some of the ceramide produced at the plasma membrane by the action of exogenous sphingomyelinase was able to gain access to the Golgi-sites of glycosphingolipid synthesis.

To investigate this problem the resynthesis assay was used once again. Breakdown of surface sphingomyelin can also be looked at as a means to supply the plasma membrane with free ceramide. By comparison to the incorporation of fluorescent ceramide the plasma membrane is literally flooded with ceramide so that one would expect to see a distinct rise in the concentration of glycosphingolipids if there existed a major pathway for transport of ceramide from the plasma membrane to the Golgi apparatus. A report by Renkonen et al.[256] found glucosylceramide and GM3 as the major glycosphingolipid components of BHK-21 cells. Van Echten and Sandhoff recently demonstrated that these glycosphingolipids are synthesised in the *cis-/medial*-Golgi [340]. It was therefore decided to study the incorporation of ceramide into these lipids subsequent to sphingomyelinase action. As Figure III-7 shows little change rise was detected in the cellular levels of glucosylceramide, while a slight increase in GM3 from 1.5% to about 1.85% of total lipid was detected following treatment with sphingomyelinase. Since breakdown of sphingomyelin in these experiments amounted to 3.6% of total lipid on average, maximally 10% of the ceramide produced at the plasma membrane, or less than 5% per hour, could have leaked into glycosphingolipids during the resynthesis period. This figure for the leakage of endocytosed membrane into the Golgi is 4-5 fold less than that found with NBD-ceramide [175], but very similar to results obtained using ricin [338], NBD-sphingomyelin [197] or NBD-glucosylceramide [145]. Thus, endogenous ceramide in the plasma membrane appears to follow non-selective membrane-flow and does not seem to be transported to Golgi cisternae via proteins.

C.III.2.7. Disruption of the cytoskeleton and sphingomyelin resynthesis

The results presented in the foregoing suggest that endocytosis is mandatory for resynthesis of plasma membrane sphingomyelin. One way to interpret the results further is to assume a location of the resynthesis site on the endocytic pathway. Exogenously added fluorescent-labelled sphingomyelin and glucosylceramide has been shown to colocalize with the transferrin receptor in a perinuclear, para-Golgi region [144,152]. Late endosomes are also located in this region. However, transport from the early endosomes, the compartment that internalized material meets first, to the late endosomes by endosomal carrier vesicles has been found to depend on an intact microtubular system [105]. Therefore the effect on sphingomyelin resynthesis of nocodazole which depolymerises the microtubules [2] was examined, since it was reasoned that resynthesis of sphingomyelin in the late endosomes would be slowed down by depolymerisation of the microtubules.

Cells labelled to equilibrium were preincubated for 30 minutes with nocodazole (10 μM) before the start of the resynthesis assay. This treatment disrupts the microtubules within minutes [145]. Nocodazole was present in all solutions during the experiment. Figure III-8 shows that nocodazole did not inhibit resynthesis of sphingomyelin in accordance with the assumption that the early, but not the late endosomes are the site of resynthesis of plasma membrane sphingomyelin. Cytochalasin D, which depolymerizes the cellular actin filaments [2] has been reported to decrease endocytosis at the apical, but not the basolateral membrane of polarized Caco-2 cells [180]. Although it is the basolateral membrane which is generally regarded to be more like the plasma membrane of unpolarized cells [282], we wanted to know whether cytochalasin D would have any effect on the resynthesis of sphingomyelin. As also demonstrated in Figure III-8 cytochalasin D did not have an inhibitory effect on the resynthesis of sphingomyelin.

C.III.2.8. Effect of sphingomyelin resynthesis on cholesterol ester

It has already been pointed out in the two preceding chapters (see C.I. and C.II.) that disturbances of the cholesterol:sphingomyelin balance in the plasma membrane

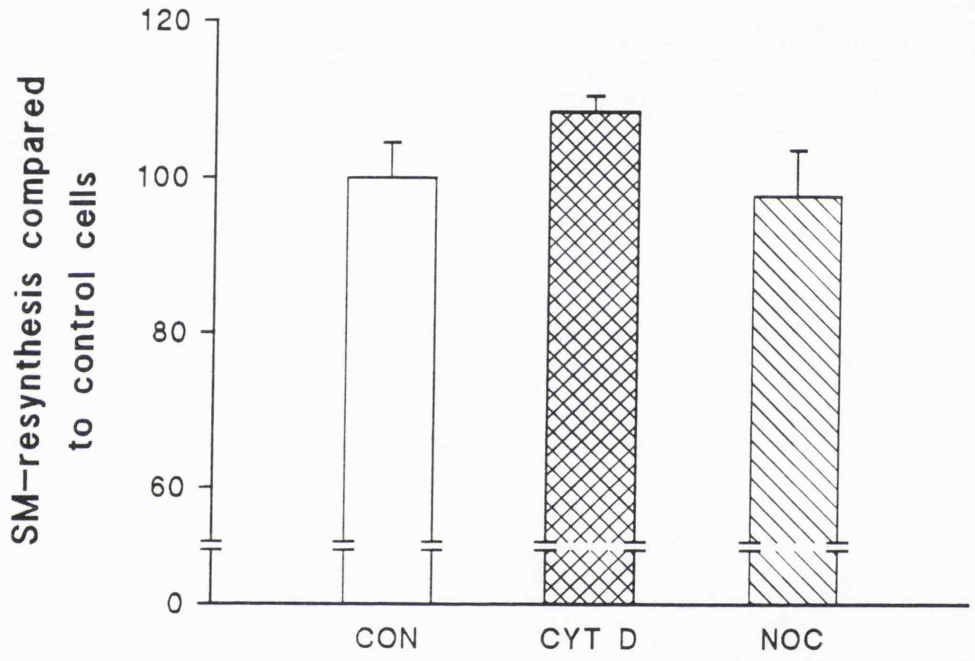


Figure III-8: Anti-cytoskeletal agents and sphingomyelin resynthesis. Cells labelled to equilibrium were pretreated with either nocodazole or cytochalasin D as described in B.13.4.. Cells were then degraded by exogenous sphingomyelinase and resynthesis followed for 3 hours in the presence of nocodazole or cytochalasin D.

by sphingomyelinase or drug treatment lead to rapid esterification of "excess" cholesterol. One obvious question to ask is therefore whether restoration of this balance, e.g. by resynthesis of sphingomyelin, would reverse cholesterol esterification.

The results of this investigation are shown in Figure III-9. Sphingomyelinase treatment of BHK-21 cells labelled to equilibrium with ^{3}H-acetate for 48 hours caused a 3-4 fold increase in the mass of cholesterol ester. Whereas around half of the degraded sphingomyelin was resynthesized within the first hour of recovery (Figures II-5) cholesterol ester levels were not affected during this time, but further incubation for two hours reduced cholesterol ester almost back to the original level. Qualitatively similar results were also obtained by Slotte and colleagues [294]. However, if cells were treated with NaF and aluminium chloride during recovery which largely suppresses resynthesis of sphingomyelin (Figure III-6) normalization of the cholesterol ester concentration was also prevented (Figure III-9).

These observations are in line with the conclusion derived from the combined effect of forskolin and BFA on cholesterol ester (C.I.3.6.), namely that esterification of cholesterol is already prominent when changes either in cholesterol or sphingomyelin are scarcely discernable. This may suggest an exquisite sensitivity of BHK-21 cells towards imbalances of the cholesterol:sphingomyelin ratio in the plasma membrane.

C.III.3. Discussion

C.III.3.1. The second sphingomyelin synthesis site must be distal to the TGN

The studies with monensin on the *de novo* synthesis and resynthesis of sphingomyelin revealed the existence of a second site of sphingomyelin synthesis distal to the *medial*-Golgi (C.II.). Neither monensin nor BFA inhibit the resynthesis of sphingomyelin and the appearance thereof at the outer leaflet of the plasma membrane (C.III.2.). Although the rate of sphingomyelin resynthesis is clearly reduced at low temperatures (C.III.1.) this treatment does not prevent access to the surface of resynthesised sphingomyelin. Considering these results it seems unlikely

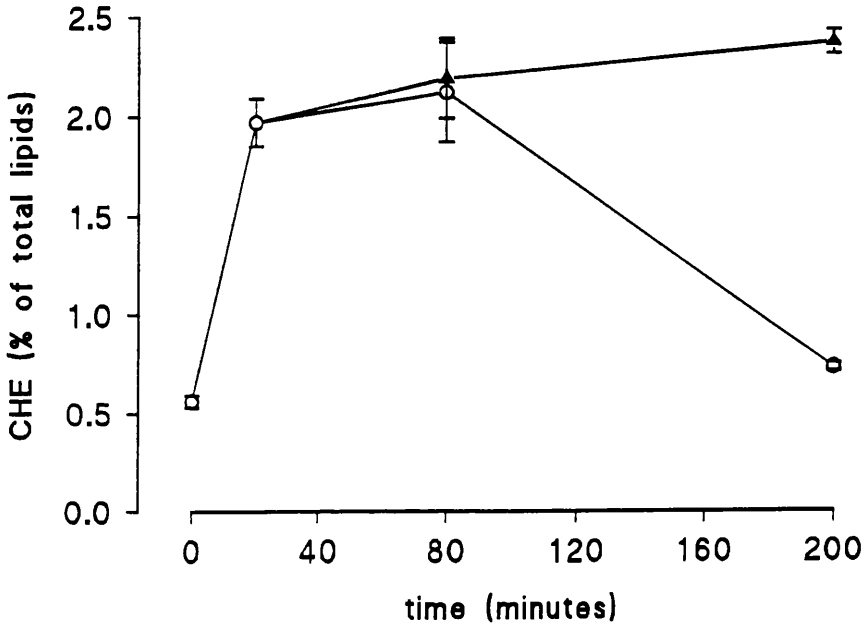


Figure III-9: Deesterification of cholesterol during sphingomyelin resynthesis. Cells were treated as in Figure II-5. Levels of cholesterol ester in control cells (○) were measured during resynthesis and compared to that of cells treated with AlF_4^- (▲) as in Figure III-5.

that the resynthesis site is located in either the *trans*-Golgi cisterna or the TGN, since both BFA [209] and incubation at 17°C block exit from the TGN to the plasma membrane [72,209]. Thus, in contrast to the findings presented here, resynthesized sphingomyelin would not be accessible to an extracellular sphingomyelinase if the resynthesis site were in the TGN or *trans*-Golgi.

A quantitative analysis of the membrane turnover during sphingomyelin resynthesis supports this conclusion. Resynthesis of plasma membrane sphingomyelin occurs with a half-time of $t_{1/2}$= 64 ± 18 minutes (C.II.2.4.). If there was a resynthesis site in the *trans*-Golgi/TGN, this would mean that an area equivalent to around 50% of the plasma membrane would have to leave the TGN per hour. This theoretical value is plainly incompatible with measurements by Griffiths et al.[102] who concluded that the rate of membrane transport from the TGN to the plasma membrane can only account for the export of newly synthesised membrane (~8%/hour of the existing plasma membrane area), but not for internalized plasma membrane recycling from the endosomes.

C.III.3.2. Resynthesis of sphingomyelin - an intracellular phenomenon?

For several reasons, it seems more likely that resynthesis of plasma membrane sphingomyelin occurs at an intracellular site and not at the cell surface. Firstly, there is practically no resynthesis of sphingomyelin in mitotic cells (Figure III-6) in which all vesicular membrane traffic including endocytosis is halted [83,364]. This observation is consistent with the assumption that beyond the early Golgi, ceramide moves by vesicular transport in BHK-21 cells [C.II.4.1.]. Consequently, ceramide transported by vesicles would not gain access to the putative intracellular site of sphingomyelin resynthesis. This is no contradiction to synthesis of sphingomyelin from NBD-ceramide at an intracellular site in mitotic cells [143], since NBD-ceramide can diffuse across aqueous phases [229] and thus gain access to the *cis-/medial*-Golgi site of sphingomyelin synthesis [174,175] or alternatively the (endosomal) resynthesis site. In accordance with the orientation of intracellular sphingomyelin in the exoplasmic leaflet of intracellular organelles [4] the newly synthesised NBD-sphingomyelin, however, is not transported to the cell surface in

mitotic cells which indicates its dependence on vesicular transport [143].

Secondly, ATP-depletion (Figure III-5) which prevents endocytosis in BHK-21 cells [144,209] clearly reduces the resynthesis of sphingomyelin even though the PCCP reaction itself has no obvious energy requirement [186,188,190,191,361]. It is remarkable that AlF_4^- reduces resynthesis of sphingomyelin much more than the apparantly rigorous regime of 50 mM DOG combined with 100 μM cyanide (Figure III-5). Originally, it was tempting to interpret this effect as reflecting a stimulation of heterotrimeric GTP-binding proteins which have recently been reported to inhibit budding [253] and fusion of endosomes [47]. However, the experiment shown in Figure III-5 clearly demonstrated that the effect was simply due to energy depletion by NaF. Steinman et al.[308] have previously demonstrated a correlation between the suppression of ATP-levels and the rate of endocytosis. Thus the inhibition of sphingomyelin resynthesis in AlF_4^--treated cells appeared to be the consequence of a more radically suppressed endocytosis due to energy depletion rather than the activation of an inhibitory GTP-binding protein. The only partial reduction of the rate of endocytosis in DOG-treated cells which showed an only intermediate level of energy depletion could explain why the inhibition of sphingomyelin resynthesis in DOG-treated cells waned towards the end of the incubation period.
Since the ATP-levels drop dramatically even after only 15 minutes of incubation with 30 mM NaF (see C.III.2.4.) one must express doubt as to whether the standard protocol for AlF_4^- treatment of intact cells [199] employed here can really deliver reliable information on the potential involvement of heterotrimeric GTP-binding proteins as claimed by a variety of researchers [69,173,262,313].

The inhibition of sphingomyelin resynthesis in mitotic cells and ATP-depleted cells can be well explained by an inhibition of endocytosis. The effects of NEM and digitonin on sphingomyelin resynthesis are consistent with this hypothesis, but since both drugs could have numerous side effects as explained above, their inhibitory effect on sphingomyelin resynthesis does not supply positive evidence for the requirement of endocytosis for resynthesis.

One *caveat* that has to be applied to the concept of an intracellular resynthesis of sphingomyelin stems from the inhibition of other non-endocytic vesicular transport processes in energy-depleted and mitotic cells [152,364]. In principle, the inhibition

of sphingomyelin resynthesis under those conditions could be attributed to either an inadequate delivery of a precursor, i.e. phosphatidylcholine, to a plasma membrane synthesis site or alternatively to insufficient removal of an inhibitory product of the reaction, i.e. diacylglycerol, from the plasma membrane.

Against the first objection one has to say that transport of phosphatidylcholine from its site of synthesis in the ER [26] to the plasma membrane is extremely quick [134,353] and does not follow the routes of vesicular transport as it is unaffected by energy depletion, monensin and anti-cytoskeletal drugs [134]. However, translocation of phosphatidylcholine from the inner to the outer leaflet of the plasma membrane ("flop", $t_{1/2} < 20$ minutes) has recently been demonstrated to be selective, protein-mediated by and energy-requiring, but not to be affected by NEM [11]. Although delivery of phosphatidylcholine to the cell surface could thus be inhibited in energy-depleted cells, it is difficult to see why the phosphatidylcholine translocator should be inhibited in mitotic cells. It is therefore highly unlikely that inadequate supply of phosphatidylcholine is the reason for the diminished sphingomyelin resynthesis in mitotic cells.

Besides sphingomyelin, diacylglycerol is the second product of the PCCP-reaction. This diacylglycerol does not pile up in the cells, but is quickly removed by an essentially unknown mechanism [225]. The inhibitory influence of diacylglycerol on the PCCP is well-documented [186,187], so that failure to remove diacylgycerol from the plasma membrane could cause inhibition of sphingomyelin resynthesis. However, whereas such a mechanism could contribute to the inhibition of sphingomyelin resynthesis in ATP-depleted cells, it cannot be the reason for the lacking sphingomyelin resynthesis in mitotic cells, since the lack of sphingomyelin resynthesis also prevents generation of "inhibitory" diacylglycerol.

Thus the most likely reason for the inhibition of sphingomyelin synthesis in mitotic cells appears to be the block of ceramide delivery to an endocytic sphingomyelin resynthesis site.

C.III.3.3. Kinetics of sphingomyelin resynthesis

A mean half-time of $t_{1/2}$= 64 ± 18 minutes was calculated for the resynthesis of surface sphingomyelin (C.II.2.4.). Consequently, the initial rate of the resynthesis of surface sphingomyelin is around 50% per hour. It has been pointed out before that this value could be consistent with resynthesis of sphingomyelin in an endocytic compartment followed by recycling to the cell surface [202]. Quantitative analysis of endocytosis in BHK-21 cells by Griffiths et al.[102] has revealed that BHK-21 cells take up 0.5 ± 0.08 μm^3 of fluid per minute in about 1000 ± 150 coated vesicles. Hence, a BHK-21 cell internalizes a total vesicle surface area of ~30 μm^2 per minute. With a total plasma membrane area of 2200 ± 470 μm^2 [102] this means that a BHK-21 cell internalizes and recycles its complete cell surface in around 75 minutes. This is close to the reported half-time of ~40 minutes for the recycling of NBD-sphingomyelin in CHO-cells [151], but roughly double the rate of sphingomyelin resynthesis,

Comparing the kinetics of sphingomyelin resynthesis to those of membrane recycling it is important to note that treatment with sphingomyelinase itself does not affect the rate of endocytosis in BHK-21 cells [4,295]. Nevertheless, the rate of sphingomyelin resynthesis appears to be in reasonable agreement with the rates of membrane internalization and recycling, if one considers the error progression in the calculation of the internalized surface area (estimated error of the half-time ~40%). Also, lipids are capable of rapid lateral diffusion with diffusion coefficients of 5-9 · 10^{-9} cm^2s^{-1} [164,231]. It follows that lipids diffuse across the surface of a BHK-21 cell with half-times of 20-40 minutes and this may well explain the slower rate of sphingomyelin resynthesis by comparison to the recycling of plasma membrane. Accordingly, the kinetics of sphingomyelin resynthesis would also support the concept that this process is dependent on and probably even limited by endocytosis. It is also noteworthy that the capacity of the putative endocytic resynthesis site which is able to synthesize 33% of the total sphingomyelin per hour is at least 4 fold higher than the rate of *de novo* synthesis of sphingomyelin which is around 8% per hour in BHK-21 cells. Thus the presumed endosomal site is not a minor activity.

C.III.3.4. The resynthesis pathway is highly conservative

Virtually all the ceramide produced at the cell surface by sphingomyelinase treatment is utilized for resynthesis of sphingomyelin (Figures II-5, III-2). Maximally 10% of the surface ceramide seemed to gain access to the *medial*-Golgi site of glucosylceramide and GM3 synthesis during the 3 hour resynthesis period (Figure III-8). This suggests that about 10% of the ceramide could have been lost to the lysosomes during resynthesis. From the lysosomes ceramide could be transported to the Golgi, a pathway discussed by several authors [103,153], where it would then be metabolized to glucosylceramide and GM3 [90,130,278].

The finding presented here that around 10% of the surface ceramide is lost on the "plasma membrane recycling pathway" [153] within three hours accords reasonably well with studies on membrane recycling using fluorescent-labelled sphingolipids. NBD-sphingomyelin and NBD-glucosylceramide were reported to leak from the recycling pathway to the lysosomes with rates of 8% per hour [152] and $<10\%$ per hour [144] respectively. This is some what higher than the "leak rate" found here which is most probably due either to the unphysiological fluorescent analogues used in these studies or to an underestimation of the leak for ceramide in the present study since only glucosylceramide and GM3, but not higher gangliosides were measured. Furthermore, some of the ceramide arriving at the Golgi would inevitably be turned into sphingomyelin, but the amount would be too small to be visible against the background of labelled sphingomyelin. Some ceramide might also leak to the TGN and study by van Deurs et al. suggested that around 5% of surface bound ricin was found in the TGN after endocytosis [338].

However, the results presented here are difficult to reconcile with data of Lipsky and Pagano [175,176] who found that $>90\%$ of NBD-ceramide added to the plasma membrane was metabolized in equal amounts to NBD-sphingomyelin and NBD-glucosylceramide, with around half of the two fluorescent metabolites residing in an intracellular pool not in commerce with the plasma membrane. In the present work, by contrast, most of the plasma membrane ceramide was turned into sphingomyelin and very little was delivered to the Golgi apparatus (Figures II-5,II-6,III-2,III-7). The clear inference is that natural ceramide cannot diffuse across aqueous phases

since in that case a substantial proportion of the plasma membrane ceramide should be recovered in resynthesized glycosphingolipids and only part of the resynthesized sphingomyelin would be degradable in monensin and BFA-treated cells.

The fact that the existence of the resynthesis site distal to the Golgi and the TGN was not indicated in studies carried out with NBD-ceramide emphasizes that the use of NBD-ceramide which is capable of rapid transbilayer movement and monomeric diffusion [229] may have led to a rather erroneous perception of cellular sphingomyelin metabolism. A new fluorescent ceramide analogue, C_5-BODIPY-ceramide, has recently been claimed to be more similar to the biophysical characteristics of endogenous ceramide and therefore better suited than NBD-ceramide to study transport and metabolism of sphingolipids [227]. The prominent red shift shown by the fluorophore upon concentration in lipid membranes (which is most likely due to excimer formation) allows one to detect where the fluorophore concentrates in cells. However, no metabolism to glucosylceramide [227] and GM3 was seen in intact cells treated with BODIPY-ceramide, but conversion occured easily in broken cells (personal communication, Simone Howitt, UCL). It is also unclear to which extent BODIPY-ceramide is capable of diffusion across aqueous phases. Before any reliable conclusions can be drawn from experiments with BODIPY-ceramide these imponderables have to be clarified.

C.III.3.5. Where does sphingomyelin resynthesis take place?

In the foregoing paragraphs the inhibition of sphingomyelin resynthesis in ATP-depleted and mitotic cells as well as the kinetics of sphingomyelin resynthesis have been interpreted as evidence for a location of the resynthesis site somewhere on the recycling pathway (see above). The question which endocytic compartment is involved in this process was left open.

A location of sphingomyelin resynthesis in the lysosomes can practically be excluded since this compartment contains an acid sphingomyelinase [152] which would counteract the resynthesis process. Furthermore, BFA is known to greatly slow transport from the endosomes to the lysosomes [173], but an effect of BFA on the resynthesis of sphingomyelin could not be detected. Finally, NBD-sphingomyelin

which does not undergo transbilayer movement and therefore appears to be a trustworthy agent to analyse vesicular transport of sphingomyelin, does not colocalize with the lysosomal markers after internalization by endocytosis [151].

Gruenberg et al.[104] have reported that in BHK-21 cells depolymerization of the microtubules by nocodazole leads to trapping of internalized molecules in the spherical vesicles which supposedly mediate transport between early and late endosomes [99]. Thus the inability of nocodazole to inhibit resynthesis of sphingomyelin (Figure III-8) would argue against the late endosomes as the site of this process. Consequently, the early endosomes appear as the most likely resynthesis site on the recycling pathway. Neither BFA [173,209] nor anti-microtubular agents [104,173,191,371] interfere with vesicular traffic between plasma membrane and the early endosomes which presents a plausible explanation for the lack of effect of BFA and nocodazole on sphingomyelin resynthesis.

Marggraf and colleagues [190,191] measured a pH-optimum (~ 6.5) of the PCCP which is rather close to the pH of the early endosomes (pH ≈ 6.0 - 6.2) [105]. The activity of the enzyme is sharply reduced below pH = 6.0 and above pH = 7.0 [190,191] which further advocates the early endosomes as the site of resynthesis. This pH optimum is not in keeping with a PCCP location in the late endosomes (pH $\approx$ 5.5 - 6.0), lysosomes (pH $\leq$ 5) or the plasma membrane (extracellular fluid pH = 7.4).

Several authors have reported a colocalization of fluorescent labelled transferrin-receptor and NBD-sphingomyelin in various cell types [146,151]. After an internalization period of five minutes most of the transferrin and NBD-sphingomyelin fluorescence is seen in a punctate pattern at the cell periphery, whereas after 30 minutes both transferrin and NBD-sphingomyelin appear in a perinuclear region associated with the centrioles [151]. Kok et al.[144] reported that in BHK-cells NBD-glucosylceramide similarly colocalized with fluorescent transferrin at the beginning of the receptor internalization, but that their fate diverged at later stages of the endocytic pathway with the NBD-glucosylceramide appearing in large spherical vesicles (which were tentatively identified as late endosomes) and later in the Golgi. According to the authors internalization of NBD-glucosylceramide follows the routes of receptor-mediated endocytosis, but not those of fluid-phase endocytosis.

In undifferentiated HT29 adenocarcinoma cells NBD-glucosylceramide seemed to follow the same pathway and eventually appeared in the Golgi, whereas NBD-sphingomyelin again colocalized with transferrin and also rhodamine-labelled ricin in a perinuclear compartment distinct from the Golgi [146]. This discrepancy was not observed in differentiated HT29 cells where NBD-glucosylceramide followed the pathway of NBD-sphingomyelin and transferrin [146].

Miller et al.[209] demonstrated that the perinuclear compartment of transferrin localisation is different from the TGN, while Koval and Pagano [151] found that nocodazole dissipates the punctate perinuclear fluorescence of NBD-sphingomyelin, but does not affect recycling of the lipid. It seems very likely that endogenous plasma membrane ceramide follows NBD-sphingomyelin on its endocytic pathway since it is mainly transported vesicularly (see C.II.4.1. and C.III.3.4.) and in contrast to NBD-glucosylceramide has very little commerce with the Golgi as reasoned above (C.III.3.4.). In other words this could mean that the endocytic pathway for resynthesis of sphingomyelin is also the pathway for endocytosis and recycling of transferrin.

The compartments involved in the endocytosis and recycling of transferrin in CHO cells have been studied *in extenso* by Yamashiro and colleagues [372]. Transferrin and α_2-macroglobulin (α_2-M) are internalized through clathrin coated vesicles (372 and ref.therein) and initially pass through small vesiculo-tubular structures in the periphery of the cells reminiscent of the morphological description of early endosomes by Gruenberg and Griffiths [99]. Whereas α_2-M is subsequently found in large vesicular structures of pH 5 to 5.5 [372], probably the compartment of uncoupling of receptors and ligands (CURL) described by Geuze [92], and eventually degraded in the lysosomes, transferrin never seemed to get access to a compartment with a pH below 5.5 in CHO-cells [372], although CHO cells efficiently accumulate iron [141]. Such an encounter of transferrin with a low pH compartment had been described previously in erythroleukemia cells [351].

Instead, transferrin rapidly segregated from α_2-M and was retrieved in a perinuclear, vesiculo-tubular compartment on the exocytic limb of the transferrin recycling pathway [372]. Measurements using transferrin labelled with the pH-sensitive fluorescein group revealed that this compartment actively maintains a pH

of 6.4 ± 0.1 which corresponds strikingly to the pH-optimum of the PCCP [190,191].

It seems very likely that the resynthesis of sphingomyelin from plasma membrane ceramide takes place in this compartment since

1. the kinetics of recycling of NBD-sphingomyelin to and from this compartment [151] fit in well with the kinetics of sphingomyelin resynthesis.
2. resynthesis is not inhibited by monensin, nocodazole [151] and BFA [209].

A location of the PCCP in this compartment would also explain why treatment of intact cells with trypsin did not affect the activity of this enzyme, in contrast to treatment of isolated plasma membranes [191]. Notwithstanding, the plasma membrane fraction still contained unidentified vesicles which were probably contaminating endocytic vesicles and wrongly assumed to have resulted from the membrane isolation procedure by sonication [191]. Furthermore, a location of a sphingomyelin synthetic activity in an endosomal compartment situated near the Golgi might explain the discrepancies in the literature with regard to a putative location of the enzyme in either *cis-/medial*-Golgi [90,129] or plasma membrane [190,191,334,361]. Finally, it would explain why there is no sphingomyelin synthetic activity found in red blood cells [191, personal observation] which do not have intracellular organelles.

C.III.3.6. How is cholesterol esterification linked to the plasma membrane sphingomyelin content?

The inhibition of anterograde transport of newly synthesized sphingomyelin through the secretory pathway by BFA and monensin causes rapid esterification of cholesterol (see C.I. and C.II.). Similarly, treatment of cells with sphingomyelinase for a comparatively short period (20 minutes) results in a rapid esterification of cholesterol which is reversed during resynthesis of sphingomyelin (Figure III-9). Very similar results were obtained by Slotte and coworkers regarding the inverse relationship between sphingomyelin resynthesis and cholesterol esterification [294]. By incubating BHK-21 cells for 48 hours with ^{3}H-cholesterol these authors

demonstrated that the sphingomyelinase treatment causes a mobilization of plasma membrane cholesterol which is partly turned into cholesterol ester. This accords with the results of the present study as the rise in cholesterol ester radioactivity was smaller than the decreases in sphingomyelin radioactivity (C.III.2.8.). Depletion of the plasma membrane sphingomyelin pool by BFA had a similar effect on surface cholesterol (C.I.). During resynthesis of sphingomyelin the plasma membrane pool of cholesterol was replenished and the cholesterol ester formed originally was degraded again [294].

The esterification of cholesterol to cholesterol ester is catalysed by the acyl-CoA:cholesterol acyltransferase (ACAT) [39,78,127,292,306] in an ATP-dependent reaction [312]. Whereas Chesterton originally assigned the ACAT-activity to the endoplasmic reticulum [43], subsequent work more specifically localised this function to the rough endoplasmic reticulum [113,255], although most of the newly synthesized cholesterol ester was claimed to be in the smooth endoplasmic reticulum [113].

Transport of radioactively-labelled plasma membrane cholesterol to the endoplasmic reticulum where the ACAT resides is very slow [260,298,299] with reported half-times of $t_{1/2}$= 45 - 125 hours [299]. This contrasts with the rapid cholesterol esterification following depletion of plasma membrane sphingomyelin [this work,296,297,293]. One obvious explanation is that sphingomyelin is able to restrict efflux of cholesterol from membranes which would be in accord with a putative cholesterol-sphingomyelin complex as already discussed in A.2.3.. The rapid esterification of "liberated" cholesterol might also point to the existence of a hitherto unidentified transport pathway from the plasma membrane to the endoplasmic reticulum.

Additionally, a decrease of plasma membrane sphingomyelin elicits a downregulation of the HMG-CoA-reductase [see C.I.,106] the key enzyme of cholesterol biosynthesis by a yet scarcely understood mechanism which seems to involve formation of oxysterols [106]. It is also completely unknown how the cell monitors the obviously very important ratio between plasma membrane sphingomyelin and cholesterol nor is it clear what advantage the formation of cholesterol ester offers to the cell by comparison to the accommodation of "free"

cholesterol.

Conspicuously, however, the flow of cholesterol released in the plasma membrane seems to always be directed towards the cell interior [297], even in the presence of powerful cholesterol scavengers like high density lipoproteins. Support for an involvement of an endocytic compartment in the esterification of cholesterol came from the observation that the lysosomotropic drug chloroquine strongly inhibits cholesterol esterification, whereas cytochalasin B or colchicine did not have an effect on the reaction [298]. However, severe ATP-depletion of the cells did not seem to adversely affect cholesterol esterification. In view of the ATP-dependence of the formation of acyl-CoA [312] and also of cholesterol ester [298] this result seems somewhat surprising.

One possibility might be that esterification of plasma membrane cholesterol is not performed by the RER-ACAT, but by a different enzyme that works by a mechanism analogous to that of the LCAT-reaction [312]. It was already pointed out in section C.I. that this idea appeared better to explain the distribution of radioactivity between cholesterol and acyl-chain in cholesterol ester. The fact that several authors reported inhibition of the esterification of plasma membrane cholesterol by the ACAT-inhibitor Sandoz 58-035 [292,299,306] does not necessarily contradict this hypothesis since the study describing the inhibition of ACAT by the Sandoz compound 58-035 did not investigate the alternative presented above.

D. Discussion

D.1. Scope of the discussion

In the present work the synthesis and transport of endogenous sphingomyelin was studied in BHK-21 cells, mainly using ^{3}H-acetate as a lipid precursor molecule. Chapter C.I. analyzed the influence on sphingomyelin metabolism of BFA, a fungal metabolite which merges the Golgi apparatus with the endoplasmic reticulum and blocks exit of vesicles from the merged compartment [142]. Treatment of BHK-21 cells with this drug greatly increased the synthesis of sphingomyelin, glucosylceramide and cholesterol ester. BFA almost completely prevented transport of the newly synthesized sphingomyelin to the plasma membrane pointing to a vesicular nature for sphingomyelin transport.

Monensin is an ionophore which in contrast to BFA inhibits vesicular transport through the secretory pathway between *medial-* and *trans*-Golgi [100]. It was shown in chapter II that monensin inhibited *de novo* synthesis of plasma membrane sphingomyelin. Apparently this was due to the effect of monensin to prevent vesicular transport of the sphingomyelin precursor ceramide to an unknown sphingomyelin synthesis site distal to the monensin block and the previously described *cis-/medial*-Golgi synthesis site. However, monensin did not inhibit resynthesis of sphingomyelin which had been degraded at the plasma membrane by an exogenous, bacterial sphingomyelinase. This was interpreted as evidence for the existence of a second site of sphingomyelin synthesis in BHK-21 cells. It was concluded that the first, "pre-monensin" site might be responsible for the synthesis of the intracellular pool of sphingomyelin [250], whereas the second site produces plasma membrane sphingomyelin (C.II.3.5.).

Chapter C.III. attempted to decide whether resynthesis of sphingomyelin occurred on the plasma membrane surface as described formerly or at an intracellular site after endocytosis of ceramide. The suppression of sphingomyelin resynthesis in ATP-depleted and mitotic cells strongly suggested that functioning vesicular transport and endocytosis were necessary conditions for sphingomyelin resynthesis. In addition, arguments were given for a location of the sphingomyelin

resynthesis site in a perinuclear, mildly acidic compartment previously described by Yamashiro et al.[372].

In the following section these results will be discussed with regard to their implications for our understanding of the regulation of sphingomyelin and sphingolipid metabolism, their possible connection with cellular cholesterol homeostasis and their relation to the existing pathways of vesicular protein transport in cells.

D.2. A new model for sphingomyelin synthesis in mammalian cells

D.2.1. Essential features of the model

The results presented in this study thus far are partly at variance with some present views on the synthesis of sphingomyelin in mammalian cells [90,129,148,153]. They suggest that the established site of sphingomyelin synthesis in the *cis-/medial*-Golgi is the one which makes sphingomyelin for the internal pool. By contrast, a second site distal to the *cis*-Golgi and TGN (C.II.4.1, C.III.3.5.) appears to be responsible for synthesis of plasma membrane sphingomyelin. The site has characteristics of an endosomal recycling compartment and may correspond to Yamashiro's 'mildly acidic para-Golgi compartment' [372] (C.III.3.5.).

Figure D-1 illustrates a model of sphingomyelin synthesis in BHK-21 cells that can account for the experimental findings presented and discussed in the foregoing sections. Ceramide is assumed to be transported from its ER site of synthesis to the early Golgi cisterna where it is partly metabolised to internal sphingomyelin on the luminal side of this compartment. However, a significant proportion of ceramide progresses further through the secretory pathway where some of it is turned into glucosylceramide on the cytoplasmic [89,130] leaflet of the *cis*-Golgi. The fact that synthesis of sphingomyelin and glucosylceramide take place in different leaflets of the Golgi membrane does not pose a conceptual difficulty, since ceramide - by analogy to diacylglycerol [7] - is supposed to undergo rapid transbilayer movement. After reaching the plasma membrane and subsequent endocytosis the remaining ceramide is turned into sphingomyelin in the lumen of Yamashiro's perinuclear,

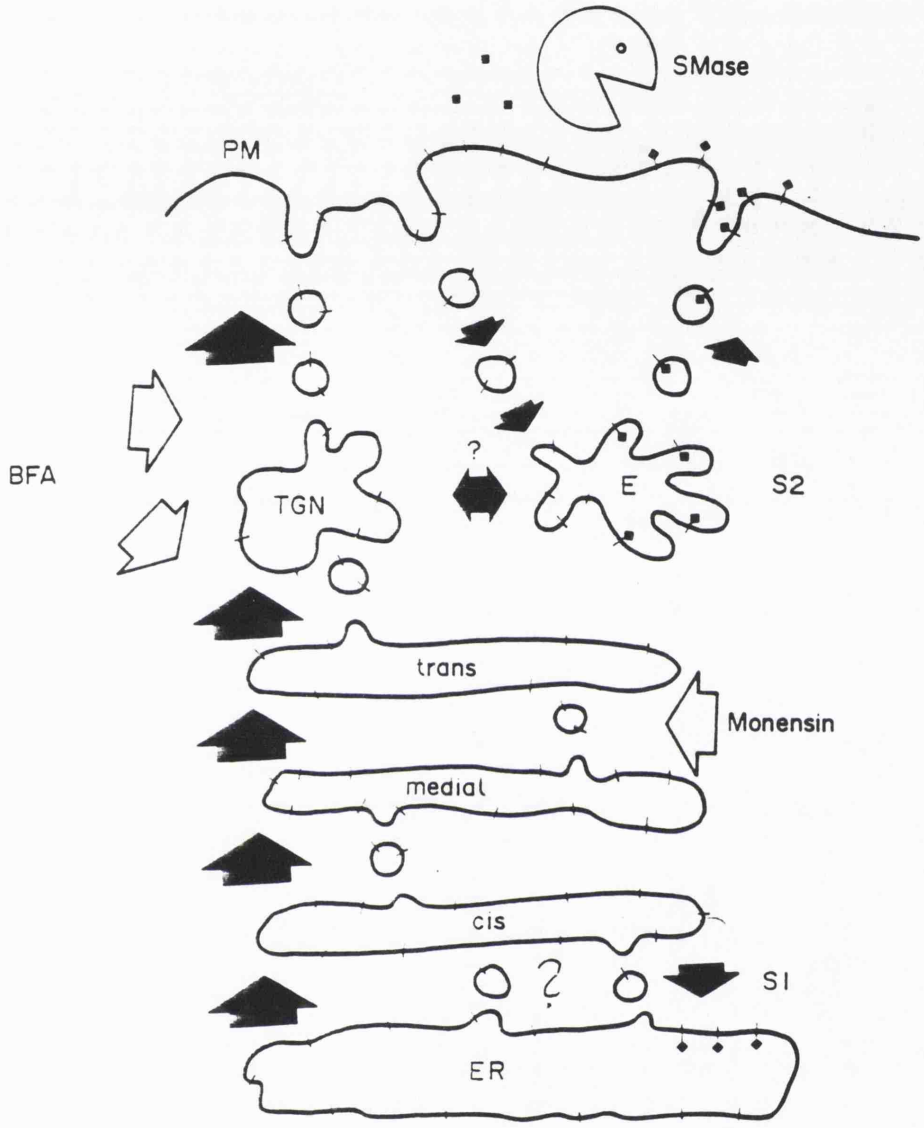


Figure D-1: The model illustrates a new, alternative concept of cellular sphingomyelin synthesis (6). The diagram shows the flow of vesicular transport from the ER through the various compartments of the Golgi apparatus to the plasma membrane (PM) and between PM and the endocytic compartment (E). The sites of action of BFA and monensin are indicated by light arrows. Ceramide is indicated as |, phosphocholine head groups as ■, sphingomyelin is shown as a composite of these symbols. S2 is the endosomal site of SM synthesis, S1 the *cis*-/early-Golgi site. The question mark between ER and *cis*-Golgi denotes that there might also be protein-mediated transport of ceramide between these compartments. The location of intracellular SM - tentatively assigned to the ER in this diagram - is essentially unknown.

para-Golgi compartment, also called 'recycling elements' by van der Sluijs et al.[337] (see C.III.3.5. for reasoning). Sphingomyelin would thus 'automatically' reach its correct destination in the outer leaflet of the plasma membrane.

In this model the 'membrane recycling pathway' [152] would be used for the production of sphingomyelin from ceramide that has been transported from the ER to the plasma membrane. As already pointed out before (C.III.3.4.) the 'recycling pathway' is not wholly conservative, so that some ceramide might be diverted to the lysosomes and thence to early Golgi-compartments as previously suggested by Koval and Pagano for NBD-ceramide [153].

D.2.2. Explanatory power of the new model

Apart from explaining the experimental results obtained in this study in a concise way, the model presented above may also offer a way to explain some previously poorly understood observations. It could explain the different kinetics of the labelling with ^{3}H-choline of the internal and plasma membrane pool of sphingomyelin described by Allan and Quinn [250], since the phosphorylcholine moiety used for sphingomyelin synthesis by the PCCP will obviously be derived from different pools of phosphatidylcholine which need not necessarily have the same specific activity. The existence of such pools was recently reported by Vance and colleagues [353]. The model would resolve why Lipsky and Pagano [175] were not able to detect the existence of two sphingomyelin synthesis sites (since NBD-ceramide would have gained access to both sites) and also why sphingomyelin synthesis was enhanced in monensin-treated cells by comparison to control cells (see C.II.3.). It would also explain why in CHO and rat liver cells sphingomyelin appears to be delivered to the cell surface with a time lag of 15 and 30 minutes respectively by comparison to glucosylceramide and GM3 [347].

Depending on the cell fractionation technique used, the two organelles accommodating the two sites of sphingomyelin synthesis might be separated or might sediment in the same fraction. Thus, one group noted explicitly that their method did not allow separation of Golgi-fractions from endocytic material [79] while others reported that their membrane preparation were significantly contaminated by

lipoprotein [122] (which contains considerable amounts of cholesterol and sphingomyelin [289]) or stated that the different fractions isolated by them could not be assigned to any specific membrane [136]. Probably, these technical difficulties can explain why various workers localised the PCCP-activity to the plasma membrane [186,187,343,361], the Golgi [90,129] or the Golgi and the plasma membrane [184].

D.2.3. Transport of ceramide to the distal Golgi?

The model presented above stipulates that a substantial proportion of newly synthesized ceramide travels by vesicular transport through the Golgi to the plasma membrane. The only evidence to support the first claim stems from the monensin experiments presented in section C.II.. There are no data available on the concentration of ceramide in different intracellular membrane fractions. One study [361] reported a plasma membrane ceramide concentration of around 1-2% of total lipid. This concentration might suffice for the needs of the above model, since synthesis of sphingomyelin is quick and hence there is little reason to expect high concentrations of intermediary compounds. Fluorescent ceramide showed accumulation in distal Golgi membranes [151,228] and it has been speculated that newly synthesized ceramide might be transported from the ER to the distal Golgi and undergo reverse transport through the secretory pathway Golgi to the early Golgi site of sphingomyelin synthesis [263]. Studies on the entry of toxins into eukaryotic cells point to potential reverse transport in the Golgi [237,273], but its existence has not been explicitly demonstrated thus far [266]. Clearly, more detailed information on the concentration of ceramide in different membrane fractions are necessary to decide whether there is accumulation of endogenous ceramide in the distal Golgi similar to that of fluorescent labelled ceramide.

Delivery of newly synthesized ceramide from the ER to the distal Golgi - if it is real - could occur either by an as yet unknown vesicular transport pathway or by transport proteins. Evidence in mitotic cells that ceramide is still able to reach the sites of glucosylceramide and (to a lesser extent) sphingomyelin synthesis could be interpreted in favour of protein-mediated ceramide transport [46]. However, it has

also been pointed out [346] that the results of this study could be influenced by redistribution of Golgi enzymes to the ER. Differential redistribution of glucosylceramide and sphingomyelin synthases from Golgi to ER has been demonstrated in BFA-treated hepatocytes [349]. Further studies are needed to explore whether there are indeed proteins capable of transporting ceramide and to explore how these proteins are involved in the synthesis of ceramide transport and sphingolipid synthesis.

D.2.4. The TGN is an unlikely site of sphingomyelin synthesis

In this work it has been supposed that ceramide is turned into sphingomyelin at a second synthesis site only after having reached the plasma membrane and not in a distal Golgi compartment. This conclusion is based on the ability of BFA to greatly inhibit appearance of newly-synthesised sphingomyelin at the cell surface and its inability to affect arrival of resynthesized sphingomyelin at the plasma membrane. Pivotal for this argument is the observation by Miller et al.[209] that in BHK cells BFA inhibits transport to the cell surface of VSV-G-protein that has accumulated in the TGN, while it does not effect recycling of the protein. Furthermore,the authors could demonstrate that the compartment containing VSV-G-protein and endosomes were distinct.

If sphingomyelin was synthesised in the TGN, it would be difficult to explain arrival of resynthesized sphingomyelin at the cell surface unless one could assume a third site of synthesis. Also, the ability of BFA to largely suppress arrival of new sphingomyelin at the surface argues against transport of ceramide or sphingomyelin from a distal Golgi to an endosomal compartment, since the BFA-block would be bypassed in this case. The existence of such transport routes has been discussed previously [103]. However, although the results of Miller et al.[209] facilitate interpretation of the data presented here, fusion of TGN with endosomes was observed in other cell types (NRK-, MDCK-cells) treated with BFA [173,371]. Therefore, it would be desirable to exclude the TGN as a potential site of sphingomyelin synthesis by independent methods.

D.2.5. The concept of sphingomyelin synthesis in an endosomal compartment needs to be tested further

The suggestion that sphingomyelin resynthesis occurs in 'recycling elements' is the first time that a synthetic, rather than a sorting process has been ascribed to an endosomal compartment. Certainly, further evidence for this hypothesis needs to be obtained. One test of the hypothesis would be to demonstrate PCCP activity in highly purified endosomal fractions. Methods such as free flow electrophoresis might be appropriate for this purpose. However, this might be easier said than done since at present a speedy assay for the identification of such vesicles is not available.

A different functional approach might be possible on the basis of some recent work by Simons et al.[35] and Mellman et al.[337] on the cellular function of *rab5* and *rab4* respectively. Overexpression in BHK cells of a *rab5* mutant defective in GTP-binding resulted in a 50% reduction in the rate of endocytosis, but left the rate of recycling and also transport in the secretory pathway unchanged [35]. Particularly at early time points this would be expected to result in an inhibition of sphingomyelin resynthesis in cells overexpressing the defective mutant. By contrast, overexpression of the *rab5* wild type seemed to increase the rate of endocytosis and it would be interesting to know whether this would also result in an increased rate of sphingomyelin resynthesis. Of course this experiment tacitly assumes that endocytosis is rate limiting for resynthesis of sphingomyelin.

D.3. Corollaries of the new model

D.3.1. A possible explanation for the distribution of saturated and unsaturated phosphatidylcholine in the plasma membrane

Phospholipids are asymmetrically distributed between the two leaflets of the plasma membrane, although for reasons of convenience this asymmetry is best explored in red cells [2,5,59]. The aminophospholipids phosphatidylethanolamine and phosphatidylserine are largely confined to the inner leaflet, while phosphatidylcholine and sphingomyelin are located in the outer leaflet of the plasma membrane. A

sizeable proportion of PC is also found in the inner leaflet, but this PC contains mainly unsaturated fatty acyl chains, a characteristic common to inner leaflet phospholipids [11,212,383,384]. It is unclear how this PC 'fatty acyl chain asymmetry' is generated, but the PCCP reaction offers an easy solution to the problem. As stated before (A.3.2.) the PCCP has a ten-fold higher affinity for unsaturated than for saturated phosphatidylcholine. Thus, the reaction would automatically lead to a depletion of unsaturated PC in the plasma membrane. Recently, a 'floppase' has been identified in erythrocytes that translocates saturated PC from the inner to the outer leaflet of plasma membrane [11]. Both, the 'floppase' and the PCCP reaction might work together in generating the asymmetric distribution of saturated and unsaturated phosphatidylcholine.

D.3.2. Newly synthesized cholesterol might be delivered to the perinuclear recycling element

Depletion of sphingomyelin in the plasma membrane by BFA or monensin lead to a conspicuous increase in the esterification of cholesterol (C.I.2., C.II.2.) and a similar result was obtained by degradation of plasma membrane sphingomyelin by an outside bacterial sphingomyelinase (C.III.2.8.). Cholesterol ester produced by the cells after breakdown of surface sphingomyelin was degraded during resynthesis of sphingomyelin (C.III.2.8.). These observations confirm and expand similar findings by Slotte and coworkers [293,296,297] who concluded that depletion of plasma membrane sphingomyelin caused redistribution of cholesterol from the plasma membrane to intracellular membranes. The inference to be drawn from this is that cells tightly control the balance of sphingomyelin and cholesterol, particularly in the plasma membrane, and that any disturbances of this balance seem to elicit vigorous counter-measures by the cells e.g. redistribution and esterification of plasma membrane cholesterol and inhibition of cholesterol synthesis.

This poses the obvious question of how the cell manages to exert control over the balance of sphingomyelin and cholesterol. One problem that the cell has to overcome for doing this is to synchronise not only *de novo* synthesis of sphingomyelin and cholesterol, but also delivery of both lipids to the surface since

sphingomyelin and cholesterol seem to follow at least partly different pathways to the cell surface. Whereas this work suggests that synthesis of plasma membrane sphingomyelin depends on transport of ceramide through the secretory pathway (C.II.), Simoni et al.[133,333] demonstrated that cholesterol can bypass both the BFA and the monensin block on its way to the plasma membrane. One would be inclined to interpret these findings as evidence for the non-vesicular nature of cholesterol transport. However, Simoni and colleagues concluded that cholesterol transport was vesicular on the basis of its inhibition by ATP-depletion and reduced temperature and also from the observation that newly synthesized cholesterol accumulated in a low density, lipid rich vesicle fraction.

Figure D-2 suggests a model for the mechanism by which the cell could potentially synchronise delivery of sphingomyelin and cholesterol to the plasma membrane. The crucial assumption the model makes is that cholesterol is not directly delivered to the plasma membrane, but probably to the same endosomal compartment that synthesises sphingomyelin from ceramide, i.e. Yamashiro's para-Golgi compartment [372] or van den Sluij's recycling element [337]. Thus, the unusual lipid composition of the plasma membrane is generated, because sphingomyelin and cholesterol are added to the plasma membrane via a vesicular pathway that has little commerce with the secretory pathway.

Delivery of cholesterol from the ER to this compartment is assumed to be mediated by a soluble exchange protein. Depending on the amount of sphingomyelin synthesized in this compartment cholesterol would be delivered to the plasma membrane or returned to the endoplasmic reticulum for esterification. This model would explain why transport of cholesterol is not inhibited by BFA, monensin or anti-cytoskeletal drugs [133,333]. ATP-depletion very likely will inhibit recycling of vesicles from the para-Golgi compartment to the plasma membrane and thus also of cholesterol transport. The same explanation can be applied to the temperature effect on cholesterol delivery with accumulation of cholesterol in a vesicle fraction [133]: protein-mediated exchange will be less affected at low temperatures than recycling of vesicles to the cell surface.

If the model described here is a good approximation to the way in which the cells enrich the plasma membrane with cholesterol one would make the following

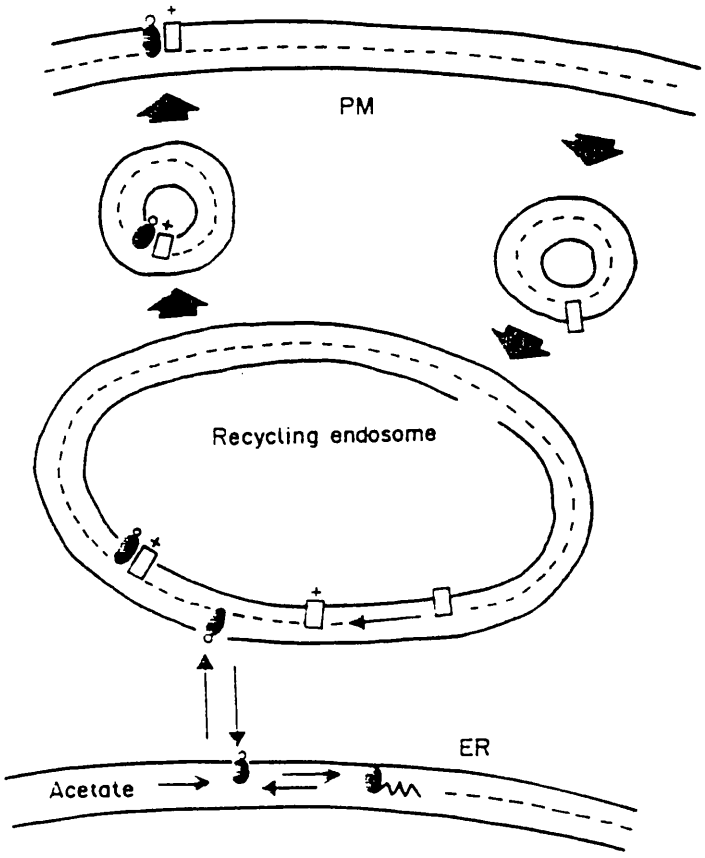


Figure D-2: A model for incorporation of cholesterol into the plasma membrane. Cholesterol (●) synthesisized in the ER passes into perinuclear endosomes where it becomes associated with SM (*ii*) newly synthesized from ceramide (□) and undergoes cotransport to the cell surface as part of normal plasma membrane recycling. Excess cholesterol in the plasma membrane is backtransported to the ER and converted into cholesterol ester. It is not exactly known in which leaflet the bulk of cholesterol resides.

prediction: vesicles derived from the trans-Golgi and the TGN ('exocytic' vesicles) should have a much lower cholesterol:phospholipid ratio since only very little cholesterol will gain access to Golgi membranes (probably by lateral diffusion into vesicles budding from the endoplasmic reticulum to the Golgi). Conversely, the equilibrium cholesterol:phospholipid ratio should very much resemble the same ratio in the plasma membrane. In fact, Helmy and colleagues [177] measured a cholesterol:phospholipid ratio in endocytic vesicles which approached that of plasma membrane, whereas exocytic vesicles had a cholesterol:phospholipid ratio close to that of ER and Golgi. While the latter finding would be in accord with the model put forward in Figure D-2, similar equilibrium cholesterol:phospholipid ratios in plasma membrane and endocytic vesicles are not surprising. However, it would be very interesting to examine in which vesicles newly synthesized cholesterol appears first. Unfortunately, such data are not available at present.

D.3.3. Sphingomyelin - determining the location of cellular cholesterol?

The exchange of cholesterol between different types of vesicles depends largely on the proportion of sphingomyelin among the lipids constituting the vesicles [179,325]. Lipid vesicles with high sphingomyelin content are less eager than vesicles with low sphingomyelin content to release their cholesterol to phospholipid vesicles not containing sphingomyelin. This propensity probably reflects the high affinity of sphingomyelin for cholesterol [57,179,355]. Consequently, one might suspect that similar rules apply on a cellular level, so that membranes with high amounts of sphingomyelin also contain a high amount of cholesterol. The model described above certainly allows for such a mechanism. However, is there any evidence for it in mammalian cells?

According to Lange et al. 90% of the sphingomyelin and 90% of the cholesterol are found in the plasma membrane of human skin fibroblasts [160,163]. Apparently contradictory figures were calculated by van Meer [344] who concluded that maximally 40% or 24% of the total cholesterol were located in the plasma membrane of rat hepatocytes and BHK cells, respectively. With regard to BHK cells, however, the calculation was partly based on erroneous stereological data and also conflicts

with experimental data of Slotte's group [294]. These researchers found that in BHK-21 cells around 70% of the total cholesterol resided in a cholesterol oxidase sensitive pool which indicates that this pool resides in the plasma membrane plasma membrane [160]. Treatment with sphingomyelinase reduced this oxidase sensitive pool to around 10% of total cholesterol which was interpreted to reflect removal of cholesterol from the plasma membrane [294]. In contrast, newer results indicate that not all cholesterol is redistributed from the plasma membrane in sphingomyelinase-treated cells (personal communication of J.P.Slotte to D.Allan). Qualitatively very similar results were obtained in human skin fibroblasts and a neuroblastoma cell line [248]. However, the kinetics of sphingomyelin resynthesis and cholesterol esterification were much slower in these cells than in BHK-21 cells which indicates that a certain precaution has to be applied to generalization of results obtained in this cell model to other cell types.

D.4. An unsolved enigma - where is the internal pool of sphingomyelin?

Around 30 - 40% of total cellular sphingomyelin resides in an intracellular location where it is not accessible to degradation by exogenous sphingomyelinases [250]. This internal sphingomyelin pool reaches the cell surface of BHK cells only extremely slowly, with a half-time of $t_{1/2} \approx$ 80 hours [250]. Moreover, it seems to be synthesized in a way different from that of plasma membrane sphingomyelin [250,C.II.]. So where is this pool of considerable size hidden in a cell?

It has previously been suggested that the internal sphingomyelin pool resides in the ER [250]. However, there are no experimental data directly supporting this assumption. In contrast, Pagano & coworkers could not detect fluorescent sphingomyelin in the ER [263 and ref.therein], but small concentrations could potentially have escaped detection. Furthermore, adding exogenous fluorescent ceramide to cells might not be the appropriate method to label the internal pool.

However, if one compares the relative membrane areas of intracellular organelles in BHK-21 cells as determined by Griffiths et al.([102], see table D-1) to the relative sphingomyelin concentration of each organelle as compiled by Koval & Pagano [153], it seems unlikely that internal sphingomyelin can reside in a compartment

Table D-1: Comparison between the relative area covered by different cellular membranes in BHK-21 cells and the average sphingomyelin content of these membranes obtained in various cell types. [1] data taken from Griffiths et al.[102], [2] data taken from Koval & Pagano [153]

compartment	surface area of compartment[1] [μm^2]	average SM-content (% of total lipid phosphorus)[2]
plasma membrane	2200 ± 470	17.7 (11.8-23.2)
ER & nuclear envelope	5870 ± 990	3.5 (2.0-4.9)
Golgi stack	1960 ± 540	9.5 (6.3-12.3)
endosomes	430 ± 105	14.3 (13.6-15.0)
lysosomes & prelysosomes	370 ± 113	18.9 (16.0-21.8)
mitochondria outer membrane inner membrane	 1080 ± 280 3950 ± 711	 1.2 (0.0-2.4)
total internal membrane area	13660 ± 2739	
total membrane area	15860 ± 3209	
total internal area without Golgi and lys.	11330 ± 2086	

other than the Golgi or the ER. A location of the internal sphingomyelin pool in the Golgi would fit in with fluorescence data of Pagano & coworkers [263] who have consistently observed accumulation of fluorescent labelled ceramide and sphingomyelin in the (*trans*-) Golgi, while there was little fluorescence in the ER. However, other workers found little sphingomyelin in the Golgi [321]. Some workers also think [David Allan, personal communication] that the internal pool of sphingomyelin could reside in an endosomal compartment that does not participate

in membrane recycling. In my opinion this unorthodox view is difficult to reconcile with the data listed in table D-1 since this hypothetical compartment either had an unexpectedly large surface area or a very high concentration of sphingomyelin.

Fractionation of cells labelled to equilibrium with radioactive ^{3}H-acetate and treated with exogenous sphingomyelinase should allow to identify the compartment hosting the internal pool of sphingomyelin. Performing a similar experiment with fluorescent labelled ceramide or sphingomyelin should clarify whether any inside fluorescence observed after this treatment is in the same location as the internal pool of sphingomyelin.

D.4.1. Consequence of restricted sphingomyelin movement for the 'bulk flow'concept

BHK cells have a doubling time of around 12 hours. Hence, roughly 183 μm^2 of plasma membrane, equivalent to 3.1% of the ER surface area, have to be synthesised in the ER and exported to the cell surface per hour (Table D-1). Assuming that intracellular sphingomyelin equilibrates within the ER and the vesicles budding from the ER, this means that internal sphingomyelin will reach the cell surface with a half-time of $t_{1/2} \approx$ 22 hours. This has to be compared to the measured half-time of $t_{1/2} \approx$ 80 hours [250] from which it can be derived that an area of around 0.9% of the ER leaves the ER per hour. Considering that the stereological data are at best a good approximation to the real values [compare 377 and 102] and that some lipids are not transported via vesicles [143] it is difficult to decide whether the difference between these values is real or whether it reflects the accumulation of experimental error. Since sphingomyelin appears to be the ideal bulk flow marker, the first interpretation would mean that sphingomyelin transport is restricted and does not follow bulk flow. This conclusion is even more obvious if one assumes that the internal sphingomyelin pool resides in the Golgi. If sphingomyelin transport to the surface was by bulk flow, then a $t_{1/2} \approx$ 7 hours would be predicted which is 11 times smaller than the one measured. Still, this figure is several times greater than the half-time of 20 minutes for transport of proteins by bulk flow from the ER to the plasma membrane [367].

If the speculated location of the internal pool of sphingomyelin in the ER or the Golgi is correct, it appears difficult to maintain the bulk flow concept in its present form. Based on studies on the varying concentrations of marker proteins in membranes of the secretory pathway, the validity of the bulk flow model has recently been questioned [14,285], while other authors have argued in support of it [378]. There are at present no data to suggest that sphingomyelin is transported by other than vesicular means. Precise knowledge on the exact location of the internal pool of sphingomyelin is therefore necessary,since -as indicated above- this knowledge will certainly bear heavily on the discussion on the validity of the bulk flow model.

D.5. Concluding remarks - cutting the Gordian knot

The work presented in this study provides evidence in support of the concept of vesicular transport of sphingomyelin and ceramide between different cellular membranes. In addition the experimental results suggest the existence of two sites of sphingomyelin synthesis, one in an early-Golgi compartment as suggested previously by other authors and one that could reside in an endosomal compartment with a perinuclear/para-Golgi location. It was suggested that this latter site is responsible for the synthesis of plasma membrane sphingomyelin from ceramide which had been transported from the endoplasmic reticulum through the secretory pathway. Consistent with the work of other authors, depletion of the plasma membrane of sphingomyelin decreased synthesis and increased esterification of cholesterol. It was suggested that the putative endosomal synthesis site of plasma membrane sphingomyelin might have an important role in adjusting the correct sphingomyelin/cholesterol ratio in the plasma membrane.

Several possibilities to test the hypothesises put forward in this thesis have been outlined. However, it was also pointed out that the most urgent task in the view of the author is the purification of the enzymes involved in lipid biosynthesis and in particular, sphingolipid synthesis. Without any doubt, this is a formidable task. However, this approach will make possible the precise intracellular location of the various steps of lipid biosynthesis by immunohistochemical methods. There is every

hope that this knowledge will help to illuminate many of the conflicting ideas concerning the mechanism and sequence of lipid synthesis and transport. In polar cells, insight into the ways of lipid sorting between apical and basolateral membrane should be opened. Ultimately, this will lead to a better understanding of membrane traffic and its connection to other important areas of cell biological research, such as the involvement of sphingomyelin and ceramide in cell signalling.

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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 μM) prevented the changes in labelling induced by 0.25 μg of BFA/ml, but in the presence of 1 μg 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 μCi of [^{3}H]acetate (NEN Dupont)/dish or 5 μCi of [^{3}H]choline (Amersham International)/dish, with or without addition of BFA (1 μg/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 μg/ml and cells were extracted after incubation for 4 h.

The ability of forskolin (100 μM, added from a 100 mM stock in dimethyl sulphoxide; Sigma) to block the effects of BFA (either 0.25 or 1 μg/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 μCi of [^{3}H]acetate or 2 μCi 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 μg/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 μg/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. 60–80 °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 [^{3}H]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 μg/ml and were maximal at 1 μg/ml (Figure 2).

Addition of forskolin (100 μM) blocked the changes in labelling produced by 0.25 μg of BFA/ml (Table 2). However, in the presence of 1 μg 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 [^{3}H]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, glycero-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-

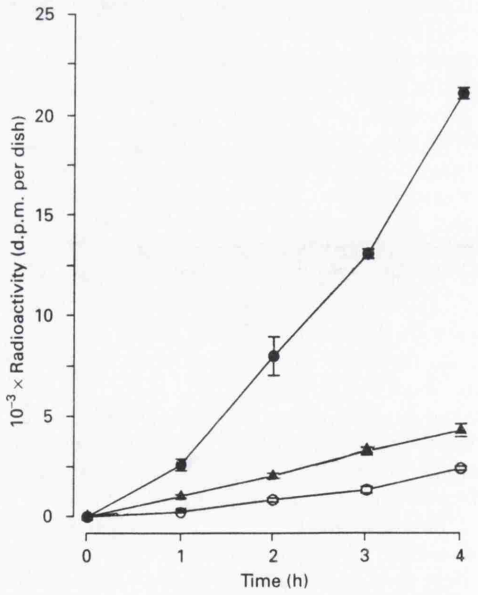

Figure 1 Incorporation of [^{3}H]acetate into lipids of BHK cells

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

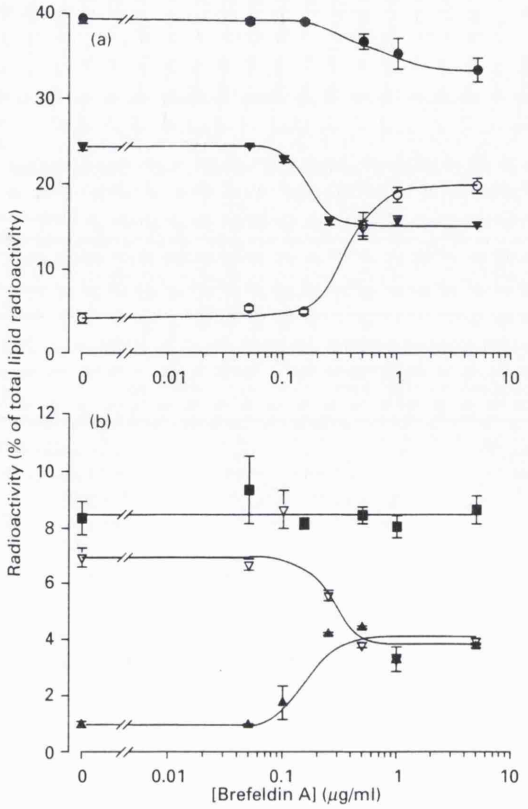

Figure 2 Effect of BFA concentration on incorporation of [^{3}H]acetate into lipids of BHK cells

Cells were incubated for 4 h with [^{3}H]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 ± 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 [^{3}H]acetate into BHK cells exposed to BFA and/or sphingomyelinase

Incorporation of [^{3}H]acetate was measured at 4 h in the absence or presence of 2 μg 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 ± 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 ± 0.1	3.1 ± 0.4**	21.1 ± 0.9***	21.6 ± 1.3
PC	43.6 ± 0.6	43.6 ± 1.5	37.5 ± 1.8*	35.5 ± 1.4
PS/PI	3.6 ± 0.1	4.2 ± 0.4	4.4 ± 0.4	4.5 ± 0.7
PE	9.1 ± 0.6	9.0 ± 0.1	9.1 ± 0.5	9.1 ± 0.3
CHOL	23.7 ± 2.2	22.8 ± 1.9	13.6 ± 1.7**	15.2 ± 0.5
CER	4.2 ± 0.7	6.9 ± 0.5**	2.4 ± 0.2**	3.5 ± 0.3
TAG	7.3 ± 1.3	8.6 ± 0.5	5.7 ± 1.2	6.1 ± 1.3
GCER	1.2 ± 0.1	1.1 ± 0.2	4.0 ± 0.2***	3.7 ± 0.3
CHOL. EST.	0.6 ± 0.1	ND	2.4 ± 0.3***	ND
Total d.p.m.	34645 ± 3833	43191 ± 8022	33007 ± 2751	33998 ± 3308

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

Cells were incubated for 4 h in 3.5 cm dishes in the presence of [^{3}H]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 ± 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 (μg/ml)	0	0.25	1	0	0.25	1
SM	6.3 ± 0.3	10.7 ± 0.2	16.4 ± 0.3	7.9 ± 0.5	7.4 ± 0.4	18.9 ± 0.3
PC	35.6 ± 0.9	35.1 ± 0.8	33.3 ± 0.4	36.3 ± 0.5	36.4 ± 0.6	33.4 ± 0.6
PS/PI	8.5 ± 0.3	9.6 ± 0.3	8.6 ± 0.2	7.4 ± 0.5	7.9 ± 0.1	8.8 ± 0.3
PE	9.5 ± 0.3	10.5 ± 0.4	9.7 ± 1.0	9.2 ± 0.3	10.4 ± 0.8	10.2 ± 0.8
CHOL	18.9 ± 0.3	11.7 ± 0.9	12.3 ± 0.6	18.1 ± 2.8	18.9 ± 0.4	14.6 ± 1.3
TAG	8.2 ± 0.1	6.5 ± 0.2	3.5 ± 0.1	9.3 ± 1.6	7.1 ± 0.4	3.4 ± 0.1
CHOL. EST.	0.7 ± 0.1	3.7 ± 0.2	3.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	1.6 ± 0.1

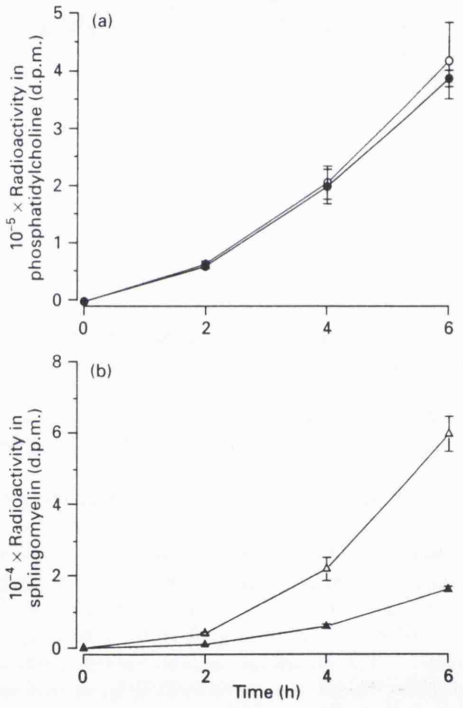

Figure 3 Incorporation of [^{3}H]choline by BHK cells in the presence or absence of BFA

Cells were incubated with [^{3}H]choline for up to 6 h in the absence (●, ▲) or presence (○, △) of BFA (5 μg/ml) and lipids were extracted and analysed as described in the Materials and methods section. Values shown represent means ± 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 [^{3}H]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 ± 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±0.1	7.5±0.3	2.8±0.2	7.9±0.1	4.3±0.2*
PC	48.0±1.0	48.1±0.6	52.1±0.3	44.9±0.3	47.8±0.6
PS/PI	13.1±0.8	13.4±0.2	14.1±0.2	14.5±0.2	15.4±0.2
PE	26.5±0.2	27.1±0.9	27.1±0.2	28.4±0.3	27.9±0.9
CL	4.8±0.2	3.9±0.1	3.9±0.2	4.7±0.3	4.4±0.2
(b) nmol of phospholipid					
PL	185±7	258±10	256±12	246±11	240±15
SM(T)	13.9	19.3	7.2	19.4	10.3
SM(S)	8.8	12.1		9.1	

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 μg of BFA/ml for 4 h as described in the Materials and methods section. Values represent (a) means ± 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 ± 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±0.3	9.6	6.6±0.2	10.1
PC	32.5±1.1	49.6	32.4±1.1	49.8
PS/PI	7.8±0.6	11.9	8.2±0.3	12.6
PE	15.5±0.2	23.7	14.8±0.5	22.7
CL	3.4±0.3	5.2	3.1±0.4	4.8
CHOL. EST.	1.1±0.1		3.0±0.3*	
CHOL	22.4±0.8		20.4±0.7	
CER	0.38±0.07		0.26±0.03	
NEFA	0.25±0.03		0.24±0.02	
DAG	0.8±0.1		1.0±0.1	
TAG	6.1±0.4		7.7±0.2	
CELL AQU.	2.7±0.2		2.6±0.2	
Total d.p.m....	50205±3341		50236±4258	
Choline label				
SM	8.8±0.5		9.4±0.5	
PC	39.8±1.7		40.3±1.0	
CELL AQU.	38.5±1.9		40.0±0.8	
Medium	12.9±0.6		11.3±0.8	

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 μg of BFA/ml 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 [^{3}H]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 [^{3}H]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 [^{3}H]acetate or [^{3}H]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 [^{3}H]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 μg/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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Rapid Report

Monensin inhibits synthesis of plasma membrane sphingomyelin by blocking transport of ceramide through the Golgi: evidence for two sites of sphingomyelin synthesis in BHK cells

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The monovalent cationophore monensin, which is known to interfere with vesicular transport through the Golgi apparatus, inhibits synthesis of sphingomyelin in BHK cells by up to 40%. The monensin-sensitive component of sphingomyelin synthesis appears to be the pool which normally reaches the cell surface since treatment of cells with exogenous sphingomyelinase causes an almost identical loss of sphingomyelin. Monensin causes increases in ceramide and glucosylceramide labelling which together are equivalent to the decrease in sphingomyelin labelling. Monensin also increases synthesis of cholesterol ester, probably due to the decreased delivery of sphingomyelin to the plasma membrane. However, monensin has no effect on resynthesis of plasma membrane sphingomyelin which has been degraded by extracellular sphingomyelinase. The results support the idea that synthesis of sphingomyelin destined for the plasma membrane does not occur in the *cis*- or *medial*-Golgi but depends on vesicular transport of ceramide to a second synthesis site which is distal to the *medial*-Golgi.

Introduction

It is now widely accepted that proteins destined for secretion or for insertion into the plasma membrane proceed by a vesicular transport mechanism from their site of synthesis in the endoplasmic reticulum (ER) to the Golgi apparatus and thence to the cell surface [1]. Vesicular transport necessarily involves a concomitant movement of lipid but it is only recently that evidence has emerged which shows that some lipids bound for the plasma membrane may also need to pass through the Golgi [2]. Thus, it has been demonstrated that monensin and brefeldin A (BFA) which block protein translocation through the Golgi cisternae, also prevent conversion of ceramide and cerebrosides to cell surface gangliosides [3]. Monensin has also been shown to prevent the transport from the Golgi to the cell surface of sphingomyelin synthesized endogenously from a fluorescent ceramide derivative added to the extracellular medium [4,5]. BFA similarly prevents the translocation of newly-synthesized sphingomyelin to the cell surface [6].

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Most workers currently favour the idea that sphingomyelin is synthesized in the *cis*- or *medial*-Golgi [2,7–9] and that it then progresses through the vesicular transport pathway to the cell surface [10,11]. The work presented here using monensin to probe the synthesis and transport of sphingomyelin in BHK cells shows that there are two sites of sphingomyelin synthesis, one proximal and the other distal to the monensin block. The latter appears to be the site which synthesizes sphingomyelin for delivery to the plasma membrane.

Materials and Methods

BHK 21 cells were cultured in 3.5 cm dishes in Glasgow MEM supplemented with 5% foetal calf serum as described previously [12]. Cells were incubated in the presence of 20 μC [^{3}H]acetate (NEN) with or without addition of 1 μM monensin (Sigma, Poole, Dorset, UK). After 4 h, some samples (with or without monensin) were incubated for 20 min with *B. cereus* sphingomyelinase (0.1 unit) in order to determine the amount of radioactive sphingomyelin which had reached the surface [6]. All the samples were then extracted with 1.9 ml (2:1, v/v) methanol/chloroform. In other experiments, the amounts of monensin were varied between 0–10 μM and cells were again ex-

tracted after 4-h incubation. Some experiments were carried out using [^{3}H]choline instead of [^{3}H]acetate. Lipids were separated and their radioactivity measured as described previously [6].

Cells were also labelled to equilibrium with [^{3}H]acetate as previously described [6,12] and were treated with 0.1 units of sphingomyelinase for 20 min to degrade surface sphingomyelin. The resynthesis of sphingomyelin was measured in control cells and in cells incubated with 1 μM monensin over a 3 h period. At the end of this incubation the cells were treated for a second time with sphingomyelinase to determine the amount of sphingomyelin which was on the surface after resynthesis.

Results and Discussion

In BHK cells labelled with [^{3}H]choline for 4 h, addition of monensin caused an approx. 30% drop in incorporation into sphingomyelin (Fig. 1). A very similar decrease in radioactivity was seen when untreated cells were exposed to sphingomyelinase, suggesting that monensin was preventing the synthesis of sphingomyelin which normally reached the cell surface where it was accessible to the sphingomyelinase. Consistent with this hypothesis, exposure of cells to sphingomyelinase after they had been incubated with monensin caused no more than the 30% loss of labelling seen with monensin or sphingomyelinase added separately. Conversely, the radioactivity of the sphingomyelinase-insensitive pool of sphingomyelin which was assumed to be inside the cells was not affected by incubation with monensin.

Comparable effects of sphingomyelinase and monensin on sphingomyelin radioactivity were obtained using cells labelled with [^{3}H]acetate (Fig. 2). However, the proportion of total sphingomyelin which was affected by treatment with sphingomyelinase or monensin was up to 50%, considerably more than with the choline-labelled cells. This was probably due to the fact that labelling of surface sphingomyelin with choline is relatively slow compared with labelling of internal sphingomyelin [13] whereas the rate of labelling of these pools with acetate is more similar.

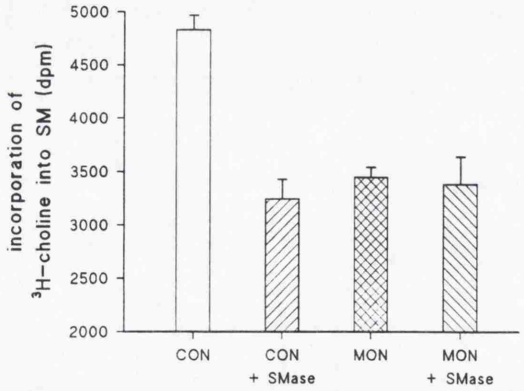

Fig. 1. Effect of sphingomyelinase and/or monensin on [^{3}H]choline labelling of sphingomyelin in BHK cells. Cells were labelled with [^{3}H]choline for 4 h in the presence or absence of monensin as described under Materials and Methods. For the last 20 min of the incubation, cells were treated with sphingomyelinase (0.1 units) before extraction and analysis of lipids. Results are expressed as means ± S.D. of triplicate determinations in one experiment which was repeated twice with similar results. CON, control; SMase, sphingomyelinase; MON, monensin.

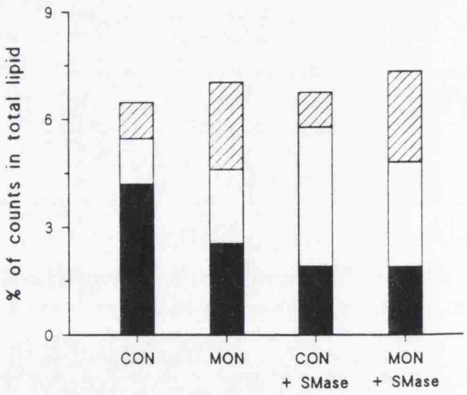

Fig. 2. Effects of sphingomyelinase and/or monensin on [^{3}H]acetate labelling of sphingomyelin, ceramide and glucosylceramide in BHK cells. The experiment was carried out as for Fig. 1. except that ceramide and glucosylceramide were measured in addition to sphingomyelin. Results are expressed as the percentage of total lipid radioactivity present in sphingomyelin (solid bars), ceramide (plain bars) and glucosylceramide (hatched bars). Values represent the means of four experiments which were each based on triplicate samples corresponding to the different conditions employed. Variation from the mean values shown was no more than ± 10%.

Breakdown of surface sphingomyelin by sphingomyelinase treatment after 4 h incubation with [^{3}H]acetate caused an equivalent rise in labelling of ceramide (Fig. 2) as expected. Sphingomyelinase treatment also caused a 2–3-fold increase in labelling of cholesterol ester (Table I) as reported previously by Slotte and co-workers [14,15] but it did not cause any change in labelling of glycosphingolipids (Fig. 2).

Addition of monensin (1 μM) alone caused little change in radioactivity of the major phospholipids or neutral lipids (Table I). In contrast, sphingomyelin radioactivity was decreased by about 40% in monensin-treated cells, while radioactivity in ceramide increased about 60% and that in glucosylceramide by about 2–3-fold (Fig. 2). Additionally, monensin caused a 2–3-fold increase in labelling of cholesterol ester (Table I). These effects of monensin were maximal at about 3 μM and half-maximal at about 0.1 μM (Fig. 3).

It was clear that the loss of sphingomyelin label from control cells treated with sphingomyelinase was quantitatively very similar to the loss of label from sphingomyelin in monensin-treated cells (Table I, Figs. 1,2). Furthermore, a combination of sphingomyelinase

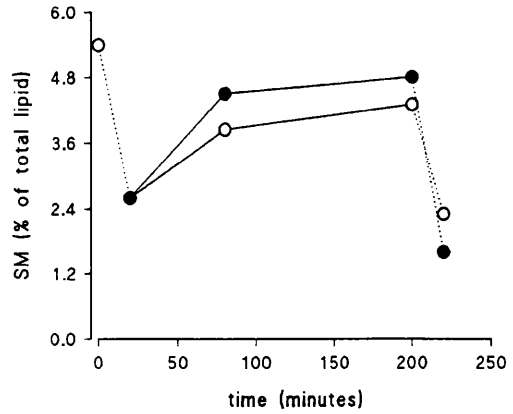

Fig. 4. Resynthesis and return to the surface of sphingomyelin degraded by exogenous sphingomyelinase is not affected by monensin. Cells were labelled to equilibrium with [^{3}H]acetate and cell surface sphingomyelin was broken down by addition of extracellular sphingomyelinase (see Materials and Methods). After removal of the enzyme, resynthesis of sphingomyelin was followed in the presence or absence of 10 μM monensin. A second treatment with sphingomyelinase was used to determine the amount of surface sphingomyelin at the end of the incubation. The dotted lines indicate the periods when sphingomyelinase was present. Results represent the means of triplicate determinations in a single experiment which was repeated twice with the same result. Control, open circles; + monensin, closed circles.

cell surface sphingomyelin biosynthesis can take place. Thus, in BHK cells at least, cell surface sphingomyelin is not made in the early Golgi as previously thought (7–9, 10) but at a later stage in the exocytic pathway. This conclusion is at variance with a previous report concluding that monensin inhibits transport but not de novo synthesis of sphingomyelin [4]. We do not know the precise reasons for this discrepancy but one explanation could be the use of a fluorescent-labelled ceramide precursor (C_6-NBD-ceramide), known to be capable of monomeric diffusion. As recently pointed out by Bishop and Bell, a caveat must be attached to results derived from such an approach [21].

Confirmation of a distal site for synthesis of sphingomyelin comes from experiments on the resynthesis of sphingomyelin which has been degraded at the surface by an exogenous sphingomyelinase (Fig. 4). In contrast to its effect on de novo synthesis of sphingomyelin, monensin had no effect on resynthesis of sphingomyelin broken down by exogenous sphingomyelinase. A second treatment with sphingomyelinase demonstrated that the resynthesized sphingomyelin is on the cell surface. Thus, it is not produced by the synthesis site proximal to the monensin block (Fig. 4) and therefore monensin does not inhibit synthesis of cell surface sphingomyelin itself so long as surface ceramide is available. Evidence for a major plasma membrane site of sphingomyelin synthesis has previously been presented by several other authors [22–24]. The assumption of two sites of synthesis for sphingomyelin as suggested by Malgat et al. [25] may resolve the conflicting data indicating *cis-/medial*-Golgi [7–9] or plasma membrane as the prime site of synthesis [22–24]. The relative importance of the two sites is likely to differ between cell types.

Our results suggest that in the normal synthesis of cell surface sphingomyelin, it is ceramide and not sphingomyelin which undergoes vesicular transport through the Golgi. Thus, by blocking the transport of ceramide between *medial-* and *trans*-Golgi, monensin prevents synthesis at a distal site of sphingomyelin destined for the cell surface and causes an accumulation of ceramide in the *medial*-Golgi, where some is converted into glucosylceramide.

Acknowledgement

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TABLE I

Distribution of [^{3}H]acetate label among lipids of BHK cells

Radioactivity in individual lipids was measured after 4 h incubation with [^{3}H]acetate with or without addition of monensin (1 μM) or sphingomyelinase as described under Methods. Values represent the percentage of total lipid radioactivity (means ± S.D. of triplicate determinations) and are derived from a single experiment which was repeated three times with substantially the same results. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; NL, total neutral lipid; CE, cholesterol ester.
* Significantly different from control value, $P < 0.01$.

	Control	+ Monensin	+ Sphingomyelinase
SM	5.6 ± 0.2	3.4 ± 0.3 *	3.2 ± 0.1 *
PC	47.1 ± 1.8	46.4 ± 2.1	48.2 ± 2.3
PS/PI	10.2 ± 0.5	10.3 ± 0.3	10.6 ± 0.5
PE	12.5 ± 0.7	12.7 ± 0.8	12.2 ± 0.3
NL	25.5 ± 1.9	27.3 ± 2.5	26.7 ± 1.7
CE	0.5 ± 0.1	1.2 ± 0.1 *	1.3 ± 0.1 *

and monensin caused little more loss of sphingomyelin than did either of these agents applied separately. These results again suggested that the pool of sphingomyelin whose synthesis was prevented by monensin was the same pool which normally made its way to the cell surface and was therefore accessible to the sphingomyelinase. Monensin, which is thought to act selectively to block vesicular transport between *medial-* and *trans*-Golgi in BHK cells [16] therefore had the effect of preventing the conversion of ceramide to that pool of sphingomyelin which normally supplied the cell surface.

Although we found a clear increase of ceramide in monensin-treated cells, the increase did not match the decrease in sphingomyelin radioactivity measured in these cells. However, the remainder of the radioactivity missing from sphingomyelin in the presence of monensin appeared in glycolipid (largely glucosylceramide).

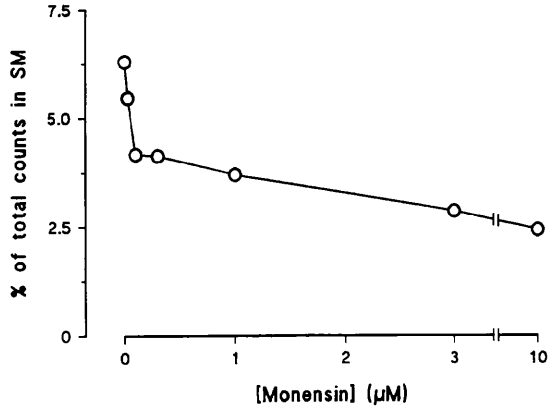

Fig. 3. Effect of different monensin concentrations on labelling of sphingomyelin cells were incubated as in the legend to Fig. 2. but using different concentrations of monensin up to 10 μM. Results are expressed as means ± S.D. of triplicate determinations in a single experiment which was repeated twice with similar results.

Thus the total radioactivity in sphingolipids was not altered by monensin; it was redistributed from surface sphingomyelin to ceramide and glycolipid. This suggests that the ceramide is accumulating in the early Golgi as a result of monensin treatment, since it is available for conversion to glycolipids and UDP-glucose:ceramide glucosyltransferase, the enzyme catalysing the initial step of glycolipid synthesis, is present in an early Golgi compartment [7–9].

These observations may explain why in other cells, monensin causes an increase in labelling of glucosylceramide and some other lower glycolipids [3,18]. Thus the prime cause of glucosylceramide accumulation in these cases could be due to the failure of ceramide to progress further through the Golgi in the presence of monensin, so that more ceramide is available for glucosylation by the glucosyltransferase. This would be in addition to glucosylceramide accumulation resulting from inhibition of synthesis of higher gangliosides, as suggested previously [3].

It is interesting to note that monensin, which like BFA [6] and treatment with exogenous sphingomyelinase [13,14], causes a decrease in plasma membrane sphingomyelin, also promotes the conversion of cholesterol to cholesterol ester (Table I). This can be seen as a generalised response of the cell to a decreased ratio of sphingomyelin to cholesterol and emphasises the importance which is attached to the maintenance of sphingomyelin/cholesterol ratio in the plasma membrane of normal cells. It suggests that an interaction between sphingomyelin and cholesterol usually limits the amount of unbound cholesterol in the plasma membrane and that the cell responds to even relatively small amounts of unbound cholesterol by esterifying it.

The radioactive sphingomyelin remaining after treatment of control cells with sphingomyelinase (Table I, Figs. 1,2) corresponds to the internal pool of sphingomyelin which we have previously identified [12,13]. The radioactivity in this pool is quantitatively similar to the radioactivity found in sphingomyelin in monensin treated cells. Further treatment of these cells with exogenous sphingomyelinase shows that sphingomyelin synthesized in the presence of monensin is almost entirely intracellular (Fig. 2). As synthesis of the internal pool of sphingomyelin seems unaffected by monensin, it is likely to be occurring at a site prior to the monensin block, i.e., in the ER or *cis*-Golgi. This could correspond to the early Golgi site of sphingomyelin synthesis which has been identified by other workers [2,7–9,19]. However, our findings indicate that this is not the site responsible for synthesis of plasma membrane sphingomyelin.

The observation that monensin blocks the synthesis of this sphingomyelin is a novel finding. It suggests that monensin inhibits the movement of ceramide from the early Golgi to a site distal to the *medial*-Golgi where

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TRANSPORT OF LIPIDS TO THE PLASMA MEMBRANE IN ANIMAL CELLS

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CONTENTS

ABBREVIATIONS

BFA	Brefeldin A	PE	Phosphatidylethanolamine
DAG	Diacylglycerol	PG	Phosphatidylglycerol
ER	Endoplasmic reticulum	PI	Phosphatidylinositol
GSL	Glycosphingolipid	PS	Phosphatidylserine
PC	Phosphatidylcholine	SM	Sphingomyelin

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I. INTRODUCTION

Because of its significance in the regulation of the overall spatial and metabolic organisation of cells, membrane biosynthesis remains one of the most interesting areas of study in cell biology. In the last decade, important advances have been made in this field, particularly regarding the manner in which membrane proteins are distributed from their site of synthesis in the ER to other regions of the cell. Of especial interest has been the elucidation of the steps involved in the processing of proteins which are destined to form part of the plasma membrane or to be exocytosed.[165,166] However, in spite of the fact that lipids account for almost as great a proportion of most membranes as proteins, it is only recently that much attention has been turned to the mechanism of lipid transport between the ER (the main site of lipid biosynthesis) and the membranes of other organelles. This interest has been reflected in the publication of many excellent reviews of this subject area.[24,54,89,116,140,153,177,207,208,215] Here we restrict ourselves to consideration of lipid transport to the plasma membrane, with special emphasis on those lipids which are most characteristic of the surface membrane of mammalian cells.

II. THE CHARACTERISTICS OF PLASMA MEMBRANE LIPIDS

A. *The Distinctive Lipid Composition of Plasma Membranes*

The plasma membrane is the only part of the cell which faces the outside world and which has to receive and conduct external messages. Associated with these specialisations the lipid composition of the plasma membrane is different from that of other membranes and accordingly the synthesis of plasma membrane lipids presents particular problems. There is evidence from a variety of sources[221] that plasma membranes are relatively rich in SM, glycosphingolipids (GSL), lipids, cholesterol and PS and that these lipids are uncommon in the membranes of the other major cellular compartments. It is probable in view of their role in transmembrane signalling that polyphosphoinositides (but not PI) are also concentrated in plasma membranes. Consequently the kinases which produce the polyphosphoinositides from PI are mainly localised in plasma membranes.[43,93,160,170]

Other cellular compartments which have direct commerce with the plasma membrane such as secretory granule membranes,[52,68] and endosomes[17,63,132] have membrane lipid compositions which resemble to some extent that of plasma membrane probably because part of the lipid in these compartments is derived by endocytosis of plasma membrane. In the case of secretory granule membranes it seems that those which undergo most frequent recycling (e.g. nerve terminals) may have a composition more like plasma membrane than those which undergo less recycling (e.g. chromaffin cells).[119,155] Membranes which do not have direct vesicular commerce with the plasma membrane such as ER, nuclear membrane and mitochondrial membranes have relatively large amounts of PC and PI but little of the characteristic plasma membrane lipids, even though ER is a major site of synthesis of some lipids like cholesterol and PS which mainly reside in the plasma membrane. Although lysosomes have a membrane composition which resembles to some extent that of plasma membrane[84] they are generally poor in PS and enriched in lysobisphosphatidate, a lipid which does not appear in other cell membranes.[29]

The overall lipid compositions of various cells seem to depend to a large extent on their different proportions of intracellular membranes and plasma membranes. This in turn depends on their size and shape so that for instance, the plasma membrane makes a relatively small contribution to the overall lipid composition of a large round cell such as an hepatocyte, whereas elongated cells with extensive surface membrane extensions and convolutions such as those in the brain have a composition which quite closely resembles that of plasma membrane. In some cell types it appears that as much as 90% of cellular cholesterol and SM is present in the plasma membrane[120,125] but this may be true generally only for cells having relatively small amounts of secretory granules and endocytically-derived compartments. A significant amount of surface membrane constituents may be present as internal vesicles recycling through the endocytic system which may or may not

be measured as surface membrane depending on the techniques employed.[120,206] However, van Meer's calculation of the amount of surface cholesterol as 24% of the total in BHK cells[206] is too low since it was based on an underestimate of the proportion of plasma membrane in the cell. Using more recent stereological data[73] gives a figure of about 50%. This is still considerably less than the 60–65% of total BHK SM which is located on the surface[157,180] but within the range of 50–60% of total cholesterol inferred from the experiments of Slotte *et al.*[180]

The obverse of their enrichment in PS and SM is that plasma membranes have much less PC and PI than typical internal membranes such as ER and nuclear membranes. Indeed, it appears that some plasma membranes e.g. those derived from MDCK cells by viruses[209] or ruminant red cells[149] can exist with little or no PC so that the role of this normally ubiquitous phospholipid must be assumed by other lipids (particularly SM and GSL). Based on evidence from red cells, myelin, enveloped viruses and vesicles released from BHK cells,[7] it appears that PI represents no more than 1–3% of plasma membrane phospholipids, much less than in ER. Presumably a residual amount of PI must exist in plasma membranes as a dynamic reservoir for synthesis of the polyphosphoinositides and in the form of GPI anchors for certain proteins attached to the exoplasmic surface.[65,130]

B. *Is There a Universal Plasma Membrane Lipid Composition?*

Many of the analyses of plasma membrane lipids from various cells are open to question due to the technical problems of obtaining clean separations between plasma membranes and intracellular smooth membranes. There are only three cases where we can be reasonably sure that we have reliable figures for plasma membrane lipid composition; in mammalian red cells where there is no significant internal membrane, in myelin (which appears to be derived largely from the plasma membrane of a Schwann cell) and in viruses which bud from the plasma membrane of their host cell taking with them a representative sample of the plasma membrane lipids (but not the proteins) of the host cell.[26] Significantly, it appears that the lipids of viruses budding from a variety of different cell types[6,26] look rather similar not only to each other but also to the lipids of mammalian red cells[149] and to myelin, suggesting that there may indeed be a 'universal' plasma membrane lipid composition. This approximates on a molar basis to about 15% each for PE, PC and SM, 8% for PS, 1% for inositol phospholipids, 2% for GSLs and 45% for cholesterol. Similar values for phospholipid and cholesterol have been derived for plasma membrane vesicles isolated from intact cells.[220] However, it appears that the relative proportions of SM and GSLs can vary considerably (e.g. myelin has relatively much more GSL and correspondingly less SM) but the total sphingolipid content is rather constant at about 20%. Possibly the large variations in GSL content between different cells are related to the role of GSLs as surface antigens which are important in governing immunological distinctions between cells.

In red cells from different animal species there is considerable variation in the proportions of PC and SM but the total choline phospholipid is fairly constant.[149] It has been argued that this may reflect a general feature of cell surfaces where the ratio of PC:SM could have a strong influence on the membrane fluidity[113] or on transbilayer mobility.[20]

Such generalisations must take account of the fact that many cells are polar, having more than one surface membrane domain, each of which can potentially separately provide the lipid envelope for a virus.[210] Experiments have shown significant differences between apical and basolateral domains of MDCK cells[209] and there is evidence for differences in lipid composition in the three surface membranes of liver cells (blood sinusoidal, bile canalicular and basolateral).[117,138] Even so, the differences in lipid composition between different surface domains of the same cell is considerably less than the differences between surface membranes and internal membranes.

C. *Transbilayer Distribution of Plasma Membrane Lipids*

The similarities between plasma membranes from different cells extend also to the transbilayer distribution of the lipids: there is considerable evidence that SM and the major GSLs are virtually always found on the external surface whereas PS and most of the PE is generally found on the cytosolic face of the plasma membrane.[150,213,226] PI and the polyphosphoinositides are mainly located in the cytosolic face but a small proportion of PI must be exoplasmic where it is involved in covalent anchoring of GPI linked proteins to the outer face of the membrane.[65,130] About two thirds of PC is in the outer lipid leaflet in red cell membranes and in a virus budding from the surface of BHK cells.[6] Based on these values it appears that approximately 55% of total phospholipid is localised on the outer leaflet of the bilayer. However, the results of this calculation do not appear to be consistent with claims that 80% of total cholesterol is in the inner leaflet of the plasma membrane bilayer.[27,78] Assuming that the area occupied by protein is the same for both leaflets, such a distribution of cholesterol would imply a large excess of inner lipid leaflet area. The fluid mosaic model of membrane structure implies equal areas for inner and outer lipid leaflets and differences in area which are as little as 1% are associated with large alterations in membrane curvature.[3] Thus based on the observed distribution of phospholipids, it seems more likely that about 55% of the cholesterol should be in the inner leaflet. A relatively even distribution of cholesterol between the two leaflets is also more consistent with the electron density distribution seen in myelin.[199]

The importance to the cell of the particular asymmetrical arrangement of plasma membrane lipids is uncertain, but it seems reasonable to place the more reactive anionic phospholipids, with their susceptibility to oxidation and propensity to undergo ion-dependent changes in association and phase behaviour, in the more sheltered and controlled cytosolic environment. The anionic phospholipids PS and PI are also involved in *trans*-membrane signalling events and need to be in the cytosolic surface of the plasma membrane to be able to exercise this function. Sphingolipids may be concentrated on the exterior surface of the cell because their sphingoid backbone, relatively saturated *N*-acyl chains and unreactive headgroups offer more resistance to the intrinsically more variable and oxidising environment of the extracellular medium than would normal glycerolipids. Furthermore, the GSLs are specifically involved in cell recognition and adhesion and could not exercise these functions unless they were on the cell surface.

It has been noted that those lipids normally found on the cell surface (SM, PC, GSLs) are also the main 'bilayer forming' lipids and are less likely to allow fusion events to occur than those anionic lipids on the cytoplasmic face of the plasma membrane which can adopt the H_{II} hexagonal phase.[51] This may help to explain why cell–cell fusion events are rare but not why most cells can indulge in constant endocytotic activity, which also necessitates fusion of external membrane faces.

Clearly in some circumstances, breakdown of the normal asymmetrical arrangement of anionic phospholipids is associated with dramatic changes in morphology and cell surface interactions. Thus a marked rise in surface exposure of PS is seen in activated platelets and is associated with platelet aggregation and the initiation of blood coagulation.[22] Likewise, increased exposure of PS on red cells is associated with ageing changes[172] and with an increased removal of affected cells from the circulation by phagocytes.[186]

III. MODES OF LIPID TRANSPORT WITHIN CELLS

A. *Vesicular Transport*

One of the keys to understanding plasma membrane biosynthesis has been the discovery that proteins are incorporated into the cell surface as a result of vesicular transport processes. This means that proteins in vesicles budding from the ER are transferred to the early Golgi cisternae as a result of fusion of the vesicles with Golgi membranes and then progress by a similar budding and fusion mechanism through successive compartments of the Golgi to the plasma membrane.[165] In this process proteins undergo progressive

post-translational modifications e.g. glycosylation or proteolytic cleavage, before they reach their destination in the plasma membrane or (in the case of secreted proteins) the extracellular medium.

Now it is clear that because vesicles must contain lipids, vesicular transport of proteins necessarily implies a concurrent cotransport of lipids. Indeed, it has been assumed in the past that vesicular transport represented the major route whereby lipids generally reached the cell surface. However, it is only recently that it has been confirmed that some lipids (particularly those which form the exoplasmic lipid leaflet) move to the plasma membrane by vesicular transport. Firstly, it has become clear that GSLs undergo elongation of their carbohydrate chains in successive compartments of the Golgi apparatus in a sequence which has analogies with the well-known mechanism for processing of proteins.[192,225] Indeed there is some evidence that proteins and GSLs are processed in parallel perhaps utilising the same vesicles for transport and possibly the same enzymes in some cases.[146, 217] Secondly, these elongation steps occur largely on the luminal surface of the Golgi and it is difficult to imagine any other process besides vesicular transport which could translate mature GSLs from a luminal compartment to the cell surface, bearing in mind that these GSLs show negligible rates of transbilayer migration.[226] Thirdly, it has been demonstrated that movement of sphingolipids to the surface is blocked in cells which are in mitosis and where consequently, vesicular transport has ceased.[107] Finally it has been shown that monensin and brefeldin A (BFA) which (by different mechanisms) inhibit the vesicular transport of proteins, also block the appearance of sphingolipids at the cell surface. In particular, GSLs and SM synthesised endogenously from fluorescent derivatives of ceramide added to cells are prevented from reaching the surface in the presence of monensin[126] which blocks between the medial and *trans*-Golgi.[74] Monensin and BFA (which blocks vesicular transport out of the *trans*-Golgi and probably the *trans*-Golgi network)[142] also prevent the appearance at the cell surface of SM synthesised *de novo* from acetate.[98, 99]

Clearly, vesicular transport involves directed sequential membrane budding and fusion events and the control mechanisms of these membrane fusion processes are currently under active study. Recent work has revealed that it is possible to examine the mechanism of vesicular transport in cell-free systems.[13,82,165,175] These experiments have demonstrated a requirement for ATP, palmitoyl CoA, GTP-binding proteins and a protein which is sensitive to *N*-ethylmaleimide and[146,166,219] for maintenance of lipid trafficking between the ER and Golgi and it is likely that future work will show further parallels between the cytosolic requirements for vesicular transport of lipids and for proteins.

Since vesicular transport depends on membrane fusion events and most membrane lipids diffuse quickly enough to ensure rapid equilibration between compartments undergoing fusion or fission, it might be expected that the lipid composition of these compartments would be rather similar. The fact that marked lipid compositional differences do occur between various organelle membranes and between different surface domains in polar cells, suggests that other more selective processes are available to modify the lipid composition originally imposed by synthesis and vesicular transport. This goes some way to explaining the current interest in lipid sorting in vesicular transport pathways and in the activity of specific lipid transporting proteins (LTPs). However, the main reason why processes other than vesicular transport must be involved in lipid transport to the plasma membrane is that PC, PE and PS translocation is quite unaffected by treatments which inhibit vesicular transport (Section III.B.1).

B. *Lipid Transport by Carrier Proteins*

1. *Specificity and Activity of LTPs*

Because of the hydrophobic nature of natural membrane lipids there is a very low concentration of free molecules which could potentially diffuse through the aqueous phase and thus equilibrate between membranes. Nevertheless, there is evidence for the simple

diffusion of lipid monomers between membranes[32] although it is unknown whether this is physiologically significant. However, lipid diffusion between membranes is enormously enhanced by the action of the lipid transfer proteins (LTPs) which are found in the soluble fraction of many cell types.[223] These LPTs may be of high specificity (e.g. those that carry only PC or those which will only exchange PC for PI) or they may have a rather general ability to bind and transfer hydrophobic molecules e.g. sterol carrier protein 2, which appears to be identical to the non-specific LPT described by others[37] and which has recently been shown to be present in peroxisomes.[104,193] There are a wide variety of proteins able to transport free fatty acids[137] but these will not be considered in this review.

The significance of the peroxisomal localisation of the non-specific LTP is not understood; it seems unlikely that this protein could be involved in the translocation of lipids other than those within peroxisomes. However it appears that most of the specific LTPs are confined to the cytosol where they can only have access to lipids on the cytosolic faces of membranes. This fits in with the apparent specificity of LTPs for glycerophospholipids which usually predominate on the cytosolic faces of membranes. There are reports of LTPs (generally in transformed or foetal cells) which can transfer SM[61] and GSLs[59,168] but it is not obvious how this ability can be utilised since SM and most GSLs are generally found on the luminal faces of membranes and so would not be expected to be accessible to cytosolic LPTs. The possibility of luminal LTPs cannot be discounted but there is at present no evidence for such entities. However, cytosolic LTPs could have access to glucosylceramide which appears to be synthesised on cytosolic membrane surfaces.[70,96,191]

2. *Role of Lipid Transfer Proteins in Vesicular Transport Pathways*

It is an attractive idea that LTPs could act to modify the lipid composition of membranes involved in vesicular transport pathways in order to achieve the differences in composition which are observed between various organelles. In theory, this could occur either by exchanging different lipids or by mediating net mass transfer between different membrane surfaces. Exchange of phospholipids is well-characterised, particularly exchange of PC for PI, but mass transfer is difficult to demonstrate using LTPs in model membrane systems. However, net mass transfer must be involved in some situations (e.g. during cell growth) although the mechanism is not well understood. Associated with this problem is the question of how the activity of LTPs might be coordinated with vesicular traffic so as to cause an orderly and directed flow of lipids and proteins from their site of synthesis in the ER to other organelles.

The possible involvement of LTPs in mediation of vesicular transport between ER and plasma membrane has been convincingly demonstrated by recent studies of yeast mutants.[40] Yeast possesses a PI-specific LTP and mutations which destroy the function of this protein are lethal,[1] suggesting that it has an important function. The discovery that the yeast PITP was identical to the product of SEC14, a gene previously shown to be essential for normal secretory function in yeast, confirmed that the ability to transfer PI is necessary for maintenance of functional vesicular transport to the cell surface.[15,42]

The picture was made more complicated by the observation that the viability of cells lacking PITP could be restored if the CDP-choline pathway for PC synthesis was blocked so that the cells could only synthesise PC by the methylation of PE.[15,42] Models put forward to explain the curious dependence of secretory activity on PC synthesis and PI transport have been based on the hypothesis that normal function of the Golgi apparatus depends on its membranes having an elevated ratio of PI:PC. The role of PIPT is to maintain this ratio by removing excess PC (synthesised either in the Golgi or derived from the ER) and exchanging it for PI.[41] A similar effect on Golgi PI:PC ratio may also be achieved by inhibiting normal PC synthesis (see above).

The precise role of the PI:PC ratio on Golgi function is unknown but it has been suggested on the basis of the bilayer couple hypothesis,[171] that a high PI:PC ratio favours the increased membrane curvature which is a necessary precondition for budding of Golgi

membrane.[176] Budding of the Golgi is of course an essential part of the vesicular transport pathway.

3. *Possible Effects of Unilateral Lipid Insertion on Transport of Lipids*

The above implication that vesicular transport can be modulated by the relative amounts of certain phospholipids in the budding surfaces introduces the possibility that specific insertion of lipids into these surfaces can influence their ability to bud. There have long been indications that membrane curvature[171] and budding[3] can be increased by differential expansion of the two leaflets of a membrane bilayer and it is possible that insertion or removal of lipids unilaterally in a membrane surface could play a part in controlling the tendency of that membrane to vesiculate.[9,66] The action of LTPs in exchanging PL between different membrane surfaces could unilaterally alter lipid packing and thus affect membrane curvature and vesiculation. Vice versa, changes in membrane curvature could alter the tendency of LTPs to transfer lipids between organelles. Thus it has been suggested that the movement of cholesterol out of vesicles is faster when the curvature is greater i.e. when the vesicles are smaller and this might explain why cholesterol moves from small intracellular vesicles to the plasma membrane which generally has a much lower curvature.[189] Possibly the rate of transport of cholesterol could be influenced by variations in the size and hence the curvature of these vesicles.

Insertion of new lipids into the cytoplasmic face of the ER is a consequence of normal lipid biosynthesis and would be expected to result in a unilateral increase in the area of the cytoplasmic lipid leaflet. This in turn would be predicted to result in an enhanced tendency of the ER to vesiculate. Is it possible that lipid biosynthesis could provide the motor for the continuous vesiculation of this membrane which initiates the entire vesicular transport pathway?

The question of what drives vesicular transport has not generally been addressed. It is well-known that energy is required to sustain vesicular transport but this requirement does not seem to be associated with protein synthesis since vesicular transport proceeds in the presence of cycloheximide which prevents synthesis of protein. However, the possibility must be considered that part of the energy requirement may reflect a vital role for lipid biosynthesis in the process. An important feature of this model is that lipid synthesis is seen as the driving force for vesicular transport from the ER to the cell surface whereas the proteins which are also necessary have an essentially regulatory role.

The magnitude of any such effect would depend on the rates of biosynthesis, transbilayer migration and cytoplasmic transport of new lipids. Rates of biosynthesis of lipids have been measured for many mammalian cells and can be as much as 10% of total lipids per hour, which means that if the ER represents 30% of total cellular lipid,[73] then about 1% is added to the area of the cytoplasmic leaflet of the ER in each minute. Addition to the outer leaflet[3] or removal from the inner leaflet[66] of similar amounts of material results in vesiculation of red cells, suggesting that vesiculation of ER induced by lipid biosynthesis is feasible. Present evidence suggests that the half-time for 'flip–flop' of phospholipids in the ER is about 20 min[85] which is comparable to the rate at which proteins undergo vesicular transport to the surface, so that flip–flop is unlikely to act quickly enough to dissipate the vesiculation potential represented by the increased area of the cytoplasmic leaflet of the ER. LTPs will only be able to prevent the potential vesiculating effects of lipid biosynthesis if they can rapidly effect net mass transfer of lipids. However, the suggestion that lipid biosynthesis drives vesicular transport can be tested by examining the effects of inhibitors of lipid biosynthesis on vesicular transport. Support for this hypothesis comes from the recent observations that the transport of proteins through the secretory pathway in CHO and BHK cells is coupled to lipid biosynthesis[162] and that inhibition of ceramide synthesis blocks axonal outgrowth.[79]

4. *Role of LTPs in PI Turnover*

LPTs may also be involved in the now well-known process by which breakdown of inositol lipid at the cell surface is utilised for the generation of intracellular signals in response to many extracellular agonists.[21] The problem in this case is that there is a relatively small pool of plasma membrane-inositol lipid (mainly PIP_2) which is rapidly degraded and which must then be replenished by phosphorylation of PI, which can only be synthesised in the ER.[18] 1,2 Diacylglycerol (DAG), the lipidic degradation product of PIP_2 breakdown, can be readily phosphorylated in many cell types by a plasma membrane DAG kinase, but in order to complete the cycle, the PA which is formed must reach the ER to be converted into PI (via CDP–diacylglycerol) and this PI must shuttle back to the plasma membrane where it can be phosphorylated by the kinases present there. Thus efficient means must exist both for the transport of plasma membrane PA to the ER and for the transport of PI to the plasma membrane. It seems unlikely that vesicular transport can be involved in either of these steps since resynthesis of PIP_2 is too fast to be explained in this way without assuming incredibly rapid and specific membrane cycling between the plasma membrane and ER. The alternative is to suppose that LTPs are available to allow PA and PI to move down their concentration gradients between the ER and cell surface, thus supplying the surface with PI synthesised in the ER from PA transported from the plasma membrane. However, it is possible that even before any PA reaches the ER, an agonist-stimulated drop in the inositide level in the plasma membrane causes an immediate increase in transfer of PI from the ER by a specific LTP. Indeed, there is recent evidence for such a mechanism.[188] Clearly it would be of interest to know if genetic deletion of PITP would prevent resynthesis of PIP_2 at the cell surface in yeast.

Agonist-dependent breakdown of PIP_2 can in extreme circumstances lead to significant mass changes in the amounts of the phospholipids involved in the PI cycle and there is the possibility that these changes in lipid ratios in the plasma membrane and ER could alter not only membrane curvature and propensity to vesiculate but could also change the rate of movement of lipids by LTPs (III.B.3).

Recently there has been great interest in the agonist-dependent activation of phospholipases which attack PC instead of inositides.[23] There is a clear possibility that access of substrate to these enzymes may also depend on LTPs.

IV. LIPID SYNTHESIS AND TRANSPORT TO THE PLASMA MEMBRANE

A. *Glycerolipids*

1. *Sites of Synthesis*

The topology of glycerolipid synthesis has been reviewed previously[18] and this topic will only be outlined here.

As for most cellular lipids, those glycerophospholipids eventually destined to reach the plasma membrane are originally synthesised on the cytosolic face of the ER.[14,18] This is consistent with the information that the substrates for lipid synthesis (choline, inositol, serine, nucleotides) are concentrated in the cytosol and there is as yet no evidence for any mechanism which would allow substantial amounts of these hydrophilic molecules to cross the ER bilayer and enter the luminal compartment. The major phospholipids PC and PE are largely made from diacylglycerol and the corresponding CDP derivatives of choline and ethanolamine. Particularly in liver, a mechanism exists in the ER for the methylation of PE to provide an alternative pathway to PC, but this may be mainly relevant for synthesis of secreted lipoproteins rather than for membrane lipids[195,198] and seems to be of minor significance in most cell types.

The energy-independent exchange of basic headgroups between glycerophospholipids can potentially vary the relative proportions of these lipids in membranes and appears to be the major route of PS synthesis in mammalian cells.[16] Base exchange is mainly a rough ER activity[205] but has also been described in mitochondria, nuclei and recently in plasma

membrane.[173] One report suggests that base exchange activity for PS and PE resides on the luminal aspect of the ER[34] although it is not obvious what role this activity could have when most PS and PE is found on the cytosolic face.

PI and PG are synthesised on the cytosolic face of the ER but by a route distinct from the other phospholipids involving the reaction of CDP–diacylglycerol with inositol or glycerol 3-phosphate respectively. Polyphosphoinositide synthesis has a fairly wide distribution, having been reported to occur in nuclei,[141] Golgi,[97] lysosomes,[45] secretory granule membranes[118,155] and endocytic coated vesicles.[36] However, the chief site for PIP_2 synthesis seems to be in the plasma membrane[43,93,160,170] and the presence there of high levels of inositide kinase activities may explain why plasma membranes generally have very low amounts of PI[77,93] (Section II.B).

2. *Transport to the Plasma Membrane*

In principle, glycerolipids synthesised in the cytosolic face of the ER could arrive in the cytosolic face of the plasma membrane either by vesicular transport or by utilising LTPs. However, the fate of these lipids could be complicated if there is rapid transbilayer migration across the ER membrane, leading to the deposition of PC, PE and PS in the luminal leaflet. There are indications that PC synthesised on the cytosolic leaflet of the ER can readily cross the bilayer through the action of a translocase[12,25] although other evidence implies that the rate is fairly slow.[87] More recent data suggests the existence of a non-specific translocase which transports PC, PE and PS across the ER bilayer with a half-time of about 20 min.[85] This rate is considerably slower than transport by LTP, which is correspondingly likely to be the dominant factor in distribution of these newly-synthesised lipids.

Moreover, it has been shown that newly-synthesised PE and PC reach the plasma membrane much faster than membrane proteins[101,178] and this process is not affected by monensin,[194] BFA,[197] ATP depletion or metabolic poisons which block vesicular transport, suggesting that the major route of transfer is mediated by LTPs. This idea is supported by the observation that in mitotic cells where vesicular transport is halted, PE is still transported normally.[107] Little is known about the precise nature of the LTPs involved in transport of PC, PE and PS in intact cells but Voelker[215] has put forward evidence that it is not the non-specific LTP present in the peroxisomes which is involved.

Studies comparing the labelling of various cell fractions from liver after an injection of labelled lipid precursor fail to show the expected pattern of falling specific activity of labelled lipid in going from ER to Golgi to the plasma membrane.[196] Indeed, for some precursors, the specific activity of plasma membrane PC was greater at all time points than that of ER PC. This could be consistent with the selection by LTPs of newly-synthesised ER PC which had not had time to mix with the unlabelled pool on the luminal surface of the ER. However, it could also mean that LTPs selectively carry lipids from areas of the ER actively involved in lipid biosynthesis, perhaps because of increased membrane curvature in these regions (see Section III.B.3).

If newly-synthesised PC can be selectively removed from the cytoplasmic surface of the ER by an LTP before much of it has a chance to migrate into the luminal leaflet of the ER bilayer, then relatively little of the newly-synthesised lipid would enter luminal membrane compartments. However, some PC must be presumed to reach luminal compartments in order to provide the phosphorylcholine headgroup for synthesis of SM in the Golgi apparatus or further along the exocytic pathway (see below). PC is the only glycerolipid which commonly appears on the cell surface; it may arrive there as a result either of vesicular transport or by cytosolic transport to the inner leaflet of the plasma membrane followed by active transbilayer migration to the cell surface under the influence of a specific translocase.[10,46] At present the evidence is insufficient to discriminate between these alternatives.

B. *Sphingomyelin*

1. *Sites of Synthesis*

The exact site of SM synthesis is the subject of active investigation.[116,140] Originally it was supposed by analogy with PC that SM was made from ceramide and CDP–choline in the ER but it became clear from kinetic studies that SM mainly derived its phosphocholine headgroup from preformed PC in a reaction mediated by ceramide–PC phosphocholine transferase (CPCT).[60,62,216] A similar pathway involving PE instead of PC appears to be present in rat liver and brain.[133]

Although the plasma membrane was initially proposed to be the site of the CPCT reaction[136, 200, 216] there is more recent evidence based largely on subcellular fractionation of rat liver, that SM is made from ceramide and PC in the *cis* or *medial* Golgi.[71,95,96] However, it is difficult to be sure that fractions of early Golgi are not contaminated with other smooth membranes including endosomes and plasma membrane vesicles which may possess synthetic activity for SM. Indeed, Futerman *et al.*[71] did find 12–13% of total activity in plasma membrane and Malgat *et al.*[133] have concluded from their cell fractionation data that there are two sites of SM synthesis, one in the plasma membrane and one which is microsomal.

Recent work from our laboratory confirms the existence of two sites of SM synthesis, one which could be in the early Golgi and the other which appears to be on the endosomal pathway which is responsible for recycling endocytosed plasma membrane back to the surface.[4,99,100] In contrast to earlier assumptions, the Golgi activity is not responsible for synthesis of surface SM but appears to synthesise the SM which remains inside the cells and which corresponds to the internal pool identified previously.[98,157] It seems unlikely that this pool which represents 30% of cell SM[157] could be accommodated in the Golgi which accounts for only about 5% of total BHK cell lipids,[73] so it probably resides in a large membrane pool like the ER. It may appear in the ER as a result of retrograde vesicular transport from the Golgi.[98] However, there is still a possibility that this internal pool of SM is in the 'secondary endosomes' which accumulate non-recycling glucosylceramide added to the BHK cell surface.[111] This could be similar to the non-recycling pool of receptors for wheat germ agglutinin which accumulates at a non-lysosomal site in CHO cells.[158]

Synthesis of surface SM depends on the transport of ceramide to a post-Golgi site which seems to be on the endosomal plasma membrane recycling pathway.[4,99,100] Support for the idea of an endosomal site of synthesis for surface SM comes from experiments on the resynthesis of surface SM after its degradation by an external sphingomyelinase.[4,100] This process has the capacity to convert ceramide back into surface SM about five times as rapidly as in normally growing cells and therefore represents a major site of SM biosynthesis.[5,100] Resynthesis of SM and its return to the cell surface is largely complete in 2 hr and the kinetics of resynthesis are similar to those of plasma membrane recycling as noted previously.[140] Furthermore, the process is completely unaffected by monensin or brefeldin A, which block protein and lipid processing through the Golgi cisternae but which do not affect plasma membrane recycling.[114,142] Nocodazole, which dissociates microtubules but which does not affect membrane recycling in BHK cells[111] likewise has no effect on resynthesis.[100] Resynthesis of SM is inhibited under conditions where endocytosis is blocked, either when cells are in mitosis or when they are depleted of ATP.[4,100] The results indicate that resynthesis of surface SM is independent of the Golgi and occurs at a site on the plasma membrane endosomal recycling pathway.

These findings are consistent with the observations that NBD–SM,[114] glucosylceramide[110] or glycoproteins[148] inserted into the plasma membrane are almost completely recycled back to the surface along the pathway of receptor-mediated endocytosis with little leakage of membrane constituents from this pathway into the early Golgi. Only in undifferentiated cells is there any evidence that glucosylceramide can reach the Golgi.[109] Calculations based on measurements of receptor turnover have indicated that the vast bulk of endocytosed membrane appears to be rapidly recycled back to the surface[81, 139] so it is

to be expected that lipids inserted into the cell surface would show similar behaviour. This places the site of resynthesis of surface SM somewhere on the recycling pathway, perhaps in the compartment associated with the centriole where exogenous NBD–SM concentrates.[114] This compartment which has been described as 'juxtanuclear endosomes'[91] is functionally distinct from the Golgi[114,215] although it is difficult to differentiate by morphological criteria since both compartments are perinuclear and probably interdigitated with each other. There is evidence that the pH of this juxtanuclear endosomal compartment is close to 6.5[222] which is similar to the pH optimum for SM synthesis determined *in vitro*.[135]

Although a large proportion of endocytosed surface membrane is recycled, there is evidence that small but significant amounts of membrane are transferred to lysosomes for degradation. The results of Koval and Pagano[115] suggest that about 8%/hr of surface NBD–SM reaches the lysosomes, where it is broken down to NBD–ceramide which can then reach the Golgi and be converted into GSL. This is consistent with our observations that about 5% of ceramide generated at the cell surface by exogenous sphingomyelinase is converted into short chain GSLs.[100] A similar proportion of endocytosed ricin is not recycled in BHK cells[202] and this suggests that NBD–SM and endogenous plasma membrane ceramide are following a well-defined endocytic pathway which splits about 20:1 in favour of recycling compared with transport to lysosomes and thence to the Golgi. These results support the idea that these lipids are reliable endocytic markers which move about the cell only by vesicular transport.

2. *Movement of SM to the Cell Surface*

Past work has generally supported the idea that SM is made in the early Golgi and then undergoes vesicular transport to the surface.[116,207,215] However, our evidence that plasma membrane SM is synthesised at a site on the endocytic plasma membrane recycling pathway conflicts with this interpretation and suggests that synthesis of SM destined for the cell surface depends on the vesicular transport of ceramide to a site in the endocytic pathway where it can be converted into SM.[4,99,100]

Our proposed model for the synthesis of SM is outlined in Fig 1. It presumes two sites of SM synthesis, one in the early Golgi which produces internal SM and the other which is part of the plasma membrane recycling pathway and synthesises SM for delivery to the surface. This model explains how BFA and monensin block the translocation of newly-synthesised SM to the surface (because they interfere with vesicular transport of ceramide through the Golgi) but not SM synthesis through the recycling pathway (because these drugs do not affect plasma membrane recycling). It also explains why most of the ceramide produced by attack of exogenous sphingomyelinase does not gain access to the early Golgi but is largely restricted to the endocytic pathway.

The diagram emphasises that there is still some uncertainty regarding the extent to which there is vesicular commerce between early endosomes and the *trans*-Golgi network (TGN). Ceramide could potentially reach the endosomes following the route from the TGN used by the mannose-6-phosphate receptor,[208] although unless there is a specific sorting of ceramide this possibility would mean that bulk membrane flow would have to follow the same route rather than going directly to the cell surface. More likely, most of the ceramide enters the endosomal recycling pathway after it has reached the plasma membrane as part of normal exocytic flow so that it can then undergo endocytosis and be transported to the site of SM synthesis: this would be consistent with the relatively slow labelling of surface SM with ^{3}H choline.[47,157] Such a mechanism would imply that the normal localisation of SM on the outer surface of the plasma membrane is a consequence of its synthesis in the lumen of vesicles in the endosomal plasma membrane recycling pathway. SM-synthesising activity in endosomes may also be important in controlling the amount of ceramide which remains in the cell surface because recent work suggests that ceramide may possess molecular signalling activity.[112,113]

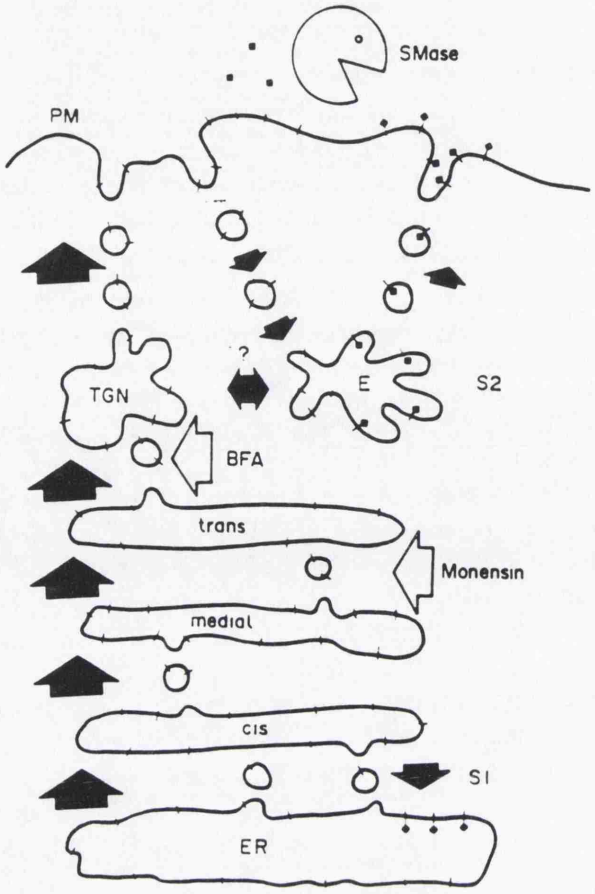

FIG. 1. A model to illustrate the synthesis of SM in BHK cells. The diagram shows the flow of vesicular transport from the ER through the various compartments of the Golgi apparatus to the plasma membrane (PM) and between the PM and the early endocytic compartment (E). The sites of action of BFA and monensin are indicated by light arrows. Ceramide is indicated as -- and phosphocholine head groups as ■, so that SM is shown as a composite of these symbols. S1 is the site of SM synthesis prior to the monensin block and is likely to be either in the early Golgi or the ER. S2 is the endosomal site of SM synthesis. The "Pacman" symbol represents sphingomyelinase.

3. *The Fate of the Diacylglycerol Residue Derived from PC during SM Biosynthesis*

One interesting corollary of the unusual mode of synthesis of SM is that for each molecule of SM made, there should also be production of a molecule of diacylglycerol (DAG) derived from PC. In view of the importance of plasma membrane DAG as an activator of protein kinase C[21] there has been some speculation regarding the possible significance of DAG arising from SM synthesis and the metabolic fate of this DAG.[116,152] A common response of cells to the introduction of DAG into their plasma membrane is to convert the DAG to phosphatidic acid, but such an effect was not observed in BHK cells resynthesising SM after treatment with sphingomyelinase,[5] suggesting that if DAG was produced it did not reach the plasma membrane.

Work from the authors' laboratory using ^{3}H palmitate-labelled cells has shown that resynthesis of plasma membrane SM is associated with production of new triacylglycerol in amounts comparable with the reduction in PC.[5] Thus DAG produced as a result of SM synthesis in the endosomal recycling pathway may be eliminated by conversion to triacylglycerol. An effective way of removing this potentially large amount of DAG must be necessary since arrival of any significant amounts of DAG at the cell surface could compromise the normal signalling function exercised by plasma membrane DAG.[21]

C. *Glycosphingolipids*

1. *Sites of Synthesis*

As noted by Hoekstra and Kok,[89] the more complex the glycolipid, the more distal is its site of synthesis. The simplest GSL, glucosylceramide, appears to be synthesised in the early Golgi where the glucosyltransferase catalysing the initial step in GSL synthesis is found,[48,96,191] although there may also be synthetic activity in the intermediate compartment between the ER and *cis*-Golgi.[89] The observation that monensin stimulates an increase in glucosylceramide at the expense of SM synthesis[99] may indicate the presence of glucosylating activity in the medial Golgi.

Recent work shows that enzymes which can synthesise glucosyl- and lactosylceramide are localised on the Golgi membrane surface facing the cytoplasm.[49,70,96,191] This observation fits in with other data which reveals the presence of GSL-specific transport proteins that could potentially cause rapid movement of glucosylceramide between cytosolic-facing membrane surfaces.[59,68] However, the third step in synthesis of GSLs, mediated by sialyltransferase I which adds sialic acid to lactosylceramide to give the simplest ganglioside GM3 (neuraminyllactosylceramide) occurs exclusively on the luminal face of the Golgi.[96,116,192] Thus glucosylceramide (and possibly lactosylceramide) would need to undergo transbilayer migration across the Golgi membrane in order to be converted into more complex GSLs. Such a process has not yet been demonstrated directly.

Since treatment with monensin or BFA, which block vesicular transport out of the Golgi, causes an almost complete inhibition of synthesis of the complex GSLs[167,169,203,204,225] it appears that these are synthesised at more distal sites in the exocytic pathway. A corresponding accumulation of glucosylceramide, lactosylceramide, GM3 and GD3 (disialyllactosylceramide) is observed with monensin which blocks transport out of the medial Golgi.[74] These experiments confirm that galactosyltransferase I and sialyltransferase I activities are localised in the early Golgi. Other galactosyltransferase and sialylating activities are present in the *trans*-Golgi[143,225] or *trans*-Golgi network.[39] Enzymes which add *N*-acetylgalactosamine to growing chains seem to be mainly localised in the TGN.[192, 225] This inference is supported by the observation that BFA, which blocks vesicular transport out of the *trans*-Golgi, prevents synthesis of GSLs containing *N*-acetylgalactosamine.[203,225]

2. *Transport of GSLs to the Plasma Membrane*

As noted above (III.A), GSLs reach the cell surface probably exclusively by vesicular transport. Based on the detailed localisation of the enzymes involved in synthesis of the GSLs (IV.C.1) it appears that ceramide synthesised in the ER passes by vesicular transport to the early compartments of the Golgi where it is converted into glucosylceramide and possibly lactosylceramide on the cytosol-facing leaflet. After undergoing transbilayer migration to the luminal leaflet of the medial Golgi, lactosylceramide is sialylated to give GM3 and GD3. Vesicular transport of these lipids to the TGN allows the sequential addition of *N*-acetylgalactosamine, galactose and sialic acid to form the formation of the higher gangliosides.[89]

Such a process does not exclude the possibility that some of the 'precursor' GSLs such as glucosyl- and lactosylceramide might reach the cell surface without being further modified but the compositional evidence for the presence of these lipids in the plasma membrane is ambiguous because of the probability of contamination of plasma membrane preparations with smooth internal membranes. However, viral membranes, which ought to contain a representative sample of host cell plasma membrane lipids, contain little of these precursor GSLs.[26]

There is evidence that glycoproteins from which sialic acid groups have been removed at the surface can be resialylated and returned to the surface after endocytosis[184] so that it is possible that some of the terminal sialyltransferases are localised in the endocytic recycling pathway. This could mean that as for the synthesis of surface SM, the terminal

events of ganglioside synthesis also occur in the recycling pathway and may therefore require endocytosis of incomplete GSLs initially delivered to the cell surface.

However, resialylation of proteins is a relatively slow process and may involve transport to the TGN.[184]

3. *Transport of SM and GSLs to the Cell Surface Domains of Polarised Cells*

Epithelial cells possess distinctive surface domains which have different lipid and protein compositions.[147,174] Generally the differences in lipid composition are confined to those lipids normally localised on the surface (sphingolipids in the case of apical regions and PC in the case of basolateral domains) whereas the amounts of the lipids which are characteristic of the cytosolic leaflet of the plasma membrane are rather similar in different domains.[174] This situation has been explained by supposing that tight junctions only prevent diffusion of exoplasmic lipids between domains[210] although it may also be due to the likelihood that the lipids of the cytosolic leaflet are subject to transfer by LTPs and this factor promotes equilibration between cytosol-facing domains. Recent work indicates that in neurons there is a diffusion barrier to phospholipids at the axonal hillock which maintains compositional differences between the axonal and somatodendritic domains.[108]

Delivery of SM and GSLs to the surface of epithelial cells is clearly dependent on vesicular transport[19,211] but there is a marked tendency for GSLs to concentrate in the apical domain while SM is more evenly distributed.[90,211] In MDCK and Caco-2 cells, disaggregation of the Golgi by nocodazole reduced overall transport of sphingolipids but did not change the pattern of glucosylceramide and SM distribution. However monensin reduced glucosylceramide transport to the apical surface and BFA increased apical delivery of SM (only in Caco-2 cells).[212] These experiments are not easy to interpret because they utilised NBD–ceramide which may not be metabolised like endogenous ceramide (Section IV.D).

It has been suggested that hydrogen-bonding interactions between GSLs and membrane proteins in transport vesicles bound for the apical domain could cause lateral segregation of GSLs and account for their specific localisation.[217] This may apply particularly to GPI-linked proteins which also show priority targeting to apical surfaces.[2] The relative enrichment in PC in the basolateral surface is seen as a negative consequence of the preferential deposition of GSLs in the apical domain.

D. *Ceramide*

Synthesis of ceramide occurs on the cytosolic face of the ER.[88,134] Ceramide itself is a relatively hydrophobic lipid and like diacylglycerol,[8,72] can readily cross the lipid bilayer to enter the luminal leaflet of the ER.[126] There is no evidence that ceramide can be transported between membranes by LTPs so probably it undergoes vesicular transport and enters the exocytic pathway where it can potentially provide the backbone for synthesis of all the sphingolipids. However, a recent report[145] suggests that ceramide passes from the ER to the Golgi by a non-vesicular mechanism; if confirmed, this would require a revision of our thinking on ceramide transport.

It seems likely that SM synthesis may be limited by the availability of ceramide since ceramide stimulates production of SM *in vitro*[136] but is generally only a minor component of cellular membranes[100] (unlike PC, the other substrate necessary for the synthesis of SM). Significantly, mutation of the enzyme in CHO cells which catalyses serine palmitoyltransferase, the rate-limiting step in ceramide biosynthesis, is associated with failure of sphingolipid synthesis and cessation of cell growth.[105] This may suggest that synthesis of ceramide has a similar crucial role in controlling sphingolipid synthesis to that played by its analog diacylglycerol in controlling the rate of glycerolipid synthesis.[28] Serine palmitoyltransferase may be as important as phosphatidate phosphohydrolase in determining the overall rate of lipid biosynthesis and thus of vesicular transport (Section III.B.3). Recent work by Rosenwald and Pagano[162,163] has raised the intriguing possibility that ceramide

levels may modulate the rate of vesicular transport through the secretory pathway. This is also suggested by Harel and Futerman's observation that inhibition of ceramide synthesis prevents axonal outgrowth in hippocampal neurons.[79]

Brefeldin A, which fuses ER and Golgi membranes, causes a decrease in cellular ceramide and an increase in SM and GSL synthesis.[33,98] This could be because enzymes normally resident in the early Golgi which can synthesise sphingolipids suddenly have access to ceramide at its site of synthesis in the ER.[134] The fact that ceramide is utilised normally at a much lower rate supports the idea that natural long-chain ceramides probably do not easily cross aqueous barriers between membranes but depend on vesicular transport for passage between organelles. This idea is supported by the evidence that little of the ceramide introduced into the plasma membrane by treatment with sphingomyelinase reaches the Golgi.[100] Thus sphingolipid synthesis is likely to be limited not only by the rate of ceramide synthesis in the ER but also by the rate at which ceramide progresses through the vesicular transport pathway.[69]

The dependence of natural ceramide on vesicular mechanisms for its transport is in contrast to the behaviour of some truncated ceramides which have been used in fluorescence studies and which gain access to the Golgi apparatus by diffusion processes which are independent of membrane fusion events. NBD–ceramide diffuses into various intracellular compartments and to become trapped in the Golgi when it is converted to SM in this organelle.[114,127] However, although NBD–ceramide has been routinely used by some workers as a Golgi marker, it is not clear to us that the original experiments[127] would have differentiated satisfactorily between the Golgi cisternae and perinuclear endosomal compartments which could be a site of SM synthesis (Section IV.B.1). Thus there are possible ambiguities attached to the interpretation of experiments which have been carried out using NBD–ceramide.[164]

NBD–ceramide distributes in cells in a manner which is probably not typical of natural ceramides. Ceramide made at the cell surface from natural SM is utilised very poorly for synthesis of glycolipids (unlike NBD–ceramide[114] and therefore only a small proportion reaches the early part of the Golgi apparatus where glucosylation can occur (Section IV.B.1). Transport of natural ceramide is most likely to occur by vesicular pathways although no studies appear to have been carried out to discover if ceramide can be transported by non-specific LTPs. Interestingly, one ceramide analogue (C_5-DMB ceramide) which is less polar than NBD–ceramide and therefore is less likely to cross aqueous barriers within the cell, not only is converted largely into SM rather than GSL but synthesis of SM occurs at a similar rate to membrane recycling,[154] like natural ceramide introduced into the cell surface.[100] This compound may therefore be a better analog of natural ceramide than is NBD–ceramide and its distribution in cells may be a more accurate representation of endocytic pathways. However, the properties of C_5-DMB ceramide have not been established well enough for us to be certain of its value as an endocytic marker.

E. *GPI-anchored Proteins*

1. *Sites of Synthesis*

Many cell surface proteins are anchored in the membrane by a phosphatidylinositol residue linked to a complex glycan (GPI) which is covalently attached to the protein.[50] The PI involved in these GPI anchors must necessarily be present in the external surface of the plasma membrane and therefore may be initially incorporated into luminal surfaces of the exocytic pathway. There is evidence that the addition of the GPI residue occurs very soon after completion of protein synthesis in the ER and this presupposes that there must be a reservoir of luminal PI together with the appropriate cofactors (ethanolamine, UDP–*N*-acetylglucosamine, dolichylphosphomannose and myristoyl CoA also in the lumen) necessary for synthesis of the mature GPI precursor.[65] Nothing is known about the luminal pool of PI which provides the membrane anchors for the complex but a very small proportion of the total ER PI would be sufficient to account for the numbers of GPI-linked

proteins observed in most circumstances. Correspondingly, very little transbilayer migration of PI from the cytoplasmic leaflet would be necessary.

2. *Movement of GPI Anchors to the Plasma Membrane*

GPI anchors for cell surface proteins are probably moved to the cell surface from their site of synthesis in the lumen of the ER/Golgi by a similar vesicular transport mechanism to that utilised by the sphingolipids.[30,65] Present evidence suggests that GPI-linked proteins are transported into the lumen of the Golgi where they form a complex with sphingolipids which is insoluble in detergent.[31] This is consistent with previous suggestions that GPI-linked proteins have an association with sphingolipids,[30,53] perhaps utilising hydrogen-bonding interactions which are not available to glycerolipids.[174] Cholesterol also appears to be associated with the complex of sphingolipids and GPI-linked proteins in the recently-discovered invaginations of the cell surface known as caveolae[30,38] although it is not known if cholesterol is involved in the complex between sphingolipids and GPI-linked proteins prior to the arrival of the complex at the cell surface.

F. *Cholesterol*

1. *Sites of Synthesis*

It has been accepted for many years that the main site of biosynthesis of cholesterol is in the ER where most of the enzymes involved in its synthesis have been localised.[161] However, there is now good evidence for another major site of synthesis in peroxisomes.[103,201] Furthermore, a recent review[80] suggests the possibility that cholesterol may not undergo complete synthesis in the ER and that the final step of cholesterol synthesis (from lanosterol) could take place in a variety of cellular compartments including the plasma membrane. It is clear that in many cases it has not been established whether the measured product is cholesterol or a close precursor[67] so that some of the data in the literature regarding the site of synthesis of cholesterol may not be completely accurate. Nevertheless, the most comprehensive experimental analysis of this problem[161] argues in favour of complete synthesis of cholesterol in the ER.

2. *Movement of Cholesterol to the Plasma Membrane*

Transport of newly-synthesised cholesterol to the plasma membrane is blocked by metabolic inhibitors and by reduction of temperature below 15°[102] and this has been inferred to provide evidence for vesicular transport of cholesterol.[207] However, cholesterol transport does not proceed through the conventional pathway through the Golgi apparatus since inhibition of vesicular transport by monensin[102] or BFA[194] had no apparent affect on the movement of cholesterol to the cell surface. Thus it has been postulated that cholesterol may be contained in specific vesicles which can bypass the Golgi[128] and there is some experimental evidence for these entities.[120,194] Although LTPs exist that can carry cholesterol, there is no convincing evidence that LTPs are necessary for the transport of cholesterol to the plasma membrane.[128] Sterol carrier protein 2 does not seem to be involved since it is localised in peroxisomes and mutants which lack this protein can still transport cholesterol to the cell surface.[128]

Some of the puzzling features of cholesterol transport could depend on the possibility that its concentration in plasma membranes may be determined by the presence there of sphingolipids.[207] Evidence exists that SM binds cholesterol with a higher affinity than other phospholipids[57,122,131] and this depends on the close steric fit of SM with cholesterol which allows maximisation of van der Waals interactions.[131,199] Such interactions may explain why in Niemann–Pick disease, where there is an accumulation of SM in lysosomes, there is a parallel increase in lysosomal cholesterol.[113] Furthermore, alteration of the levels of SM in fibroblast plasma membrane is associated with pronounced changes in the distribution,

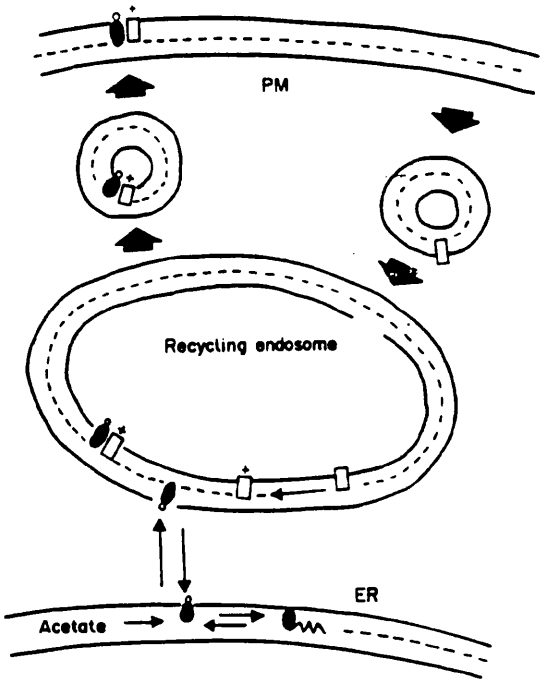

FIG. 2. A model for integration of cholesterol into the plasma membrane. Cholesterol (●) synthesised from acetate in the ER passes into perinuclear endosomes where it becomes associated with SM (🍴) newly synthesised from ceramide (□) and undergoes cotransport to the cell surface as part of normal plasma membrane recycling. Excess cholesterol resulting from too much synthesis or an imbalance of cholesterol:SM in the plasma membrane accumulates in the ER and is converted to cholesterol ester (●〰).

synthesis,[75] oxidisability and rate of esterification of cholesterol.[179–183] BFA and monensin, which prevent normal movement of SM to the cell surface[98,99] also cause an increased esterification of cholesterol[98,99,185] and a decrease in its synthesis.[98] These changes suggest that cells are very sensitive to the ratio of cholesterol:SM in their surface and respond to a drop in SM by reducing the amount of surface cholesterol. Esterification of cholesterol may be a consequence of its transfer to intracellular pools[179–183] where it is exposed to acyl CoA transferase (ACAT), which is localised in the rough ER,[161] although we have some evidence that acylation may be at the expense of preformed lipids rather than free fatty acid.[98] Either process would lead to a reduction in plasma membrane cholesterol. There is also evidence for a reciprocal effect whereby the level of cholesterol in the cell can influence the synthesis of sphingolipids.[129]

If plasma membrane SM synthesis occurs in recycling endosomes (IV.B.1), then cholesterol could be added to the endosomal membrane soon after synthesis of SM, so that cholesterol and SM arrive simultaneously at the cell surface. This scheme is represented in Fig 2. It is possible that transfer could occur via cholesterol-rich vesicles shed from the ER although the mechanism of production of such vesicles distinct from the normal vesicles on the exocytic pathway, presents many problems. More likely, cholesterol desorbs from the SM-poor environment of the ER and diffuses through the aqueous phase into the SM-rich environment of the recycling endosomes, in a process which is analogous to the transfer which has been demonstrated between unilamellar lipid vesicles.[131,190] Thus for cholesterol there might be no need for a LTP to mediate exchange between ER and endosomes. Modulation of cholesterol esterification and synthesis may depend on the balance of cholesterol movement between the ER and the endosomes; this in turn will depend on the amount of new endosomal SM which can potentially act as a sink for ER cholesterol.

The above model would explain not only why cholesterol transport is unaffected by monensin and BFA (because transport does not involve the Golgi) but why it *is* affected by reduced temperature and energy depletion (because it depends on vesicular transport through the exocytic limb of the endocytic recycling pathway). It would also suggest that

the vesicles rich in newly-synthesised cholesterol isolated by Lange[120] could correspond to endosomes bound for the cell surface or to perinuclear endosomes (IV.B.1). This relatively short vesicular pathway could also explain the shorter half time for transport of cholesterol to the cell surface (10 min)[56,102] compared with vesicular transport of proteins and sphingolipids (20 min). Others have found much longer times for transport of cholesterol, of the order of hours[124,125,144] but these discrepancies could in part be due to methodology e.g. Kaplan and Simoni[56] isolated total surface membrane whereas other workers employed chemical or enzymic modification of specific molecules in the outer surface of the plasma membrane.

The model for cholesterol movement from ER to recycling endosomes shown in Fig. 2 assumes that transbilayer migration of cholesterol is so fast that it does not represent a limiting kinetic factor. As a relatively hydrophobic lipid cholesterol, (like diacylglycerol)[8] might be expected to equilibrate rapidly between the two leaflets of the lipid bilayer although a strong interaction with SM might hinder the transbilayer movement of cholesterol (see IV.E.2). However, there is some disagreement regarding the rate of transbilayer migration of cholesterol, with several groups finding very short half-times[55,106,121,123] but a more recent determination suggesting a much slower rate.[27]

Although the above evidence provides circumstantial support for the concept that cholesterol concentrates in plasma membranes as a result of its affinity for sphingolipids, the molar amount of cholesterol in plasma membranes seems to be about three times as much as the molar amount of SM and probably about double the amount of total sphingolipids so that it is difficult to explain the observations in terms of a 1:1 complex of cholesterol with sphingolipids. Interestingly there is physical evidence that cholesterol might bind to phospholipids in the molar ratio of 2:1.[156]

V. GENERATION AND MAINTENANCE OF PLASMA MEMBRANE LIPID COMPOSITION AND ASYMMETRY

If as described above, plasma membrane SM is synthesised in recycling endosomes (IV.B.2), if cholesterol associates with the new SM in the endosomal membrane (IV.F.2) and if PS is largely transported by LTPs rather than by vesicular transport (IV.A.2) then there is no reason to suppose that the membranes in the organelles of the exocytic pathway (especially Golgi membranes) should be progressively enriched in these lipids. Such a compositional gradient would only be expected if cholesterol, PS and SM were all synthesised in the ER/early Golgi membranes and transported to the cell surface by vesicular processes which involved sorting at each step. In the following section we consider the compositional evidence which can help to resolve these alternatives.

A. *Is there A Gradient of Membrane Lipid Composition in the Secretory Pathway?*

It has been commonly accepted that in terms of membrane lipid composition, there is a gradient of increasing cholesterol, PS and sphingomyelin in passing from the ER through the Golgi to secretory vesicles and thence to the plasma membrane, implying a continuous enrichment of these characteristic surface lipids through the secretory pathway.[140,207,214] In our opinion, the evidence for a gradient of SM, PS and cholesterol in the membranes of the secretory pathway is rather weak, being based largely on old determinations of lipid composition in rat liver[44] where Golgi "membranes" are likely to have been contaminated not only with plasma membrane but also with the large amounts of lipoproteins present in the lumen of the vesicular subfractions isolated. Liver subfractions are particularly confusing in this respect, because liver is the main site not only of synthesis of lipoproteins but of their uptake from plasma. Thus lipoprotein on its way out of the cell via the secretory pathway or on its way into the cell from plasma via the endocytic pathway constitutes a significant fraction of the total lipid of a variety of light vesicular fractions. Large amounts of intravesicular lipoprotein lower the density of membrane vesicles so that

they sediment with Golgi fractions on density gradient separations.[81] Indeed, the selective purification of Golgi vesicles from liver on density gradients probably depends on the low density conferred on them by their content of lipoprotein. Since lipoproteins are rich in PC, LPC, SM and cholesterol, the overall lipid composition of these light subfractions will be skewed towards these lipids and away from PE, PS and PI, which are poorly represented in lipoproteins.[149] Thus we suggest that any apparent enrichment of Golgi "membranes" in cholesterol and SM is due to the presence of endovesicles and/or lipoprotein in luminal contents.

Only for cholesterol is there any evidence for an enrichment of a typical plasma membrane lipid in the Golgi membrane[151] but this conclusion was based on a semi-quantitative assessment of filipin association with cell fractions and is open to question since other workers have found low cholesterol contents in purified Golgi membrane fractions.[63,92,187] In cases where the luminal contents were largely removed from Golgi vesicles which were greatly enriched in *trans*-elements[92,187] cholesterol was present in amounts no higher than in ER, suggesting that no significant change in cholesterol content occurs prior to the TGN. This is supported by a recent study which shows that newly-synthesised cholesterol is not present in the galactosyltransferase-rich fraction (*trans*-Golgi) of fibroblasts and which provides other evidence for the absence of cholesterol in Golgi membranes.[120] Where separation of exocytic and endocytic vesicles from rat liver was achieved, it appeared that the lipid composition of exocytic vesicles resembled ER in terms of cholesterol:phospholipid ratio whereas endocytic vesicles resembled plasma membrane.[83]

The phospholipid content of Golgi membranes even as far as the *trans*-Golgi is very similar to that of the ER.[17,63,132] This is especially true for liver Golgi membranes which have been depleted of contents;[17,187] indeed, the amounts of PS and SM in Golgi membranes were generally less than in ER. In some cases, relatively large amounts of PS and SM have been found in Golgi fractions[86] but again this could be potentially explained in terms of contamination with endocytic vesicles.[81]

For secretory granules there is an indication that the membrane lipid composition does resemble that of the plasma membrane[11,52,68,119] but in most cases it is not clear what proportion of the secretory granule membrane is derived directly by *de novo* synthesis and how much represents recycled plasma membrane. Most secretory cells rapidly recycle surface membrane after exocytosis[28] and utilise it for repackaging more secretory product, so that after multiple cycles of this pathway the lipid composition of the granule membrane would resemble the surface membrane because each cycle would add some endocytosed plasma membrane lipid.

Thus the paucity of evidence for gradients of lipid composition in the secretory pathway suggests that cholesterol and SM destined for the plasma membrane are not added in the Golgi as has been assumed previously. This is consistent with the concept that these lipids are added in the endocytic pathway. (IV.B.2 and IV.F.2)

B. *Maintenance of Plasma Membrane Lipid Composition and Asymmetry*

The evidence that surface SM is synthesised in an endosomal compartment which is part of the plasma membrane recycling pathway (Section IV.B.2) and that cholesterol may partition selectively into membranes enriched in sphingolipids (IV.F.2), suggests that the endosomal compartment is where some of the characteristic lipids of the plasma membrane are accumulated. Whether any of the terminal reactions of GSL synthesis could also occur in this compartment is unknown. However, there is a possibility that the peculiar lipid composition and asymmetry of the plasma membrane could be partly determined by the presence of enzymes catalysing the synthesis of mature sphingolipids in the endocytic plasma membrane recycling pathway. Besides their function in *de novo* synthesis, such enzymes could also serve a role in the repair of exoplasmic lipids which had lost their terminal residues and their return to the cell surface.[64]

The synthesis of sphingomyelin and the complex GSLs on luminal surfaces (IV.B.2,

IV.C.2) could be an initial determinant of plasma membrane lipid asymmetry because these lipids show very little tendency to diffuse into the cytoplasmic leaflet of the bilayer.[227] When sphingolipids can be as much as 30% of total acyl lipids in the plasma membrane (i.e. 60% of surface lipids), this would tend to exclude glycerolipids from the outer leaflet thus encouraging their passive accumulation in the inner leaflet.

Cytoplasmic transfer of lipids by LTPs seems to be much faster than vesicular transport and this might suggest that the lipid composition of cytosolic-facing membrane leaflets would be similar throughout the cell due to the dissipation of intermembrane concentration gradients by LTPs. This appears to be true for PE but not for PI, PS and PC since the amounts of these lipids differ greatly between the cytosolic leaflets of the ER and plasma membrane. Therefore there must be other processes occurring which are capable of concentrating PS and PE and decreasing the amount of inositol lipids and PC in the cytosolic leaflet of the plasma membrane relative to the ER.

Probably the most important factor in maintaining the asymmetric distribution of anionic phospholipids is that many cells possess an ATP-dependent aminophospholipid translocase which drives PS and PE into the inner leaflet of the plasma membrane.[58,226] A similar process may drive PC into the outer leaflet of the plasma membrane and into the luminal leaflet of secretory granules.[10,46] There have also been indications that interaction of PS with cytoskeletal proteins may assist in maintaining this lipid on the cytosolic face of the membrane[76,222] although this concept has been challenged recently.[226] It has additionally been suggested that a polarised arrangement of charges on the surface of integral membrane proteins may lock anionic phospholipids into the cytosolic leaflet.[94] Another possibility is that the widely-distributed phospholipid-binding proteins collectively known as annexins,[35] which in the presence of low concentrations of Ca^{2+} have a strong affinity for anionic lipids, may help to concentrate these phospholipids on cytosolic membrane faces.

However, it should be noted that normal phospholipid asymmetry can be maintained for relatively long periods in vesicles from red cells which are not only free of spectrin and annexins but are also depleted in ATP.[159] Enveloped viruses which are derived from a host cell plasma membrane but which contain no host cell proteins or ATP also maintain the phospholipid asymmetry of the host cell plasma membrane for many hours.[6] These phenomena suggest that the energy barrier to diffusion of the hydrophilic headgroups of phospholipids through the hydrophobic core of the membrane is sufficient to maintain lipid asymmetry for periods of hours, although the asymmetry may break down over a longer time scale.

VI. CONCLUSIONS

Our understanding of the mechanism by which different lipids make their way to the plasma membrane has improved dramatically over the past few years. Until quite recently it had been assumed that all of plasma membrane lipid biogenesis depended on vesicular transport of newly-synthesised lipids from the ER through the Golgi and thence to the cell surface. This must still be the major pathway for the GSLs, which need to undergo processing of their carbohydrate chains, probably in the same compartments where the analogous processing of glycoproteins occurs. However, vesicular transport is likely to represent only a minor route for transport of lipids which occupy the cytosolic face of the plasma membrane (PE, PS, PI); these lipids are subject to transport by cytosolic LTPs, which can move lipids about the cell much more quickly than can vesicular transport. The mechanism of synthesis of plasma membrane SM has proved difficult to elucidate but we believe that the experimental evidence is best explained in terms of vesicular transport of ceramide through the ER–Golgi complex to the plasma membrane and thence to an endosomal site where conversion to SM occurs. We suggest that cholesterol adds to the endosomal membrane as SM is synthesised because of the strong affinity of these two lipids for each other, so that cholesterol and SM reach the plasma membrane simultaneously as endosomes are recycled to the cell surface. Modulation of the ratio of cholesterol:SM in

plasma membrane is achieved by regulation of cholesterol efflux from the ER to and from endosomes.

According to this scheme, the overall lipid composition and asymmetry of the plasma membrane is determined to a large extent by the synthesis of mature sphingolipids in the endocytic recycling pathway. Consequently there is no reason to expect a gradient of SM or cholesterol concentration in passing from ER membranes through successive compartments of the Golgi apparatus. Indeed, there is little convincing experimental evidence to support this expectation.

VII. PROBLEMS YET TO BE SOLVED

There remain certain outstanding problems to be decided regarding the mechanism of plasma membrane lipid assembly:

(1) Where exactly are the main sites of plasma membrane SM synthesis? Is it in the early Golgi, the endocytic recycling pathway or in both? This question is amenable to experimental analysis by cell fractionation, using endocytic markers to follow recycling vesicles and measuring directly the ability of these vesicles to synthesise SM. However, precise localisation will depend on purification of SM-synthesising activity and analysis by immunohistochemical techniques.

(2) Do vesicles on the recycling pathway possess other 'repair' activities such as the ability to add sialic acid, sulphate or phosphate groups to lipids from which these groups have been removed? For instance, are the terminal reactions of GSL synthesis endosomal?

(3) What is the significance and intracellular location of SM which is not on the cell surface? Is there an equivalent pool of cholesterol? Is this SM pool in the ER or in secondary endosomes?

(4) Which cytosolic factors are important for maintenance of lipid traffic to and from the plasma membrane? Suitable cell-free systems which can translocate lipids between organelles have already been designed but need to be refined for the study of lipid vesicular transport in permeabilised cells.

(5) How is the balance maintained between the amounts of the various plasma membrane lipids? How is cholesterol esterification linked to delivery of SM to the cell surface? Does alteration of cholesterol levels affect synthesis or breakdown of SM? Is ceramide synthesis (and particularly the rate-limiting step catalysed by serine-palmitoyl CoA ligase) affected by the amount of cholesterol in the cells?

(6) What is the fate of the diacylglycerol presumed to be produced as a consequence of SM synthesis from PC?

(7) Is lipid biosynthesis a motor for vesicular transport?

These and other problems will continue to make the analysis of plasma membrane lipid synthesis and renewal an active field of study in the next few years.

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Synthesis of surface sphingomyelin in the plasma membrane recycling pathway of BHK cells

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Abstract

Sphingomyelin, which has been degraded at the BHK cell surface by exogenous sphingomyelinase, is converted back into sphingomyelin with kinetics similar to those of plasma membrane recycling. Resynthesis of sphingomyelin under these conditions proceeds at a rate about 4-fold higher than normal biosynthesis of sphingomyelin. Neither resynthesis of sphingomyelin nor its return to the surface is inhibited by brefeldin A (BFA), which is a potent blocker of vesicular transport through the Golgi but has no effect on plasma membrane recycling. However, resynthesis of plasma membrane sphingomyelin is greatly decreased in cells undergoing mitosis or energy depletion, where endocytosis is inhibited. We conclude that the main site of surface sphingomyelin synthesis in BHK cells could be in recycling endosomes and not in the Golgi apparatus as proposed previously. We also suggest a model pathway by which cholesterol may reach the plasma membrane via recycling endosomes.

Key words: Sphingomyelin; Endocytosis; Brefeldin A; Cholesterol

1. Introduction

It has been known for some years that sphingomyelin is synthesised by an unusual process which depends on transfer of the phosphocholine headgroup of pre-existing phosphatidylcholine to ceramide [1–4]. Although it seems clear that all the steps in ceramide biosynthesis occur in the endoplasmic reticulum (ER) [5], the precise location of the site of sphingomyelin synthesis has been the subject of some dispute. Originally it was thought to be the plasma membrane [3,6] but recent work has suggested that sphingomyelin is synthesised in the early Golgi [7–10] and is delivered to the cell surface by a vesicular transport process which follows the secretory pathway [11–13]. Consistent with this interpretation, inhibitors of vesicular transport such as monensin and BFA interfere with the normal movement of sphingomyelin to the surface [13–15].

We have recently found evidence for a site of synthesis of internal sphingomyelin which could be in the ER or early Golgi [14–16], but this does not appear to be the site responsible for the synthesis of cell surface sphingomyelin. From evidence that monensin inhibits the synthesis of cell surface sphingomyelin from ceramide, we have suggested that sphingomyelin destined for the cell surface is synthesised not in the early Golgi but at a site distal to the medial Golgi [15]. We show here that in BHK cells there is a major site of sphingomyelin synthesis which is not in the Golgi, based on its insensitivity to BFA and monensin, and which appears to be part of the plasma membrane endocytic recycling pathway. This site may be responsible for the de novo synthesis of plasma membrane sphingomyelin.

2. Methods

Incubation of cells and measurement of sphingomyelin resynthesis. BHK 21 cells were cultured in 3.5-cm dishes using 2 ml of Glasgow MEM supplemented with 5% foetal calf serum and tryptose phosphate as described previously [14,15,17]. In some experiments cells were labelled for 4 h with 20 μCi of [^{3}H]acetate (NEN-Dupont) with and without addition of 5 μg/ml BFA. Half of the samples were then treated with 1 μl (0.1

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IU) *B. cereus* sphingomyelinase (Sigma) for 20 min to degrade surface sphingomyelin. The medium was removed and then all of the samples were extracted with 1.9 ml of methanol/chloroform (2:1 v/v).

In most experiments cells labelled to equilibrium (48 h) with 5 μCi of [^{3}H]acetate (NEN Dupont) were washed with cold MEM, reincubated at 37°C with serum-free MEM and then treated with 1 μl (0.1 IU) *B. cereus* sphingomyelinase (Sigma) for 20 min to degrade surface sphingomyelin. They were then washed three times with ice-cold serum-free MEM and returned to the same medium at 37°C for up to 3 h to allow them to resynthesise sphingomyelin. At various time points triplicate samples of cells were washed with 2 ml ice-cold saline and extracted with 1.9 ml methanol/chloroform (2:1 v/v). Some samples which had been allowed to resynthesise sphingomyelin for 3 h were treated for a second time with sphingomyelinase before extraction to determine if the resynthesised sphingomyelin had returned to the surface. Experiments were carried out in which the cells were allowed to resynthesise sphingomyelin in the presence of *N*-ethylmaleimide (100 μM), digitonin (50 μM), BFA (5 μg/ml), or nocodazole (10 μM) (all from Sigma). Nocodazole was added 30 min prior to addition of sphingomyelinase. In other experiments cells were energy-depleted by treatment for 2 h with 0.1 mM KCN and 50 mM deoxyglucose or with 30 mM NaF prior to breakdown and resynthesis of sphingomyelin as above.

Preparation of mitotic cells. A modified version of the method of Featherstone et al. [18] was used for preparation of mitotic cells. BHK21 cells were grown as monolayers to subconfluence in 75-cm^2 Falcon flasks. Cells from one flask were seeded out into 850-cm^2 Falcon roller bottles containing 50 ml growth medium plus 500 μCi [^{3}H]acetate. The bottles were incubated under constant rotation for 2 days at 37°C. To synchronise the cells in interphase, thymidine was added to a final concentration of 5 mM at the end of day one. Following incubation overnight for 10–12 h at 37°C the bottles were rotated for 5 min at 200 rpm to remove loosely-attached cells and cell debris. The medium was discarded and the cells washed twice with cold growth medium. Subsequently the cells were reincubated for 3 h in 50 ml radioactive growth medium (10 μCi [^{3}H]acetate/ml) plus 0.1 μM nocodazole. This treatment blocks the cells in metaphase after passing G2. Mitotic cells were harvested by rotating the roller bottles for 15 min at 200 rpm. The cells were sedimented by centrifugation of the medium at 1000 rpm for 5 min and resuspended in 20 ml of serum-free growth medium. About $1.5–3 \cdot 10^7$ cells were thus obtained from each roller bottle, equivalent to a mitotic yield of 15–30%.

Sphingomyelin resynthesis in mitotic cells. Triplicate 0.5-ml aliquots were taken from the resuspended mitotic cells for lipid extraction before and immediately after treatment of the cells with *B. cereus* sphingomyelinase (0.1 units/ml) for 20 min at 37°C. The remainder of the sample after treatment with sphingomyelinase was divided into two portions and washed twice with cold growth medium either with or without nocodazole (0.1 μM). The cells were suspended to a volume of 12 ml in growth medium with or without addition of nocodazole and triplicate 0.5-ml aliquots were incubated at 37°C for up to 4 h, before extraction and analysis of lipids.

Lipid analysis. Lipids were separated by tlc as described previously [14] and identified by comparison with standards (Sigma). Spots identifiable 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 v/v) and 2 ml of Ultima Gold scintillation fluid. Samples were counted in a Canberra Packard TC2500 scintillation counter.

ATP determinations. These were carried out on perchloric acid extracts of cells which had been allowed to resynthesise sphingomyelin as above. The method [19] employed firefly luciferase (Sigma).

3. Results and discussion

BFA prevents delivery of newly synthesised sphingomyelin to the plasma membrane but has no effect on resynthesis and surface localisation of sphingomyelin degraded at the cell surface

Fig. 1 shows that normal delivery of endogenously synthesised sphingomyelin to the cell surface is prevented by BFA [14]. Thus in the absence of BFA about 40% of newly synthesised SM can be degraded by

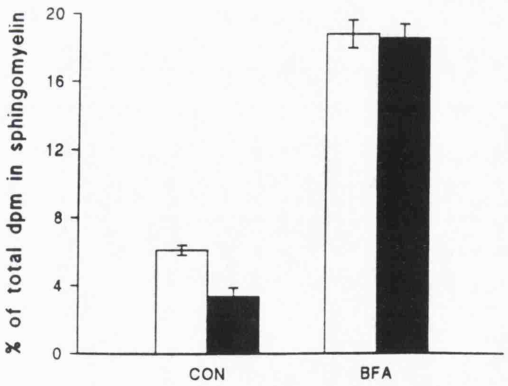


Fig. 1. BFA increases de novo synthesis of sphingomyelin but prevents it from reaching the cell surface. Triplicate dishes of cells were labelled with [^{3}H]acetate for 4 h in the absence (CON) or presence of BFA (5 μg/ml) and then incubated with (filled bars) or without (open bars) sphingomyelinase. Extraction and analysis of lipids was as described in Section 2. Results are expressed as mean ± S.D. of percentage of total lipid radioactivity in sphingomyelin. Similar results were obtained in three other experiments.

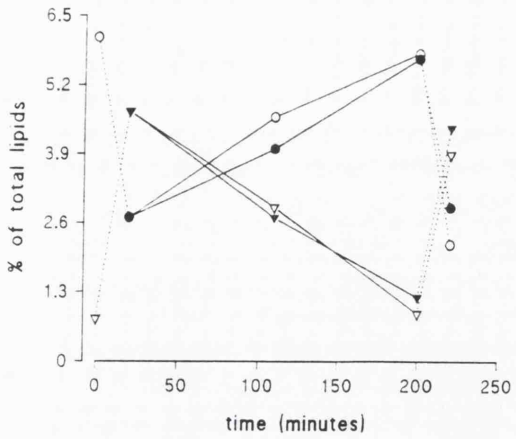


Fig. 2. BFA does not affect resynthesis and surface localisation of sphingomyellin of BHK cells exposed temporarily to sphingomyelinase. Cells were labelled to equilibrium with [^{3}H]acetate, treated with sphingomyelinase and then allowed to resynthesise sphingomyelin after removal of the enzyme, as described in Section 2. After 3 h, a further aliquot of sphingomyelinase was added to some samples in order to determine the proportion of surface sphingomyelin after resynthesis (dotted lines). Parallel determinations were carried out with 5 μg/ml BFA present during resynthesis (solid symbols). Values are expressed as the percentage of total lipid radioactivity present as sphingomyelin (circles) or ceramide (triangles) and represent the means of triplicate determinations in one experiment which gave results representative of three similar experiments.

external sphingomyelinase whereas none can be degraded in the presence of BFA. BFA also caused the characteristic increase in de novo synthesis of sphingomyelin [14,20,21] which has been considered to be diagnostic of its action in fusing Golgi cisternae with endoplasmic reticulum (ER), inducing retrograde movement of Golgi components (up to and including the *trans* Golgi) back to the ER [22].

In marked contrast, when BHK cells were exposed to exogenous sphingomyelinase and then allowed to resynthesise sphingomyelin after removal of the enzyme [17,23], BFA had no effect on this resynthesis (Fig. 2). The resynthesised sphingomyelin was also found to be on the cell surface, since a second treatment with sphingomyelinase degraded the same amount of sphingomyelin as it did originally. Similar results were obtained with monensin [15], another inhibitor of vesicular transport through the Golgi.

In BHK cells it has been reported that BFA prevents export of viral proteins from the *trans* Golgi network (TGN) to the surface but does not affect plasma membrane recycling [24] or the budding of clathrin-coated vesicles from the plasma membrane [25]. The inability of BFA to prevent plasma membrane recycling fits in with the reported failure of monensin to stop recycling of NBD-sphingomyelin introduced into the plasma membrane [26], and indicates that neither the Golgi cisternae nor the TGN are involved in recycling of plasma membrane. The absence of an effect of BFA on sphingomyelin resynthesis or its localisation at the cell surface thus strongly suggests that resynthesis occurs at a site which is distal to the TGN, perhaps even at the plasma membrane itself as suggested previously [6,8].

Resynthesis of surface sphingomyelin depends on endocytosis

For the following reasons we believe that resynthesis of sphingomyelin occurs not at the plasma membrane but at some internal site. Firstly, resynthesis appeared to depend on vesicular transport, since mitotic cells (whose vesicular transport mechanisms are inoperative [18,27]) showed no ability to resynthesise sphingomyelin (Fig. 3, solid triangles). In contrast, cells released from mitosis (open triangles) resynthesised up to 60% of the originally degraded sphingomyelin, although this was clearly less than asynchronous cells (circles) which had not been exposed to nocodazole. It should be emphasised that the cells released from mitosis in Fig. 3 were mitotic up to the end of the treatment with sphingomyelinase and were only released gradually from mitosis as the nocodazole diffused out from them during the course of the incubation in nocodazole-free medium. Thus it is likely that the absence of any resynthesis during the first hour relates to the time taken for these cells to emerge from mitosis. It is important to note also that nocodazole at up to 10 μM had no perceptible effect on resynthesis of sphingomyelin in normal cells and neither did cytochalasin D (10 μM), despite its very marked effect in

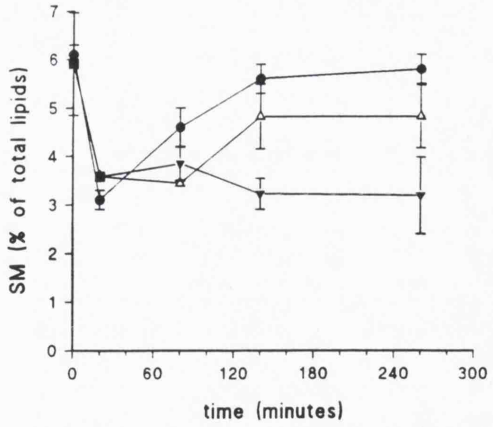


Fig. 3. Mitotic cells are unable to resynthesise sphingomyelin. Labelled mitotic cells were prepared and incubated with sphingomyelinase as described in Section 2. After removal of the enzyme, the resynthesis of sphingomyelin was measured in the presence (▼) or absence (△) of nocodazole. A parallel sample of interphase cells which had not been treated with nocodazole was also allowed to resynthesise sphingomyelin after exposure to sphingomyelinase (●). The experiment shown is one of three which gave similar results. Values represent the means ± S.D. of triplicate determinations at each time point.

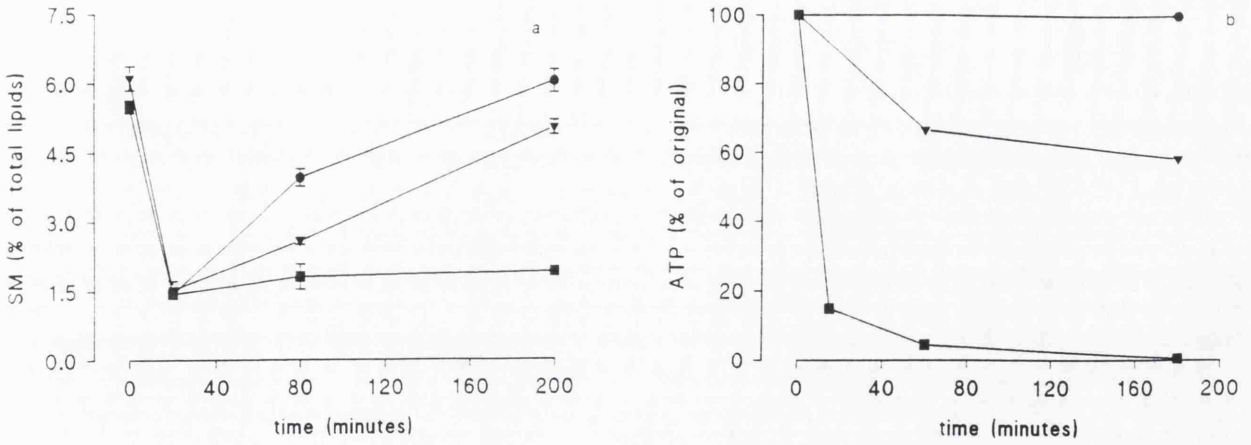
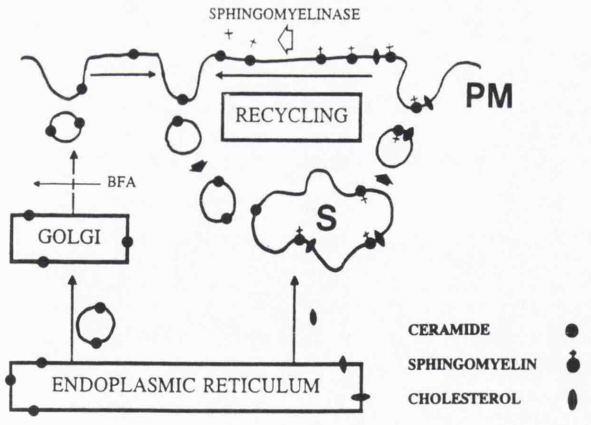


Fig. 4. Time course of resynthesis of sphingomyelin in energy-depleted cells. Cells labelled to equilibrium with [^{3}H]acetate were treated with sphingomyelinase and then allowed to resynthesise sphingomyelin after removal of the enzyme either alone (circles) or in the presence of 100 μM KCN + 50 mM deoxyglucose (triangles) or 30 mM NaF (squares). Sphingomyelin resynthesis (a) or ATP content (b) was measured in the cells at the beginning of the incubation and at various times subsequently. ATP measurements represent the means of duplicate determinations which differed by less than 5%.

causing the cells to round up (results not shown). Thus the integrity of microtubules or of actin filaments are not necessary for resynthesis of cell surface sphingomyelin.

Secondly, energy depletion of the cells by treatment with cyanide + deoxyglucose or with 30 mM NaF, which is known to inhibit endocytosis [28,29] inhibited resynthesis by 50% and 90% respectively (Fig. 4) even though the ceramide-phosphatidylcholine phosphocholinetransferase itself has no known energy requirement. Under these conditions, ATP levels were reduced by 40% in the presence of cyanide + deoxyglucose and by more than 95% in the presence of NaF. Treatment with *N*-ethylmaleimide or digitonin also caused almost complete inhibition of sphingomyelin resynthesis, but this might also be explained in terms of the ATP depletion promoted by these agents. *

We conclude from the above experiments that restoration of surface sphingomyelin after its degradation by sphingomyelinase involves endocytosis of surface membrane containing the newly-formed ceramide, movement of endocytic vesicles to a site where ceramide is converted to sphingomyelin and then transport of sphingomyelin back to the surface. Although this interpretation is consistent with previous indications that lipids inserted into the cell surface are internalised and recycled back to the surface by a pathway similar to that utilised by receptors [30,31], our results show that the site of sphingomyelin resynthesis must



CONSEQUENCES OF AN ENDOSOMAL SITE (S) OF PLASMA MEMBRANE SPHINGOMYELIN SYNTHESIS

Fig. 5. A model for the synthesis of plasma membrane sphingomyelin in BHK cells. The diagram illustrates the proposed pathway of ceramide transport from its site of synthesis in the ER to the plasma membrane (PM) and thence to recycling vesicles where it is converted into sphingomyelin, which is then delivered to the surface. This model also suggests a route by which cholesterol undergoes non-vesicular transfer from the ER to recycling vesicles in response to sphingomyelin synthesis at site S in recycling endosomes.

* Some authors [25] have ascribed morphological effects on cells incubated with 65 μM aluminium chloride in the presence of 30 mM NaF to the action of aluminium fluoride on heterotrimeric GTP-binding proteins. Clearly, under these conditions which are similar to those employed in Fig. 4b, it would be difficult to differentiate effects on GTP-binding proteins from effects due to energy depletion. Without precise determinations on ATP levels in intact cells treated with aluminium fluoride, claims for specific effects on G proteins must be regarded with caution.

also be on the recycling pathway. This is shown diagrammatically in Fig. 5.

The site of sphingomyelin synthesis could be the perinuclear area associated with the centrioles where NBD-sphingomyelin accumulates on its way through the membrane recycling pathway [30] and may correspond to the perinuclear or 'juxtanuclear' endosomes referred to by Hopkins [32] which appear to be similar to the recycling compartment described by Maxfield and colleagues [31,33]. If so, then sphingomyelin synthetic activity could be an enzymatic marker for these endosomes. Perinuclear endosomes appear to have a luminal pH which is only slightly acid (pH 6.5) [33] and interestingly, the pH optimum of the ceramide-phosphatidylcholine phosphotransferase is also 6.5 [34].

Kinetics of sphingomyelin resynthesis and plasma membrane recycling

The rate of resynthesis of sphingomyelin which had been broken down at the surface was about 50% in the first hour (Figs. 1, 4, 5) or about 30% of the original total sphingomyelin per hour. Quantitative analysis of endocytosis has shown that BHK cells internalise 0.6 μm^3 of fluid per min in about 1000 coated vesicles [35], giving a value for the vesicle surface area internalised of about 30 μm^2 per min. With a total surface area of 2200 μm^2 [35], this means that BHK cells should internalise half of their surface in about 40 min, in broad agreement with the kinetics of sphingomyelin resynthesis and recycling [31]. Thus sphingomyelin resynthesis could be limited by the rate of endocytosis and indeed, may represent a novel way of quantifying endocytosis. It should be noted that the rate of endocytosis is not affected by treatment with sphingomyelinase [17].

BHK cells grown under our conditions had a doubling time of 12 h, so that the normal rate of synthesis of all cell components including sphingomyelin was about 8% of the total per hour or about a quarter of the maximum rate of the resynthesis pathway. This difference is similar to the relative rates of de novo synthesis and recycling of NBD-sphingomyelin in CHO cells, although in these cells the recycling pathway seems faster than in BHK cells [13].

The observation that ceramide produced at the plasma membrane is so completely converted back to surface sphingomyelin indicates that little of the endocytosed membrane lipid is lost to other intracellular compartments. Thus endocytosed membrane appears to be largely directed to the site of sphingomyelin resynthesis (S in Fig. 5) and thence back to the surface. Little change was observed in the amount of glucosylceramide or GM3 (sialyllactosylceramide, the major BHK cell glycolipid [36]) during resynthesis of sphingomyelin (Fig. 6), suggesting that the ceramide produced at the cell surface was not available for conversion into

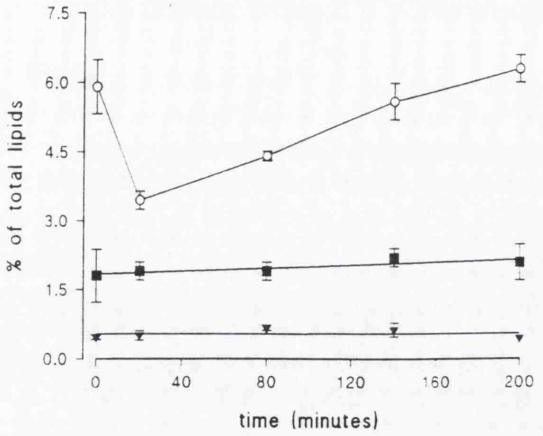


Fig. 6. Changes in glycosphingolipids during resynthesis of sphingomyelin. In an experiment similar to that shown in Fig. 1, measurements were made of sphingomyelin (○), glucosylceramide (▲) and ganglioside GM3 (■) after alkaline methanolysis of total lipids [14]. A small amount of label, equivalent to about 0.1% of the total was found in lactosylceramide and in triosylceramide, but no change was seen in either of these species in the course of this experiment. Values represent the means ± S.D. of three experiments in which triplicate determinations were carried out.

glycolipid. Since synthesis of GM3 depends on transfer of ceramide to the early Golgi [37–39], this result signifies that most of the endocytosed membrane is recycled directly and not routed through the early Golgi. That would be consistent with an estimate based on uptake of endocytosed ricin into the Golgi apparatus of BHK cells which suggests that no more than 5% of endocytosed membrane was routed through the Golgi [40]. The data also indicate that ceramide, like glucosylceramide [30] and sphingomyelin [31] inserted into the plasma membrane, follows non-selective (bulk) membrane flow through the recycling pathway.

Our observations regarding the fate of natural ceramide generated in the plasma membrane contrast with those of Pagano and co-workers [13,26] who concluded that exogenous NBD-ceramide could be converted into both glycolipids and sphingomyelin (probably in the cis-Golgi), even in cells which were in mitosis and where consequently vesicular transport processes were inoperative. This suggests that unlike endogenous ceramide, NBD-ceramide introduced into the cell surface rapidly gains access to the intracellular site of glycolipid synthesis in the early Golgi. These observations emphasise that results obtained using short-chain NBD-ceramide, which is more likely to diffuse across aqueous partitions between membranes, are not necessarily a reliable guide to the fate of endogenous plasma membrane ceramide.

The cis / medial-Golgi site for sphingomyelin synthesis

Despite the conclusion from the present work that the major site of sphingomyelin synthesis is endosomal,

there is evidence for another site of sphingomyelin synthesis in the cis/medial Golgi [7–10]. We have shown recently that BHK cells possess an internal pool of sphingomyelin which does not mix with the plasma membrane sphingomyelin pool but labels more rapidly with [^{3}H]choline [16]. The short-term labelling of this pool with [^{3}H]acetate is not inhibited by monensin [15] so that the internal pool of sphingomyelin could be synthesised at an early Golgi site. However it should be noted that the localisation of sphingomyelin synthesis to the early Golgi has only been claimed in liver, and the results obtained may not be typical of less specialised cells. Also, the liver fractionation schemes employed are unlikely to have separated early Golgi elements from other vesicular smooth membranes including the recycling vesicles, where we propose that sphingomyelin is resynthesised.

Is the endosomal site of sphingomyelin resynthesis also responsible for de novo synthesis of plasma membrane sphingomyelin?

Considering the large capacity of the recycling pathway for resynthesising sphingomyelin, it is tempting to suggest that it represents part of the normal pathway which is responsible for de novo biosynthesis of plasma membrane sphingomyelin. We have recently shown that the de novo synthesis of cell surface sphingomyelin from ceramide is blocked by monensin, demonstrating that monensin prevents the transport of ceramide to a site which is distal to the medial Golgi where ceramide can be converted into sphingomyelin destined for the plasma membrane [14]. The present work using brefeldin A suggests that this site is distal to the TGN. If the distal site of sphingomyelin biosynthesis is the same as the site of resynthesis on the plasma membrane endocytic recycling pathway, then this implies that ceramide must make its way to an endosomal site in order to be converted into plasma membrane sphingomyelin. Our hypothesis that sphingomyelin synthesis may occur in recycling endosomes represents the first suggestion that endosomes have any lipid biosynthetic capacity at all, and clearly differs from previous ideas that sphingomyelin synthesis occurs in the cis/medial-Golgi [7–10] and that sphingomyelin reaches the surface by a process involving vesicular transport through the Golgi cisternae. This hypothesis explains why monensin and BFA block the appearance of newly-synthesised sphingomyelin at the surface (Fig. 1) [10,14] whereas they have no effect on sphingomyelin resynthesis and return to the surface after treatment of cells with exogenous sphingomyelinase (Fig. 2). It also explains why monensin causes a build-up of ceramide (presumably in the medial Golgi) which can partly be converted into glucosylceramide [10], whereas most of the ceramide introduced into the recycling pathway through the action of external sphingomyelinase does not reach the Golgi and is consequently not converted into glycolipids (Fig. 6).

Although some products of the exocytic pathway can enter endosomes [41], the bulk of exocytic flow probably passes directly to the plasma membrane. Rather than invoking another site of sphingomyelin synthesis between the TGN and the plasma membrane, we conclude that most of the ceramide in the exocytic pathway is normally transported all the way to the plasma membrane and is only converted into sphingomyelin when it enters the endosomal pathway, where the sphingomyelin synthesis site appears to be localised (Fig. 5). Thus the characteristic orientation of sphingomyelin on the external leaflet of the plasma membrane would be defined by its synthesis in the lumen of endocytic vesicles involved in membrane recycling. Such a tortuous route for surface sphingomyelin synthesis would be consistent with the relatively slow rate at which newly-synthesised sphingomyelin appears at the plasma membrane [8].

Does cholesterol reach the cell surface by cotransport with sphingomyelin in endosomes?

Finally, we would like to suggest the possibility that cholesterol transport to the cell surface could take place utilising the same recycling endosomal vesicles which carry newly-synthesised sphingomyelin (Fig. 5). Such a model would explain why cholesterol transport to the cell surface is not affected by monensin [42] or BFA [43] (and thus does not involve the Golgi apparatus) but is blocked by energy depletion [42] (and thus depends on vesicular transport, probably through the endosomal pathway). Our explanation for this apparent enigma of cholesterol transport does not need to assume a vesicular bypass to the secretory pathway as proposed recently by van Meer [44]. It would also explain why purified trans-Golgi membrane vesicles [45] or exocytic vesicles [46] are not enriched in cholesterol or in sphingomyelin, as would be expected if these lipids were added in the early Golgi. The model would fit in with the evidence that sphingomyelin and cholesterol interact specifically [47] and that the synthesis and delivery to the cell surface of sphingomyelin and cholesterol are intimately connected [23]. Thus the rate of delivery of cholesterol to the plasma membrane would be determined by the rate of synthesis of sphingomyelin in the recycling pathway. The putative endosomal vesicles which carry cholesterol and sphingomyelin could correspond to the vesicles rich in newly-synthesised cholesterol which have been described by other workers [42,48]. In this case there would have to be a direct route for cholesterol transport from its site of synthesis in the endoplasmic reticulum to the recycling endosomal vesicles as shown in Fig. 5.

This proposed mechanism for the cotransport of sphingomyelin and cholesterol to the cell surface

through the endosomal pathway (which is described in more detail in a recent review [49]) has the advantage of making it easier to understand how the characteristic lipid composition of the plasma membrane is conserved, since these major plasma membrane lipid constituents are added in a closed vesicular loop which is largely independent of the secretory pathway.

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