Utilization of phosphatidylcholine and production of diradylglycerol as a consequence of sphingomyelin synthesis

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1. After the degradation of cell-surface sphingomyelin (SM) by exogenous sphingomyelinase (SMase), the resynthesis of SM by baby-hamster kidney (BHK) and human leukaemia-60 (HL-60) cells was examined in relation to utilization of substrate phosphatidylcholine (PtdCho) and generation of the expected product, diradylglycerol (DRG). Using [³H]choline-labelled BHK cells incubated in non-radioactive medium, SMase caused a release of phosphocholine, which was derived approximately equally from SM and PtdCho, consistent with the anticipated resynthesis of SM at the expense of PtdCho. However, with choline-labelled cells incubated in radioactive medium or [¹⁴C]acetate-labelled cells treated with SMase, no loss of radioactivity from PtdCho or accumulation of labelled DRG was observed, suggesting that any DRG produced as a consequence of SM synthesis must have been rapidly converted back into PtdCho. In contrast, SMase treatment of HL-60 cells caused more than a doubling of DRG levels at the expense of PtdCho,

and this appears to be the first demonstration of a rise in DRG related to the synthesis of SM. The DRG produced consisted of about 80 % 1,2-diacylglycerol and 18 % 1-*O*-alkyl-2-acylglycerol species, a similar composition to that of the DRG backbone of total cell PtdCho. 2. The requirement for cell-surface PtdCho in the biosynthesis of SM by BHK cells was also investigated. Treatment of [³H]choline-labelled BHK cells with *Bacillus cereus* PtdCho-specific phospholipase C (PLC) rapidly degraded about 6 % of the total PtdCho, which was assumed to represent the cell-surface pool. This did not appear to be the pool of PtdCho required for SM synthesis, since (a) the released phosphocholine was additional to that derived from PtdCho in cells treated with SMase and (b) treatment with PLC did not affect SM synthesis, either *de novo* or in response to degradation of cell-surface SM by SMase. These findings suggest either that there is no SM synthase in the plasma membrane or, if it is present, then it does not utilize cell-surface PtdCho as a substrate.

INTRODUCTION

Unlike the other major phospholipids, sphingomyelin (SM) is not synthesised *de novo* in the endoplasmic reticulum (ER), but in a distal membrane compartment utilizing the ceramide:phosphatidylcholine phosphocholine transferase (SM synthase), which transfers the phosphocholine group from preformed phosphatidylcholine (PtdCho) to ceramide, leaving a residual diradylglycerol (DRG) (Scheme 1). This reaction is of special interest because it interrelates DRG and ceramide, the central metabolites of glycerolipid and sphingolipid biosynthesis respectively. Indeed, it potentially represents a way in which DRG

and ceramide concentrations can be co-operatively modulated, a possibility of particular significance in view of the known or proposed action of these molecules as regulators of signalling processes in cells [1–6]. Recent work also implies a role for ceramide in the control of endocytosis and exocytosis [7,8] and for DAG derived from sphingolipid synthase activity in the regulation of vesicle budding from exocytic compartments in yeast [9]. However, attempts to measure the anticipated generation of diacylglycerol (DAG) as a consequence of enhanced SM synthesis have previously been unsuccessful [10–12]. The present work, using HL-60 cells, seems to represent the first case where at least some of this ‘missing’ DAG has been found.

The possibility that the concentrations of ceramide and DAG (or, more generally, DRG) in mammalian membranes could be reciprocally influenced as a consequence of SM synthase activity lends great significance to the question of the precise subcellular localization of this enzyme. Early attempts to establish the subcellular site of SM synthase concluded that it was concentrated in the plasma membrane [13–15], but a number of reports in the early 1990s strongly suggested that the major site was in the early/mid-Golgi apparatus [16,17]. We have proposed that the main site of plasma-membrane SM biosynthesis is in an endocytic compartment or the *trans*-Golgi network [18–20], although some new experimental evidence conflicts with this hypothesis [21], and attention has returned to the idea of a plasma-membrane localization for at least part of the total activity [21–23]. Experiments described here provide evidence against the idea that SM synthase activity is present at the cell surface.

Scheme 1 Metabolic connections between the synthesis and degradation of SM and PtdCho

Abbreviations used: SM, sphingomyelin; SMase, *Bacillus cereus* sphingomyelinase C; BHK, baby-hamster kidney; PtdCho, phosphatidylcholine; DRG, diradylglycerol; DAG, diacylglycerol; AAG, 1-*O*-alkyl-2-acylglycerol; PtdCho-PLC, *B. cereus* phosphatidylcholine-specific phospholipase C; PtdIns-PLC, *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C; ER, endoplasmic reticulum; HL-60, human leukaemia-60; GMEM, Glasgow modification of Minimal Essential Medium.

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MATERIALS AND METHODS

Materials

[2-¹⁴C]Sodium acetate and [methyl-³H]choline chloride were from Amersham Pharmacia Biotech, Rainham, Essex, U.K. *Bacillus cereus* PtdCho-specific phospholipase C, *B. cereus* SMase C and lipid standards were from Sigma Chemical Co., Poole, Dorset, U.K. *Bacillus thuringiensis* PtdIns-specific phospholipase C was obtained from ICN, Thame, Oxfordshire, U.K. Tissue-culture media and supplements were obtained from Gibco, Paisley, Renfrewshire, Scotland, U.K., Silica Kiesegel 60 TLC plates were obtained from Merck, Darmstadt, Germany. 1-*O*-alkyl-acylglycerol (AAG) was made by phospholipase C (PLC) treatment of 1-*O*-alkylphosphatidylcholine. 4-Dimethylaminopyridine and benzoic anhydride were obtained from Sigma-Aldrich, Poole Dorset, U.K.

Tissue culture

Baby-hamster kidney (BHK) fibroblasts were grown to confluence (about 10⁶ cells/dish) on 3.5 cm-diameter plastic Petri dishes in Minimal Essential Medium (Glasgow modification, GMEM) supplemented with 5 % tryptose/phosphate broth and 5 % fetal-calf serum. HL-60 (human leukaemia-60) cells were grown in suspension (up to 10⁶ cells/ml) in RPMI-1640 medium containing 10 % heat-inactivated fetal-calf serum.

Treatment of cells with phospholipases and lipid analogues

Both BHK and HL-60 cells were grown in the presence of radiolabel (1 μ Ci/ml for choline and acetate) for 48 h. The cells were then washed twice with non-radioactive RPMI/Hepes, pH 7.4 (GMEM/Hepes for BHK cells) and incubated at 37 °C in the presence or absence of sphingomyelinase, with or without addition of PtdCho-specific phospholipase C under conditions specified in the Figure legends. The extracellular medium was analysed for choline and phosphocholine using the paper-chromatographic system described previously [11]. In some experiments with [³H]choline-labelled BHK cells, cells were re-incubated with GMEM containing radioactive choline at the same specific radioactivity as was used for the initial labelling protocol.

Lipid analysis

After incubation of BHK cells the medium was carefully removed for analysis and the lipids were extracted from the cells by the addition of 1.9 ml of methanol/chloroform (2:1, v/v) to the dishes. In the case of HL-60 cells, chloroform/methanol (2:1, v/v) was directly added to the cell suspension to give a single phase. Following transfer to glass test tubes the phases were split according to the procedure of Bligh and Dyer [24]. The chloroform phase was dried using a water pump and neutral lipids were separated in the solvent system benzene/diethyl ether/ethanol/acetic acid (500:400:20:1, by vol.). Spots were imaged for 2 h on ¹⁴C-sensitive screens and quantified on a Fuji phosphorImager. After this analysis of the neutral lipids the phospholipids were then separated by running the same plate in chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) and repeating the phosphorImaging analysis for the phospholipid spots.

DRG analysis

Separation and analysis of alkyl and acyl species of acylglycerols was achieved by a modification of the method used by Warne and Robinson [25]. The ¹⁴C-labelled lipids were extracted and the chloroform phase was dried as described above. Neutral lipids

were then separated from phospholipids on small silica columns by elution with 10 ml of diethyl ether. Phospholipids were subsequently eluted with 10 ml of methanol. The neutral lipid extract was dried in a stream of N₂ and allowed to react with 5 mg of benzoic anhydride and 2 mg of 4-dimethylaminopyridine in a total volume of 120 μ l of benzene. After 3 h the reaction was terminated by the addition of 200 μ l of ammonium hydroxide. Acetonitrile (2.5 ml) and water (0.5 ml) were added and the lipids were extracted into 2 $\times$ 3 ml of hexane. The hexane was evaporated in a stream of N₂, and the residue was dissolved in 50 μ l of hexane, spotted on to TLC plates and developed in benzene/hexane/diethyl ether (50:45:4, by vol.) [26]. Radioactive spots were quantified using a Fuji phosphorImager and subsequently identified by iodine staining of appropriate standards run on the same plates.

RESULTS AND DISCUSSION

Breakdown of PtdCho as a consequence of SMase action on BHK cells

BHK cells labelled to equilibrium with [³H]choline and then incubated in non-radioactive medium slowly released radioactivity into the medium (Figure 1a) at the expense of PtdCho (Figure 1b). Chromatographic analysis of the medium confirmed that essentially all of this radioactivity was choline, probably released as a result of endogenous phospholipase D activity [27]. The observed rate of choline release corresponded to about 2 % of total radioactivity/h (Figure 1a). Treatment with SMase did not affect choline release (results not shown), but did cause an additional release of phosphocholine, largely of course at the expense of SM (Figures 1a and 1c). However, particularly at longer time points, it was clear that the amount of phosphocholine which was released was too large to be explained entirely in terms of SM breakdown and that the additional phosphocholine must have been derived from another source (Figure 1e). This source was not the intracellular pool of water-soluble choline metabolites (95 % of which was phosphocholine), since this scarcely changed in radioactivity (Figure 1d), but appeared to be PtdCho, which in SMase-treated cells lost radioactivity at the rate of about 3 %/h in excess of the control value (Figure 1b), i.e. approximately the same rate as the increase in the release of phosphocholine in excess of that derived from SM (Figure 1e). The trivial interpretation of these results is that PtdCho is degraded as a result of PLC activity that contaminates the SMase. However, no degradation of PtdCho was seen in lipid extracts or Triton-solubilized cells treated with SMase (results not shown), and SMase pretreatment did not affect the amount of cell-surface PtdCho which could be degraded by a PtdCho-specific PLC (Figures 1a and 1b). It was therefore concluded that the loss of PtdCho seen in response to SMase was due to its utilization as a substrate for SM resynthesis from the ceramide initially formed. This interpretation was supported by a measurement of the rate of PtdCho breakdown (3 % of total radioactivity/h) in the continued presence of SMase (Figure 1b) which was equivalent to resynthesis of about one-third of total SM/h and was similar to previous values for the maximum rate of resynthesis of SM following its degradation at the cell surface and subsequent removal of the SMase [10,11].

PLC treatment degrades essentially all of the cell-surface PtdCho

Exposure of cells to PtdCho-PLC caused a release of phosphocholine (Figure 1a) and loss of radioactivity from PtdCho (Figure 1b) that was equivalent to about 6 % of total PtdCho radioactivity after 20 min and 10 % after 2 h, but with little significant effect

on SM (Figure 1c). It was assumed that the initial fast phase of degradation corresponded to PtdCho on the cell surface and that the slower breakdown probably reflects the movement of PtdCho to the surface from other pools, e.g. recycling from endosomes or transbilayer migration. Previous calculations [28] based on the results of phospholipase A treatment of viruses and plasma-membrane vesicles derived from BHK cells [29,30] suggest that about 6% of the total BHK-cell PtdCho is on the outer leaflet of the plasma membrane, and this value is also similar to estimates (5%) obtained by an entirely independent method using phospholipid-exchange proteins [31]. Thus treatment of intact cells with PLC appears to degrade virtually all of the surface PtdCho.

Prior treatment with SMase did not affect the amount of PtdCho that could subsequently be broken down by PLC (6% of total radioactivity after 30 min and about 9% after 2 h) (Figures 1a and 1b) and the amounts of phosphocholine which were released by both enzymes were additive when they were present together. Thus these results indicate that the pool of PtdCho which is the immediate donor of phosphocholine to ceramide in SM resynthesis is distinct and separate from the cell-surface pool of PtdCho.

Resynthesis of cell-surface SM by BHK cells is not inhibited by PtdCho-PLC

Following treatment of [14 C] acetate-labelled BHK cells with SMase for 20 min, SM levels decreased from 5.8% of total lipid radioactivity to 3.7% (Table 1). After washing out the SMase and further incubating for 3 h in serum-free medium, SM levels rose to 5.9%. This rate of SM resynthesis was similar to that reported previously in this cell type [10,11] and was unaffected by the presence throughout the incubation of PtdCho-PLC, which completely degraded cell-surface PtdCho (Table 1). This confirms the conclusion from the experiment of Figure 2 that cell-surface PtdCho is not the pool of PtdCho which is required for the resynthesis of cell-surface SM.

De novo synthesis of SM in BHK cells is unaffected by depletion of cell-surface PtdCho

Incubation with PtdCho-PLC has previously been shown to inhibit SM synthesis in fibroblast membranes [32], so the effect of this enzyme on *de novo* SM synthesis in intact cells was measured. Figure 2 shows that, for periods up to 4 h in the presence of PLC, where cell-surface PtdCho was likely to be severely depleted, the *de novo* synthesis of SM and PtdCho was unaltered, again suggesting that cell-surface PtdCho is not involved in synthesis of

Figure 1 Changes in distribution of radioactivity between culture medium and phospholipids of [3 H]choline-labelled BHK cells treated with SMase and/or PtdCho-PLC at 37 °C in the presence of non-radioactive medium

BHK cells in 3.5 cm-diameter dishes were grown for 48 h in 2 ml of medium containing 1 μ Ci/ml [3 H]choline and then were washed twice with non-radioactive medium before incubation at 37 °C with 1 ml of serum-free GMEM. SMase (0.1 unit/ml) was added to half of the dishes and at various times up to 3 h the supernatant was removed and lipids were extracted and analysed as described above. Samples of the upper phase from the phase partition (corresponding to the intracellular aqueous compartment) were retained and analysed. After 1 h of incubation (arrowed), samples in the presence (closed symbols) or absence (open symbols) of SMase were additionally exposed to PtdCho-PLC (0.1 unit/ml) (squares) for the remaining 2 h of incubation. Values represent the percentage of total radioactivity in each fraction. (a) Extracellular medium; (b) PtdCho; (c) SM; (d) intracellular aqueous phase; (e) extracellular phosphocholine derived from SM breakdown and from breakdown of PtdCho (calculated from a and c). In (e), closed squares represent the loss of label from SM, open squares represent the total phosphocholine in the medium and triangles refer to the calculated difference between these values, which is the amount of phosphocholine assumed to derive from PtdCho. The experiment shown was repeated with the same result on two other occasions.

Table 1 Changes in lipid distribution in BHK cells following treatment with exogenous SMase and/or PtdCho-PLC: resynthesis of SM does not require cell-surface PtdCho

BHK cells were grown on 3.5 cm-diameter dishes in the presence of 1 μ Ci/ml of [14 C]acetate for 48 h before washing twice in non-radioactive medium and reincubating in serum-free GMEM at 37 °C. Samples were then incubated with 0.1 unit/ml of *B. cereus* SMase for 20 min and some of these samples were extracted with chloroform/methanol. The remaining samples were washed three times with 4 ml of cold GMEM + serum and then the cells were incubated in 1 ml of GMEM for a further 3 h in the presence or absence of 0.1 unit/ml of *B. cereus* PtdCho-PLC. Results are expressed as a percentage of total lipid radioactivity and represent the means $\pm$ S.D. of triplicate determinations in one experiment that was repeated with essentially the same results on three other occasions.

Incubation conditions	Percentage of total lipid radioactivity			
	Ceramide	DRG	SM	PtdCho
Medium alone (3 h 20 min)	0.14 $\pm$ 0.03	0.3 $\pm$ 0.1	5.8 $\pm$ 0.5	48.1 $\pm$ 0.9
+ SMase (20 min)	2.21 $\pm$ 0.07	0.4 $\pm$ 0.1	3.7 $\pm$ 0.3	47.3 $\pm$ 0.7
+ SMase (3 h)	2.43 $\pm$ 0.06	0.3 $\pm$ 0.1	3.5 $\pm$ 0.3	48.0 $\pm$ 0.5
+ SMase (20 min) then medium alone (3 h)	0.25 $\pm$ 0.02	0.3 $\pm$ 0.1	5.9 $\pm$ 0.5	47.2 $\pm$ 1.1
+ SMase (20 min) then + PLC (3 h)	0.21 $\pm$ 0.05	6.4 $\pm$ 1.0	6.1 $\pm$ 0.8	41.1 $\pm$ 0.8
Medium (20 min) then + PLC (3 h)	0.18 $\pm$ 0.05	7.3 $\pm$ 0.9	6.0 $\pm$ 0.3	40.4 $\pm$ 0.7

Figure 2 Incorporation of [14 C]acetate into SM and PtdCho in the presence of PtdCho-PLC

Triplicate samples of BHK cells in 3.5 cm-diameter dishes were incubated at 37 °C with 1 μ Ci/ml of [14 C]acetate in the absence (open symbols) or presence (closed symbols) of PtdCho-PLC (0.1 unit/ml) for the indicated times. The supernatant was removed and phospholipids were extracted and analysed as described in the Materials and methods section. Results are expressed as means $\pm$ S.D. for one experiment that was repeated on two further occasions with substantially the same results. Circles, SM; squares, PtdCho.

SM. This experiment indicates that *de novo* synthesis of SM takes place at an intracellular site which could be either in the Golgi apparatus, as has been suggested previously [16,17], or in an endocytic compartment [18–20].

DRG levels are not increased by SMase treatment of BHK cells

Treatment of [14 C]acetate-labelled BHK cells with PtdCho-PLC for 3 h caused an increase in DRG and a decrease in PtdCho which in each case was equivalent to about 7% of total radioactivity or about 14% of the label remaining in PtdCho (Table 1). This is comparable with the amount of PtdCho that is degraded in [3 H]choline-labelled cells during a 2 h incubation with PtdCho-PLC (9% of total radioactivity or 12% of the remaining PtdCho; Figures 1a and 1b, and Table 2) and suggests that, under the conditions of these experiments, there was no significant net resynthesis of PtdCho in the presence of PtdCho-PLC. However, either in the presence of SMase for 3 h or during resynthesis of SM in the absence of SMase, when the synthesis of SM corresponding to 2% of total phospholipid would have been

Table 2 Changes in distribution of radioactivity between culture medium, cell aqueous fraction and phospholipids of [3 H]choline-labelled BHK cells treated with SMase or PtdCho-PLC in the continued presence of radioactive medium

BHK cells were labelled as described in the legend to Figure 1 and were then reincubated for 1 h in serum-free GMEM containing the same specific radioactivity of [3 H]choline as used originally. Lipids were extracted from triplicate samples at this time (zero time). Further triplicate samples were incubated for 3 h at 37 °C, either with no further addition or in the presence of 0.1 unit/ml SMase or 0.1 unit/ml PLC before extraction and analysis as described in Figure 1. Values represent the percentage of radioactivity appearing in each fraction (mean $\pm$ S.D.) in a single experiment that was repeated on one further occasion with a different batch of cells but with almost identical results. *Significantly different from corresponding 3 h control, $P < 0.005$.

Time (h)	Percentage of radioactivity appearing in each fraction			
	SM	PtdCho	Cell aqueous	Medium
Zero	11.2 $\pm$ 0.3	48.0 $\pm$ 0.2	25.7 $\pm$ 0.3	15.2 $\pm$ 0.2
3 h control	11.7 $\pm$ 0.2	49.1 $\pm$ 0.6	26.8 $\pm$ 0.7	12.4 $\pm$ 1.1
3 h SMase	4.3 $\pm$ 0.5*	50.3 $\pm$ 1.6	25.5 $\pm$ 0.7	19.9 $\pm$ 2.2*
3 h PLC	11.4 $\pm$ 0.4	43.8 $\pm$ 0.9*	25.8 $\pm$ 0.8	19.1 $\pm$ 1.3*

expected to give rise to an equivalent amount of DRG derived from PtdCho [12], there was no significant change in DRG or PtdCho levels (Table 1). Thus any DRG which was produced as a consequence of SM synthesis must have been largely converted back into PtdCho. It seems, therefore, that BHK cells have the ability to convert back into PtdCho all the DRG assumed to arise from SM synthesis, but none of the DRG derived from breakdown of cell-surface PtdCho. This difference is probably because conversion of the DRG back into PtdCho can keep up with the relatively low rate of DRG production in SM synthesis (about 1–2% of total lipid radioactivity/h), but not with the maximum of about 1%/min for production of DRG by PLC (Figure 1b). However, it is not obvious why [14 C]acetate-labelled PtdCho is resynthesized whereas choline-labelled PtdCho is not apparently resynthesized under the same conditions (Figure 1b). This discrepancy is explained by noting that, when choline-labelled cells are incubated in non-radioactive medium (Figure 1), there is an efficient chase of label from phosphocholine, and any resynthesis of PtdCho from unlabelled precursors would not be measured. When cells were incubated instead in medium still containing radioactive choline during the treatment with SMase (Table 2), there was no significant decrease in the radioactivity of

Figure 3 DRG generation in response to SM breakdown in HL-60 cells

HL-60 cells were grown to a final density of 10^6 cells/ml in the presence of $1 \mu\text{Ci/ml}$ of [^{14}C]acetate for 36 h. After washing with RPMI/Hepes, cells were incubated in 0.4 ml of RPMI/Hepes, either alone (open symbols) or in the presence (closed symbols) of SMase (0.1 unit/ml). Lipids were then extracted and analysed as described in the Materials and methods section. Data are expressed as a percentage of total lipid radioactivity in each lipid class and represent the means of triplicate determinations in one experiment that was repeated twice with essentially the same results. Error bars have been omitted for the sake of clarity. Squares, ceramide; circles, SM; triangles, DRG.

PtdCho when SM was degraded, showing that resynthesis of PtdCho must have been occurring, i.e. the results were the same as with acetate labelling. In contrast, treatment with PLC caused an approx. 12% decrease in PtdCho as observed in Figure 1, confirming the absence of significant resynthesis in the presence of PLC. As with SMase treatment, the loss of radioactivity from phospholipid was reflected in an equivalent rise in the radioactivity of the medium (Table 2).

DRG levels are increased by SMase treatment of HL-60 cells

Initially HL-60 cells were found to resynthesize cell-surface SM after degradation by an exogenous SMase at a similar rate to BHK cells. Thus brief treatment of HL-60 cells with 0.1 unit of SMase decreased SM levels from 4.6% of total lipid to 2.2% and after washing out the SMase, followed by further incubation for 1 h in the presence of serum, SM levels increased to 3.2% of total lipid (results not shown). This is equivalent to a rate of resynthesis of 40% of the degraded SM/h, which is similar to that reported before for BHK cells [10,11]. However, in contrast with BHK cells, HL-60 cells did accumulate DRG as a consequence of SMase treatment. Figure 3 shows that SMase treatment of HL-60 cells for up to 2 h led to an increase in DRG to more than double the level in untreated cells. This represents an accumulation of extra DRG which is equivalent to approx. 1% of total lipid after 2 h treatment with SMase. If the cells are able to resynthesize 40% of the degraded SM in 1 h (i.e. the equivalent of about 2% of their total lipid as SM in 2 h; see above), then the accumulating DRG corresponds to about 60% of the maximum amount of SM which is synthesized. Thus it appears that the DRG which accumulates as a consequence of SMase treatment represents more than half of the DRG assumed to be produced during the resynthesis of SM. It seems unlikely that this DRG was generated by contaminating PtdCho-PLC in the SMase preparation, since no evidence for this activity was seen with intact BHK cells or lipid extracts treated with the SMase (see above). Neither were the cells adversely affected by treatment

Table 3 Comparison of the DRG composition of PtdCho of cells and cell surface with that produced as a result of SMase action

HL-60 cells were labelled with [^{14}C]acetate for 36 h and incubated in RPMI/Hepes as in the legend to Figure 3. Cells were incubated for 60 min with either PtdCho-PLC, SMase or PtdIns-PLC (each 0.1 unit/ml). Lipids were extracted and separated as described in the Materials and methods section. For the determination of DRG composition of individual lipids, the lipid extracts were digested with either PtdCho-PLC or PtdIns-PLC as appropriate in the presence of 0.01% Triton X-100. Neutral lipids were separated and benzoylated as described in the Materials and methods section. Alkyl and acyl DRG were then separated and quantified by phosphorimaging. Data are expressed as the % of total lipid radioactivity in DAG and AAG. From these values, the percentage of total DRG represented by AAG was calculated. Results represent the means $\pm$ S.D. from three separate experiments carried out in triplicate.

Incubation	DAG (% of total lipid)	AAG (% total lipid)	AAG (%)
Control	0.6 ± 0.1	0.10 ± 0.01	14 ± 2
PtdCho-PLC (intact cells)	4.7 ± 0.3	1.06 ± 0.06	18 ± 3
PtdCho-PLC (total lipid extract)	41.2 ± 2.1	9.0 ± 0.7	18 ± 1
SMase (intact cells)	1.09 ± 0.05	0.22 ± 0.02	17 ± 4
PtdIns-PLC (intact cells)	1.10 ± 0.05	< 0.02	< 2
PtdIns-PLC (total lipid extract)	5.8 ± 0.4	< 0.02	< 0.5

with SMase, since no decrease in cell viability (uptake of Trypan Blue) was observed and, after removal of the enzyme, the cells were capable of resynthesizing SM (see above).

Another way in which DRG levels could potentially be increased is if ceramide produced by SMase could activate either an endogenous PLC or a phospholipase D in conjunction with a phosphohydrolase which could convert phosphatidic acid into DRG. There is no precedent for such a PLC, and ceramide has generally been found to exert a negative effect on phospholipase D activity, although there is a report of phospholipase D activation by ceramide in fibroblasts [33]. However, we found no evidence for an accumulation of phosphatidic acid or of phosphatidylethanol in cells incubated with SMase in the presence of ethanol (results not shown). Furthermore, synthesis of DRG had a time course similar to that of SM resynthesis and there was no significant increase in DRG at 20 min, despite the large rise in ceramide at this time (Figure 3).

Alkylacyl composition of DRG produced as a consequence of SM synthesis

Previous work using Krebs II ascites cells indicated that there was much more alkylacylglycerophosphocholine in the inner leaflet of the plasma membrane than in the outer leaflet [34]. This raised the possibility of identifying the pool of PtdCho that is utilized in the formation of SM in HL-60 cells on the basis of the differential alkylacylglycerol content of the DRG generated by SM synthesis. Table 3 shows that the DRG produced as a consequence of SM degradation and resynthesis contains about 17% alkylacyl DRG, the remainder being DAG. This composition was the same as those for basal DRG and for DRG derived from the total PtdCho of HL-60 cells, but slightly different from that found in Krebs II ascites cells, which contained 23% alkylacyl DRG [34]. Analysis of cell-surface PtdCho in HL-60 cells by treatment of intact cells with *B. cereus* PtdCho-PLC gave DRG which was 18% alkylacyl and 82% DAG (Table 3). Thus, in contrast with the results with Krebs II ascites cells, HL-60 cells showed no significant difference between the alkylacyl composition of the cell-surface pool and total PtdCho. The DRG produced as a result of SM synthesis had a similar alkylacyl composition, so we were unable to derive any information about

the subcellular localization of SM synthesis from these experiments. However, the DRG composition of PtdCho contrasted sharply with both total and cell-surface PtdIns, which contained no detectable alkylacylglycerol in its backbone. Table 2 also shows that, as described previously in lymphocytes and several other cell types [35], PtdIns-PLC caused the hydrolysis of cell-surface PtdIns in HL-60 cells and the consequent generation of DRG equivalent to 0.5% total lipid or about 10% of total PtdIns. Again, as with PtdCho-PLC treatment of intact cells (see above), the amount of breakdown induced by PtdIns-PLC measured with radioactive acetate is similar to that found using the headgroup radiotracer [35], suggesting that DRG produced by phospholipase treatment of intact cells is not rapidly converted back into the parent phospholipid. This contrasts with the resynthesis of SM in BHK cells (Figure 2) and agonist-stimulated PtdCho and PtdIns(4,5) P_2 breakdown [35], where resynthesis of PtdCho or PtdIns respectively is prompt. These observations may suggest that DRG generated on the outer leaflet of the cell membrane is converted back into a phospholipid more slowly than an internal pool of DRG.

Conclusion

Pagano [12] proposed a model whereby the putative DAG produced as a consequence of SM synthesis was converted back to PtdCho, either in the endoplasmic reticulum or locally at the site of SM synthesis. This appears to be valid for BHK cells, since SMase treatment produced no increment in DRG or decrement in PtdCho in cells labelled to equilibrium with [14 C]acetate (Table 1) and caused an increase in choline incorporation into PtdCho and SM [11]. In contrast, HL-60 cells appear to be unable to completely resynthesize the PtdCho, which is utilized to sustain SM synthesis. This seems to be the first example to be described where DRG accumulates as a consequence of SM synthesis, although we do not know why HL-60 cells differ in this respect from BHK fibroblasts and other cell types [12].

Our second major finding is that cell-surface PtdCho does not seem to be necessary for resynthesis of SM degraded at the cell surface or, indeed, for any component of the *de novo* synthesis of SM (Table 1; Figure 2). This is surprising in view of previous reports [16,21] that at least some SM biosynthesis takes place at the plasma membrane, where the obvious donor of phosphocholine to ceramide would be outer-leaflet PtdCho. Thus recent work has demonstrated the presence of SM synthase activity in the basolateral (but not apical) surface of Madin–Darby canine kidney ('MDCK') cells [23] and in the surface membrane of BHK cells, where it accounted for about 10% of the total SM synthesis [21]. These activities were measured in terms of conversion of extracellular short-chain ceramide into SM at 10 °C (when vesicular transport is blocked), and it is argued by these authors that this must represent cell-surface synthesis of SM at the expense of cell-surface PtdCho.

Our present results are difficult to reconcile with the above findings unless cell-surface SM synthesis utilizes PtdCho in the inner leaflet (which seems unlikely), but they are consistent with our previous conclusion that at least one component of SM biosynthesis occurs either in an endosomal compartment or in the *trans*-Golgi network. However, the experiments of van Helvoort et al. [23] showed, in HepG2 and BHK cells, that, when the density of endosomes was increased as a result of oxidation

of diaminobenzidine by endocytosed horseradish peroxidase, there was no change in the equilibrium density of SM synthase, which was therefore unlikely to be present in the endosomes. Less compelling evidence was provided that suggested that SM synthase was not present in the *trans*-Golgi network either. Thus there is still some disagreement regarding the localization (or even the existence) of SM synthase outside the Golgi cisternae.

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