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Resynthesis of sphingomyelin from plasma-membrane phosphatidylcholine in BHK cells treated with *Staphylococcus aureus* sphingomyelinase

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About 60–65% of the total sphingomyelin in intact BHK cells is in a readily accessible pool which is rapidly degraded by *Staphylococcus aureus* sphingomyelinase. No more sphingomyelin is broken down in cells which have been fixed with glutaraldehyde or lysed with streptolysin O, suggesting that all the sphingomyelin which is available to the enzyme is on the cell surface. The inaccessible pool of sphingomyelin does not equilibrate with the plasma-membrane pool, even after prolonged incubation. Experiments using [^{3}H]-choline show that much more phosphocholine is released from the intact cells treated with sphingomyelinase than can be accounted for by breakdown of the original cell-surface pool of sphingomyelin; the excess appears to be a consequence of the breakdown of sphingomyelin newly resynthesized at the expense of a pool of phosphatidylcholine which represents about 8% of total cell phosphatidylcholine and may reside in the plasma membrane. This would be consistent with resynthesis of cell-surface sphingomyelin by the phosphatidylcholine:ceramide phosphocholinetransferase pathway, which has previously been shown to be localized in the plasma membrane. However, in [^{3}H]palmitate-labelled cells there appeared to be no accumulation of the diacylglycerol expected to be produced by this reaction, and no enhanced synthesis of phosphatidate or phosphatidylinositol; instead there was an increased synthesis of triacylglycerol. A similar increase in labelling of triacylglycerol was seen in enzyme-treated cells where the sphingomyelinase was subsequently removed, allowing resynthesis of sphingomyelin which occurred at a rate of about 25% of total sphingomyelin/h. Treatment of BHK cells with sphingomyelinase caused no change in the rates of fluid-phase endocytosis or exocytosis as measured with [^{3}H]inulin.

INTRODUCTION

Among the phospholipids of mammalian cells, sphingomyelin stands out not only as the sole common sphingophospholipid but also as the most reliable marker of plasma membrane, where it seems to be localized in the outer leaflet of the lipid bilayer [1,2]. A variety of evidence suggests that synthesis of sphingomyelin by the conventional route utilizing CDP-choline:ceramide cholinephosphotransferase is not a major pathway in most cells, except perhaps when utilizing short-chain ceramides [3,4]; sphingomyelin appears to be synthesized directly from phosphatidylcholine by transfer of the phosphocholine moiety to ceramide [5–11]. Furthermore, this unusual reaction seems to be localized in the plasma membrane [12,13], although it is not known on which side of the membrane the activity resides. If it were present on the outer surface of the plasma membrane, then sphingomyelin could be synthesized *in situ* from ceramide and outer-leaflet phosphatidylcholine.

In beginning the experiments described here, we reasoned that breakdown of surface sphingomyelin in cultured cells by an exogenous bacterial sphingomyelinase should lead to resynthesis of sphingomyelin at the expense of plasma-membrane phosphatidylcholine, and that it might be possible to assess the amount of this pool of phosphatidylcholine and the rate at which it was utilized. In addition, we had some evidence that sphingomyelinase could cause endocytosis in red cells [14], and we wanted to know if this enzyme could also induce a similar response in other cell types for which we used BHK cells as a model.

MATERIALS AND METHODS

BHK cell culture and labelling with radioactive compounds

BHK21 cells were cultured in monolayers in Glasgow MEM supplemented with 10% (w/v) tryptose phosphate, 2 mM-glutamine and 5% (v/v) foetal-calf serum (all obtained from Gibco). For determinations of lipid P, cells were grown on 10 cm-diam. dishes, but for experiments using radioactive labels, 3.5 cm dishes were generally used.

[^{3}H]Choline, [^{3}H]palmitic acid and [^{32}P]P_i (carrier-free) were obtained from Amersham International. *Staphylococcus aureus* sphingomyelinase was obtained from Sigma Chemical Co. as a solution in 50% (v/v) glycerol containing 240 μg of protein/ml and with a quoted activity of 160 units/mg of protein. For some experiments the enzyme was purified [15]. Streptolysin O was obtained from Wellcome Diagnostics and reconstituted to an iso-osmotic solution at a concentration of 2 I.U./ml. Cells were labelled to equilibrium by incubation for 40 h with [^{3}H]choline (0.5 μCi in 2 ml of growth medium) or with [^{3}H]palmitate (0.25 μCi in 2 ml). They then were washed with 2 ml of serum-free medium containing 0.2% bovine serum albumin, incubated for 2 h and finally washed and

re-incubated in 1 ml of the same medium. For studies with ^{32}P, cells were labelled for 90 min with 10 μCi of [^{32}P]P_i in 1 ml of serum-free medium and were not washed further before addition of enzyme.

Measurement of fluid-phase endocytosis, exocytosis and lipid P in cells treated with sphingomyelinase

Unlabelled cells in 10 cm dishes containing 5 ml of medium were incubated with 10 μCi of [^{3}H]inulin with or without 10 μl of sphingomyelinase at 37 °C for up to 3 h. At various times the medium was removed, the cells were washed with 4 × 20 ml of ice-cold 0.9% NaCl, and lipids were extracted from the cells by addition of 3.75 ml of chloroform/methanol (1:2, v/v). In some experiments cells were released from the plates by treatment with EDTA (0.2 mg/ml) + trypsin (0.5 mg/ml) before addition of sphingomyelinase and extraction of lipids. In other experiments cells in suspension were either fixed in 1% glutaraldehyde [0.1 M-Pipes/NaOH buffer (pH 7.0)/5% sucrose for 5 min at room temperature] or permeabilized by addition of streptolysin O (0.4 I.U./ml of cell suspension) or 0.2% Triton X-100 before treatment with sphingomyelinase and lipid extraction. After standing for 1 h at −20 °C, the organic solvent solution was removed, and to this solution was added 1.25 ml of chloroform and 1.25 ml of 0.9% NaCl which had been used to wash the cell residue. On vigorous mixing and subsequent centrifugation (500 *g* for 5 min) this mixture separated into two phases, in accordance with the procedure of Bligh & Dyer [16]. A 1 ml sample of the upper phase, which contained cell-associated inulin radioactivity, was added to 8 ml of PCS scintillation fluid (Amersham International) and counted for radioactivity in a Searle liquid-scintillation counter. The chloroform phase was dried under reduced pressure, and the phospholipids were separated on silica-gel H plates [17] and analysed for P as described previously [18]. In the same set of experiments, cells that had been incubated for 3 h in order to take up the maximum amount of [^{3}H]inulin were washed with 4 × 20 ml of ice-cold serum-free medium and were then re-incubated in 10 ml of the same medium at 37 °C. Over the subsequent 3 h, 0.5 ml samples of the medium were removed at various time intervals, and their radioactivity was measured as above in order to determine the rate of efflux of inulin. This was done in the presence and absence of sphingomyelinase and of serum in the medium.

Measurement of lipid- and water-soluble radioactivity in [^{3}H]choline-labelled cells treated with sphingomyelinase

Cells labelled to equilibrium with [^{3}H]choline in 3.5 cm dishes were incubated with or without 1 μl of sphingomyelinase at 37 °C for up to 3 h. At various times the medium was removed and lipids were extracted from the cells as described above. Experiments were also carried out with different amounts of sphingomyelinase incubated with cells for 1 h. After phase partition [17] of the lipid extract, phospholipids were separated [18], extracted from the silica with 1 ml of methanol/water/acetic acid (5:3:2, by vol.) and counted for radioactivity in 8 ml of PCS. Samples of the medium and of the aqueous phase (which corresponded to the internal aqueous compartment of the cells) were also counted. In some experiments these aqueous samples were analysed by paper chromatography in the solvent ethanol/water (17:3, v/v) to separate phosphocholine from free choline. In this system the R_F of phosphocholine, glycerophosphocholine and choline was 0.24, 0.38 and 0.65 respectively. It was clear from these analyses that phosphocholine accounted for at least 95% of the water-soluble radioactivity in the internal aqueous pool and of the increment in external radioactivity produced by sphingomyelinase action.

The effect of sphingomyelinase on the rate of synthesis of phosphatidylcholine and sphingomyelin was also investigated. [^{3}H]Choline and sphingomyelinase were added simultaneously to unlabelled cells, and the distribution of radioactivity in the intracellular aqueous compartment and in the choline phospholipids was analysed at various times up to 4 h.

[^{3}H]Palmitate-labelled cells treated with sphingomyelinase were analysed as for the choline-labelled cells, except that additional samples of the lipid extracts were separated in a t.l.c. system which resolves neutral lipids [19]. Experiments were also carried out to determine the rate of resynthesis of sphingomyelin after treatment of cells with sphingomyelinase. In this case, cells labelled to equilibrium with [^{3}H]palmitate were treated with sphingomyelinase as above for 20 min, and then the sphingomyelinase was removed by washing the cells once with 5 ml of serum-free medium containing 5 mM-EDTA, which chelated with Mg^{2+} required for enzymic activity. The cells were re-incubated in fresh serum-free medium supplemented with 0.2% bovine serum albumin for various times up to 3 h, and radioactivity was measured in phospholipids and neutral lipids. In some experiments, unlabelled cells in 10 cm dishes were treated in parallel with the [^{3}H]palmitate-labelled cells so that measurements of P and specific radioactivity could be obtained for the resynthesized sphingomyelin.

RESULTS

Sphingomyelin represented about 8–9% of the total phospholipids in BHK cells (Fig. 1*a*), but addition of sphingomyelinase resulted in the loss of about 50% of total cell sphingomyelin P within 20 min, rising to 60–65% after prolonged incubation (Fig. 1*a*). Enzyme purified to give a single band by SDS/polyacrylamide-gel electrophoresis [1] yielded identical results. Inclusion of serum in the medium caused some inhibition of sphingomyelin breakdown (Fig. 1*a*), and so in subsequent experiments incubations with sphingomyelinase were carried out in medium containing 0.2% bovine serum albumin instead of serum. During incubation with sphingomyelinase for up to 3 h there were no obvious morphological changes in the cells, and viability was at least 98% as judged by exclusion of Trypan Blue.

No significant difference in sphingomyelin breakdown was seen with cells which had been released from the plates by EDTA/trypsin treatment before addition of sphingomyelinase (Table 1), and no significant changes were seen in the amounts of other phospholipids in the treated cells. Fixation of cells with glutaraldehyde or lysis with streptolysin O resulted in faster attack by the enzyme, but in neither case did the breakdown of sphingomyelin rise to more than 65% (Table 1). Treatment with glutaraldehyde prevented extraction of most of the phosphatidylethanolamine (because this aminophospholipid was cross-linked), but no change in the extractability of any other lipid was observed. Treatment of cells with enzyme in the presence of 0.2%

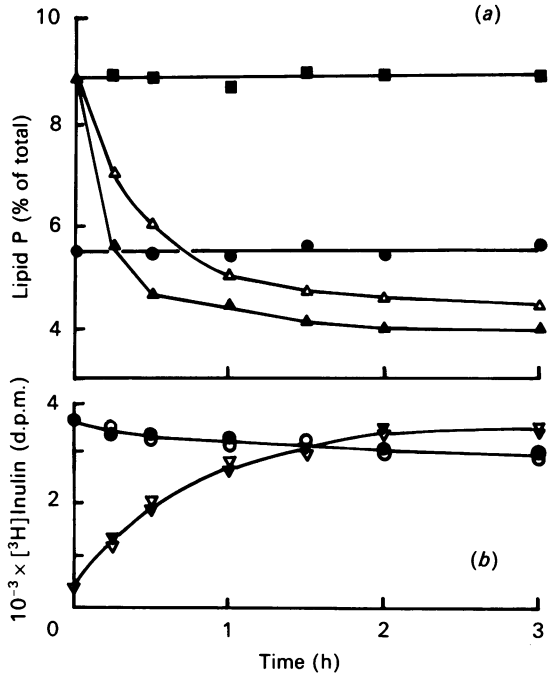

Fig. 1. Effects of sphingomyelinase on (*a*) phospholipid P and (*b*) [^{3}H]inulin uptake and efflux in BHK cells

Cells attached to 10 cm dishes were incubated with or without sphingomyelinase in the presence of [^{3}H]inulin as described in the Materials and methods section. After various times the cells were washed and extracted with chloroform/methanol. Cell-associated radioactivity was measured and phospholipid P was analysed after t.l.c. After incubation of untreated cells with [^{3}H]inulin for 3 h, efflux of the label from washed cells was measured in the presence and absence of sphingomyelinase. The experiment presented here is one of five which all gave similar results. (*a*) Sphingomyelin P in enzyme-treated cells with (△) or without (▲) serum; ■, controls; ●, phosphatidylcholine P; (*b*) ▼, ▽, uptake of [^{3}H]inulin; ●, ○, efflux of [^{3}H]-inulin. White symbols refer to samples without sphingomyelinase.

Triton X-100 caused breakdown of essentially all of the sphingomyelin (Table 1).

Addition of sphingomyelinase caused no significant alteration in endocytotic or exocytotic activity as measured by uptake and efflux of [^{3}H]inulin (Fig. 1*b*). Uptake of this marker was half-maximal at about 30 min, but subsequent efflux was only about 5%/h.

With [^{3}H]choline-labelled cells it was found that the concentration of sphingomyelinase which gave maximum breakdown of sphingomyelin was about 100 munits/ml of medium (Fig. 2*a*). When measurements were made of the water-soluble products of sphingomyelinase action, it was clear that much more radioactivity was released than could be accounted for by breakdown of the original sphingomyelin alone (Figs. 2*a* and 2*b*) and that > 95% of this radioactivity chromatographed with phosphocholine. Whereas loss of radioactivity from sphingomyelin was largely complete by 30 min, radioactivity in phosphocholine outside the cells continued to increase up to 2 h after addition of sphingomyelinase, implying that new sphingomyelin was being produced at the surface of the cells but was continually being degraded by the sphingomyelinase. After 1 h, in addition to

Table 1. Effects of glutaraldehyde, streptolysin O and Triton X-100 on the distribution of radioactivity in [^{3}H]choline-labelled BHK cells exposed to sphingomyelinase

BHK cells were labelled to equilibrium with [^{3}H]choline and either treated with sphingomyelinase while still attached to the dishes or released into suspension by addition of EDTA/trypsin before the subsequent treatments shown (see the Materials and methods section). Glutaraldehyde (1%) or streptolysin O (0.4 I.U./ml) was added before incubation with sphingomyelinase (2 μl) for 30 min. Results refer to percentage of total radioactivity in the cells (157098 ± 11234 d.p.m. in this experiment) and represent the means ± S.D. for triplicate determinations in one experiment which was repeated twice with essentially the same results. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; MED, aqueous medium outside the cells; AQU, aqueous phase inside the cells.

	Distribution of radioactivity (%)			
	SM	PC	MED	AQU
Untreated control	11.5±0.3	66±2	4±1	22±2
+sphingomyelinase	4.5±0.3	66±1	9±1	21±1
Control cells in suspension	11.4±0.4	65±1	3±1	22±1
+sphingomyelinase	4.6±0.3	64±2	8±1	23±2
+glutaraldehyde	11.2±0.4	63±3	25±1	2±1
+streptolysin O	11.8±0.5	64±2	24±1	3±1
+0.2% Triton X-100	11.1±0.4	64±2	27±2	1±1
+glutaraldehyde +sphingomyelinase	4.3±0.4	63±3	32±1	0
+streptolysin +sphingomyelinase	4.4±0.5	64±3	31±1	1±1
+0.2% Triton X-100 +sphingomyelinase	0.3±0.1	64±2	36±1	0

phosphocholine derived from the original sphingomyelin, the medium contained extra phosphocholine equivalent to about 5% of total radioactivity or about 8% of phosphatidylcholine radioactivity (Fig. 2*b*). Furthermore, there was an approximately equivalent loss of label from phosphatidylcholine, which was particularly evident at longer incubation times (Fig. 2*b*; Table 2), suggesting that a fraction of this phospholipid was being drawn upon to replace sphingomyelin disappearing from the plasma membrane. After incubation for 2 h with sphingomyelinase, about 10% of the total phosphatidylcholine radioactivity had disappeared, and the increase in radioactivity in the external medium approximately equalled the loss of radioactivity from sphingomyelin and phosphatidylcholine combined (Table 2). Radioactivity in the water-soluble pool inside the cells, which also co-chromatographed with phosphocholine, showed little change over 2 h.

If phosphatidylcholine was being utilized to replace phosphocholine removed from sphingomyelin at the cell surface, it followed that breakdown of sphingomyelin should lead indirectly to an equivalent increase in diacylglycerol derived from phosphatidylcholine. Experiments were therefore carried out with cells labelled to equilibrium with [^{3}H]palmitate in order to measure these predicted changes. Radioactivity in sphingomyelin accounted for about 11–12% of total incorporation (Table 1) and, on treatment with sphingomyelinase, about 60% of the radioactivity disappeared from sphingomyelin, with an equivalent rise in ceramide

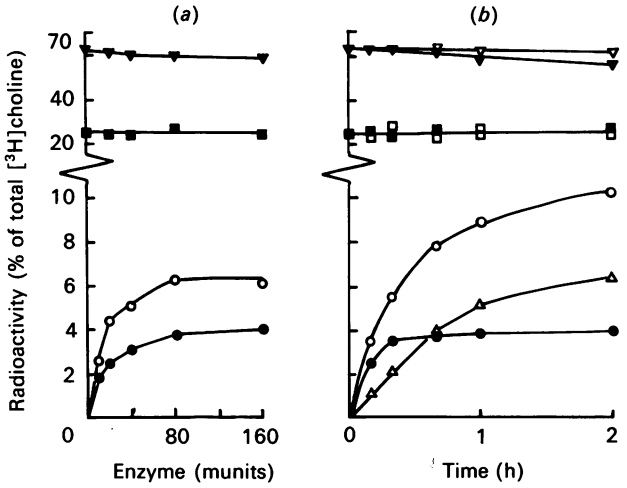

Fig. 2. Changes in the distribution of radioactivity in [^{3}H]choline-labelled BHK cells treated with sphingomyelinase

Cells labelled to equilibrium with [^{3}H]choline were treated with (*a*) various amounts of sphingomyelinase for 40 min or (*b*) 2 μl of sphingomyelinase for various times up to 2 h. Extraction and assay of radioactive phospholipids and aqueous fractions were as described in the Materials and methods section. Results illustrated are derived from one experiment of five which gave essentially the same results. (*a*) ●, Breakdown of sphingomyelin; ○, phosphocholine in the medium outside the cells (corrected for control values); ▼, radioactivity in phosphatidylcholine; ■, radioactivity in the intracellular aqueous fraction. Symbols in (*b*) are as in (*a*), with the addition of controls for phosphatidylcholine (▽) and intracellular aqueous fraction (□), and a derived curve (△) which represents phosphocholine in the medium minus breakdown of sphingomyelin. Values are expressed as percentages of total radioactivity in the cells and medium (121325±7264 d.p.m. in this experiment).

radioactivity as expected. There was a small but reproducible decrease (about 7% in 1 h) in labelling of phosphatidylcholine, but no sign of an equivalent rise in diacylglycerol or in possible phosphorylated metabolites of this lipid, such as phosphatidate or phosphatidylinositol. Experiments with ^{32}P-labelled cells (results not shown) confirmed that there was no change in labelling of the latter phospholipids. However, in palmitate-labelled cells treated with sphingomyelinase there was a rise in triacylglycerol radioactivity which accounted for at least part of the decrease in phosphatidylcholine radioactivity, suggesting that some of the putative diacylglycerol derived from phosphatidylcholine was subsequently acylated.

Support for the suggestion that diacylglycerol derived from phosphatidylcholine might be converted into triacylglycerol was obtained from experiments where [^{3}H]-palmitate-labelled cells were incubated with sphingomyelinase and the enzyme was then removed, allowing resynthesis of sphingomyelin to occur (Fig. 3). It was clear from iodine-stained t.l.c. plates that restoration of the original sphingomyelin amount occurred concurrently with a progressive diminution in the amount of ceramide in the cells, and this was confirmed by the pattern of labelling observed. During a 3 h incubation after removal of sphingomyelinase, loss of label from phosphatidylcholine approximately matched an increase of radioactivity in triacylglycerol. Over this period the

Table 2. Distribution of radioactivity in lipids and aqueous fractions from [^{3}H]choline- and [^{3}H]palmitate-labelled BHK cells incubated with or without sphingomyelinase

Cells labelled to equilibrium with either [^{3}H]choline or [^{3}H]-palmitate were incubated in the absence or presence of 2 μl of sphingomyelinase for 1 h at 37 °C before extraction of lipids. Values are expressed as percentages of total radioactivity as means±S.D. from triplicate determinations. The change in each fraction (sphingomyelinase-treated minus control) is listed under Δ. In four separate experiments with [^{3}H]choline the mean breakdown of phosphatidylcholine was 8±2% in treated cells. Total radioactivity in each dish in this experiment was 187677±12433 d.p.m. in choline and 95996±8352 d.p.m. in palmitate. Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CER, ceramide; TG, triacylglycerol; DG, diacylglycerol; NEFA, non-esterified fatty acid.

	Distribution of radioactivity (%)			
Treatment ...	0 min control	60 min control	60 min + enzyme	Δ
Choline-labelled cells				
SM	11.4±0.3	11.6±0.2	4.3±0.4	−7
PC	67±3	65±2	59±2	−6
Cell water	21±2	22±2	21±1	−1
Medium	1.0±0.4	2.0±0.5	14.1±1.2	+12
Palmitate-labelled cells				
SM	13.5±0.5	13.3±0.6	5.4±0.3	−8
PC	47.2±0.5	46.8±0.7	43.2±0.5	−3
PE	24.3±0.6	24.1±0.4	23.2±0.7	−1
PS+PI	11.4±1.1	12.4±1.3	12.2±2.2	0
CER	0.5±0.1	0.5±0.2	8.8±0.3	+8
TG	3.4±0.3	3.8±0.4	5.5±0.4	+2
DG	0.5±0.2	0.6±0.3	0.6±0.2	0
NEFA	0.3±0.2	0.4±0.1	0.4±0.2	0

original sphingomyelin content of the cells was restored at a rate approximately equivalent to synthesis of 17% of total sphingomyelin/h (1.5% of total phospholipid/h), although the rate for the first 1 h was somewhat higher (25% of total sphingomyelin/h). Similar results were obtained from phosphate determinations on the resynthesized sphingomyelin, showing that this material had a specific radioactivity close to that of the original sphingomyelin.

This rate of synthesis of sphingomyelin seems likely to be greater than the normal rate in control cells, which have a doubling time of about 12 h and thus should synthesize about 8% of their sphingomyelin in 1 h (ignoring turnover). Indeed, experiments in which the incorporation of [^{3}H]choline into initially unlabelled cells was measured with and without addition of sphingomyelinase suggested that incorporation into sphingomyelin after 1 h in cells exposed to sphingomyelinase was twice that in untreated cells (Fig. 4). However, under these conditions the mass of sphingomyelin was decreased to one-third in the presence of the enzyme, so that the specific radioactivity of sphingomyelin was raised about 6-fold compared with control cells. The corresponding

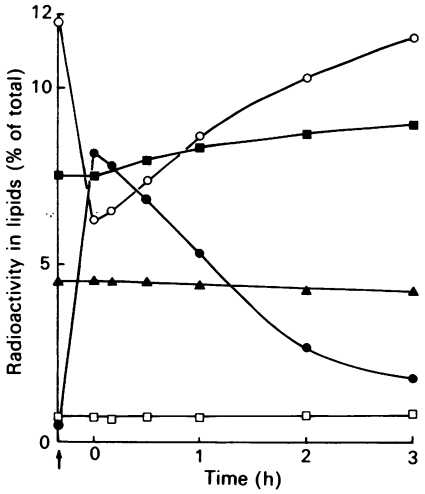

Fig. 3. Resynthesis of sphingomyelin from ceramide in BHK cells treated with sphingomyelinase and then incubated in the absence of the enzyme

Cells were labelled to equilibrium with [^{3}H]palmitate and then were treated with 2 μl of sphingomyelinase for 20 min (enzyme addition indicated by arrow). The enzyme was removed at zero time (see the Materials and methods section), and the cells were re-incubated for 3 h in serum-free medium. Lipids were extracted at various times and analysed. Values were presented as percentages of total radioactivity (89786$\pm$4656 d.p.m.) from one representative experiment of four giving similar results. Symbols: ○, sphingomyelin; ●, ceramide; ▲, phosphatidylcholine (% $\times 10^{-1}$); ■, triacylglycerol; □, diacylglycerol.

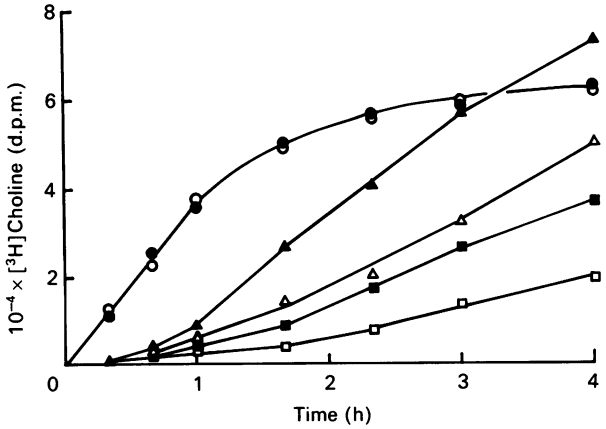

Fig. 4. Incorporation of [^{3}H]choline into BHK cells in the presence or absence of sphingomyelinase

To cells approaching confluence on 3.4 cm dishes were added either 0.3 μCi of [^{3}H]choline alone (○, △, □; controls) or [^{3}H]choline and 2 μl of sphingomyelinase (●, ▲, ■). At various times duplicate samples were washed and the lipids extracted. Radioactivity in sphingomyelin (■, □; $\times$10), phosphatidylcholine (▲, △) and the internal aqueous pool (●, ○) was measured. Similar results were obtained in three further experiments.

incorporation into phosphatidylcholine in the presence of sphingomyelinase was raised about 50% with a similar increase in specific radioactivity. It was noticeable that there was almost no incorporation of [^{3}H]choline into either sphingomyelin or phosphatidylcholine for about 20 min after addition of the label to both control and sphingomyelinase-treated cells. In contrast, the labelling of the water-soluble pool inside the cells, which was shown to be largely phosphocholine, showed no lag in incorporation and was half-maximal at about 1 h after addition of label.

DISCUSSION

When BHK cells were treated with *S. aureus* sphingomyelinase, about 60% of the total sphingomyelin of the cells was degraded within 30 min (Figs. 1–3), presumably because this portion of the sphingomyelin was at the cell surface or in a membrane pool which was in rapid equilibrium with the surface. No more than 5% more sphingomyelin was degraded even after prolonged incubation with sphingomyelinase, suggesting that the cells possessed a pool of sphingomyelin accounting for about one-third of the total which was not in equilibrium with the sphingomyelin accessible to the enzyme. Exactly the same result was obtained with cells which had been released from the plate with EDTA/trypsin (Table 1), so the resistant pool of sphingomyelin was not a consequence of a lack of access of the enzyme to areas of the cell membrane in contact with the plate.

It is possible that one effect of sphingomyelinase treatment was to decrease the rate at which membrane components, including lipids, cycle between intracellular pools and the plasma membrane, but this seems unlikely in view of the failure of sphingomyelinase to influence either endocytosis or exocytosis of [^{3}H]inulin (Fig. 1*b*). These results would also suggest either that the effect of sphingomyelinase in causing endocytosis in red cells [14] is not a general phenomenon, or that BHK cells are normally endocytosing at a maximal rate which cannot be increased. Although in red cells the process of endocytosis promoted by sphingomyelinase appears to create a pool of sphingomyelin which is inaccessible to the enzyme, such a phenomenon is apparently incapable of explaining the enzyme-resistant pool in BHK cells.

The nature and location of the inaccessible pool of sphingomyelin (about 35% of the total) is not known, but clearly the failure of this pool to be attacked in intact cells is not due to a difference in its chemical nature, since incubation of the cells with sphingomyelinase and Triton X-100 caused almost complete breakdown of sphingomyelin (Table 1). A significant fraction of the inaccessible pool could reside in the endoplasmic reticulum, nuclear membrane [20] or in lysosomes, which appear to account for a relatively large proportion of the sphingomyelin in BHK cells [21]. Another possibility is that this pool of sphingomyelin is on the cytosolic face of the plasma membrane and related organelles since transbilayer migration of sphingomyelin is extremely low [22]. However, this seems to be unlikely, in view of the observation that, when cells are permeabilized with streptolysin O, breakdown of sphingomyelin is still no more than 65% in the presence of sphingomyelinase (Table 1). Streptolysin O, which selectively permeabilizes plasma membranes and other membranes rich in cholesterol [23], caused almost complete release of lactate dehydrogenase from BHK cells in suspension, so it can be assumed that it allowed free access of the sphingomyelinase to the cytoplasmic compartment. Fixation of the cells with glutaraldehyde would be expected to prevent fusion of intracellular membranes with the

plasma membrane and thus to restrict the sphingomyelinase to breakdown of sphingomyelin originally present on the cell surface. The failure of this treatment to decrease breakdown of sphingomyelin in cells subsequently treated with sphingomyelinase suggests that essentially all the accessible sphingomyelin is on the outer face of the plasma membrane and very little of it can be derived from intracellular sources. Indeed, there is some evidence for this suggestion from the results of experiments to investigate phospholipid asymmetry in enveloped viruses [24] whose phospholipid composition and organization is thought to mirror that of the host-cell plasma membrane. It was concluded from these studies that, when virus was treated with sphingomyelinase at 37 °C (but not at lower temperatures), 100% of the sphingomyelin was degraded, indicating that all the membrane sphingomyelin was in the outer leaflet of the lipid bilayer. If 65% of total cellular sphingomyelin is on the cell surface and this lipid represents 8–9% of the total phospholipid in intact cells (Fig. 1, Table 2), it follows that about 5% of the total cell phospholipid is plasma-membrane sphingomyelin. From the work of van Meer *et al.* [24], sphingomyelin accounts for about 25% of the phospholipids of the plasma membrane of BHK cells, so that the plasma membrane should account for about 20% of the total phospholipids of these cells. This value is about 50% more than a recent estimate of the plasma-membrane area as a proportion of the area of the total membranes of the cell [25].

When the enzyme was removed after degradation of the accessible pool of sphingomyelin was complete, the resulting ceramide was smoothly converted back into sphingomyelin over a 3 h period (Fig. 3). It is not clear from our results whether the sphingomyelin is resynthesized at the cell surface from ceramide which remains there, or whether it is resynthesized internally after diffusion of the ceramide to the cell interior. The former is what would be predicted if, as previously suggested, plasma-membrane sphingomyelin is largely synthesized *in situ* at the expense of plasma-membrane ceramide and phosphatidylcholine through the agency of ceramide:phosphatidylcholine phosphocholinetransferase, which itself is localized in the plasma membrane [12,13]. Consistent with this suggestion is the observation that, although *S. aureus* sphingomyelinase has no enzymic activity against any phospholipid other than sphingomyelin, it nevertheless causes the degradation of a limited proportion of the cellular phosphatidylcholine of BHK cells (Table 1, Figs. 2 and 3). A decrease in phosphatidylcholine was only seen in cells labelled to equilibrium with [^{3}H]choline or palmitate and not in unlabelled cells, suggesting that the cells could synthesize sufficient new (unlabelled) phosphatidylcholine to maintain their normal complement of this lipid even when they could not replenish degraded sphingomyelin. The labile pool of ^{3}H-labelled phosphatidylcholine, which accounts for about 8% of the total in the cells, is steadily degraded when sphingomyelin is broken down or when ceramide is reconverted into sphingomyelin, and therefore may represent the immediate donor of phosphocholine for the synthesis of cell-surface sphingomyelin. It seems likely that it also has a localization at or near the plasma membrane, particularly because this pool reacts rapidly to degradation of the surface pool of sphingomyelin (Fig. 2*b*), although it is more than 40 min before the cell responds with a general increase in synthesis of phosphatidylcholine and sphingomyelin (Fig. 4).

Assuming that plasma-membrane phospholipid represents about 20% of the total in BHK cells and that phosphatidylcholine accounts for 30% of this [24], it can be calculated that plasma-membrane phosphatidylcholine should be about 6% of total cell phospholipid or 12% of total phosphatidylcholine. Since only about 60% of this appears to be on the outer face of the plasma membrane [24], we can account for the 8% degradation of phosphatidylcholine which results from sphingomyelinase attack on intact cells (Fig. 2) largely in terms of degradation of the outer-leaflet pool.

Transfer of phosphorylcholine from phosphatidylcholine to ceramide to produce sphingomyelin should in the first place give rise to plasma-membrane diacylglycerol equivalent to the loss of phosphatidylcholine, but experiments with [^{3}H]palmitate-labelled cells failed to reveal any change in diacylglycerol radioactivity after addition of sphingomyelinase (Figs. 3 and 4). Diacylglycerol produced in the plasma membrane of other cell types by exogenous or endogenous phospholipase C is rapidly converted into phosphatidate [26] and phosphatidylinositol [27], but neither of these phospholipids showed any increased labelling in BHK cells treated with sphingomyelinase. However, there was a significant increase in [^{3}H]palmitate radioactivity in triacylglycerol, suggesting that at least part of the putative plasma-membrane diacylglycerol resulting indirectly from sphingomyelinase action became acylated. Not all of the decrease in phosphatidylcholine could be accounted for in terms of triacylglycerol synthesis, and it is possible that some of the diacylglycerol formed could have been degraded. No increase in monoacylglycerol or non-esterified fatty acid was observed, but further degradation of monoacylglycerol to glycerol and conversion of fatty acids into acyl-CoA derivatives have not been discounted. Acylation of diacylglycerol is usually assumed to occur at the endoplasmic reticulum, so that these data may suggest that the pool of phosphatidylcholine which is the ultimate donor of phosphocholine to sphingomyelin is inside the cell, even if the proximal donor is at the cell surface.

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