Degradation of plasma membrane phosphatidylcholine appears not to affect the cellular cholesterol distribution

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Abstract To clarify the role of possible cholesterol/phosphatidylcholine interactions in cellular cholesterol distribution, we have used a phosphatidylcholine-specific phospholipase C from *Bacillus cereus* to degrade the cell surface phosphatidylcholine of cultured human fibroblasts. Of cellular phosphatidylcholine, approximately 15% was susceptible to degradation by the phospholipase. In spite of the dramatic redistribution of cellular cholesterol that can be observed after sphingomyelin depletion, the degradation of cell surface phosphatidylcholine did not affect the distribution of cholesterol in fibroblasts. In cholesterol-depleted cells as well as in cholesterol-loaded cells, the size of the cell surface cholesterol pool (susceptible to cholesterol oxidase) remained unchanged after phosphatidylcholine degradation. The rate of cholesterol esterification with [^{3}H]oleic acid and the rate of [^{3}H]cholesterol efflux from fibroblasts to high density lipoproteins also remained unchanged after degradation of plasma membrane phosphatidylcholine. An increase in the level of [^{3}H]cholesterol efflux to high density lipoproteins was observed after degradation of plasma membrane sphingomyelin with exogenous sphingomyelinase, in contrast to earlier reports, where no such effect was observed. The results suggest that interactions between cholesterol and phosphatidylcholine in the fibroblast plasma membranes are less important than cholesterol/sphingomyelin interactions for the asymmetric distribution of cellular cholesterol.—**Pörn, M. I., M. P. S. Ares, and J. P. Slotte.** Degradation of plasma membrane phosphatidylcholine appears not to affect the cellular cholesterol distribution. *J. Lipid Res.* 1993. **34:** 1385–1392.

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The cellular cholesterol distribution is thought to be asymmetric at three different levels. First, there is an intermembrane concentration gradient, with the plasma membrane being very rich in cholesterol (1), whereas the endoplasmic reticulum has an intermediate level, and the mitochondria have the lowest content of cholesterol. The intermembrane asymmetry appears to result, at least partially, from a specific affinity of certain phospholipids for cholesterol in the order sphingomyelin > phosphatidylcholine > phosphatidylethanolamine (2, 3). Second, two types of intramembrane asymmetry are thought to exist, a transbilayer and a lateral asymmetry. Depending on the method used, various results have been obtained concerning the distribution of cholesterol between the inner and the outer leaflet of cellular plasma membranes (see ref. 4, and references therein). A lateral asymmetry of plasma membrane lipids has been suggested to result in cholesterol-rich domains and cholesterol-poor domains (5, 6).

In recent years the effects of sphingomyelin depletion on the cellular cholesterol distribution have been extensively investigated. Plasma membrane sphingomyelin has been found to be of major importance for the intermembrane cholesterol gradient of several different cell types, as depletion of cell surface sphingomyelin by treatment with exogenous sphingomyelinase (a sphingomyelin-specific phospholipase C) leads to an extensive redistribution of cellular cholesterol from the cell surface to intracellular membranes (7–11). This is evidenced by a decreased oxidizability of cellular cholesterol, an increased rate of esterification, a decrease in the rate of cholesterol biosynthesis, and activation of the conversion of cholesterol to steroid hormones in steroidogenic cells. In the present study we have used a phosphatidylcholine-specific phospholipase C (PC-PLC) from *Bacillus cereus* for degradation of cell surface phosphatidylcholine, in order to clarify the role of possible cholesterol/phosphatidylcholine interactions in the cellular cholesterol homeostasis. The PC-PLC isolated from *B. cereus* has been shown to have a much lower sphingomyelinase activity than the corresponding

Abbreviations: PC-PLC, phosphatidylcholine-specific phospholipase C; BHK, baby hamster kidney; DMEM, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum; ACAT, acyl-CoA:cholesterol acyltransferase; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate.

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enzyme from *Clostridium perfringens* (12), and should therefore be more suitable for this task. The results suggest that plasma membrane phosphatidylcholine is less important than sphingomyelin for the intermembrane asymmetry of cellular cholesterol. Depletion of plasma membrane phosphatidylcholine with PC-PLC did not affect the steady-state distribution of cell cholesterol. In addition to the previously reported effects of sphingomyelinase, we found that it also increased the level of cholesterol efflux, in the presence of high density lipoproteins as extracellular cholesterol acceptors.

MATERIALS AND METHODS

Materials

[1α,2α-^{3}H]cholesterol (48.4 Ci/mmol), [9,10-^{3}H]oleic acid (10 Ci/mmol), and [methyl-^{3}H]choline (80 Ci/mmol) were purchased from Amersham. Cell culture media and supplements were obtained from NordCell (Stockholm, Sweden). Fetal calf serum was purchased from GIBCO. Cell culture dishes were from Nunc (Denmark). Phosphatidylcholine-specific phospholipase C (EC 3.1.4.3, *Bacillus cereus,* Grade I) was obtained from Boehringer-Mannheim. Ham F-12 nutrient mixture, sphingomyelinase (EC 3.1.4.12, *Staphylococcus aureus*), and phospholipids were from Sigma. Cholesterol oxidase (EC 1.1.3.6, *Brevibacterium* sp.) was purchased from Beckman Instruments.

Cell culture

Human skin fibroblasts (line GM8333) were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. BHK-21 cells are grown continuously in our department. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12% (fibroblasts) or 5% (BHK-21 cells) fetal calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 1 mM nonessential amino acids. The cells were grown to confluency in 75-cm^2 culture flasks at 37°C in 5% CO_2. Cells for experiments were seeded in 35-mm or 60-mm culture dishes, and treated as described separately.

Lipoproteins

Low density lipoprotein (LDL), high density lipoprotein (HDL), and lipoprotein-deficient serum (LPDS) were prepared from fresh human plasma (EDTA 4 mM) by sequential ultracentrifugation (13, 14). After dialysis, lipoproteins and LPDS were sterilized and stored in the dark at 4°C.

Degradation of [^{3}H]choline-labeled choline phospholipids

The choline phospholipids of subconfluent cells were labeled to constant specific activity with 2.0 μCi/ml of [methyl-^{3}H]choline chloride for 48 h. The confluent cells were then incubated with serum-free DMEM for 3 h and rinsed with phosphate-buffered saline, before exposure to PC-PLC at 37°C. Treatment of confluent cells with PC-PLC (1.0 U/ml) for 1 h did not cause any significant decrease in cell viability, as determined by trypan blue exclusion. The cellular content of phosphatidyl[^{3}H]choline and [^{3}H]sphingomyelin was determined as described separately.

Labeling of cells with [^{3}H]cholesterol

Subconfluent cells were incubated for 48 h with [^{3}H]cholesterol in fetal calf serum ([^{3}H]cholesterol in ethanol solution mixed with serum [5 μCi/ml]; 8% serum in DMEM). Prior to experiments cells were incubated in serum-free DMEM for 3 h.

Manipulations of the cellular cholesterol level

For depletion of cellular cholesterol, fibroblasts were grown in serum-free DMEM supplemented with 6% LPDS for 3 × 24 h prior to some experiments. For expansion of cellular cholesterol pools, cells were first grown in 6% LPDS 2 × 24 h and then exposed to LDL (0.5 mg protein/ml), LPDS, and the ACAT inhibitor 58-035 (5 μg/ml) for 24 h.

Determination of cell surface cholesterol

Dishes of confluent cells with or without [^{3}H]cholesterol (for thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) analysis, respectively) were rinsed with phosphate-buffered saline and treated with PC-PLC or sphingomyelinase for different times. Cells were then chilled on ice and fixed with 1% glutaraldehyde for 10 min, and after that thoroughly rinsed with phosphate-buffered saline. The amount of cholesterol at the cell surface was determined by oxidation with cholesterol oxidase (1.5 U/ml in serum-free Ham F-12) at 37°C. Sphingomyelinase (50 mU/ml) was included in the oxidation solution in order to facilitate the oxidation of cell surface cholesterol (6). After 45 min, dishes were placed on ice and the medium was aspirated. Cells were then rinsed with ice-cold phosphate-buffered saline and stored at −20°C until lipid analysis was performed.

Incorporation of [^{3}H]oleic acid into cholesteryl [^{3}H]oleate

Confluent fibroblasts were incubated in serum-free DMEM for 16 h, rinsed with phosphate-buffered saline, and then exposed to 1.5 μCi/ml of [^{3}H]oleic acid (57 mCi/mmol, complexed to albumin) in Ham F-12, for 60 min at 37°C. Thereafter 2.0 U/ml of PC-PLC or 0.1 U/ml of sphingomyelinase was added to the dishes and the esterification of cholesterol with [^{3}H]oleic acid was allowed to proceed for up to 2 h. Dishes were then rinsed with ice-cold phosphate-buffered saline and stored at −20°C until the amount of synthesized cholesteryl [^{3}H]oleate was determined.

Efflux of cellular [^{3}H]cholesterol

Confluent cells with [^{3}H]cholesterol were exposed to PC-PLC or sphingomyelinase for 30 min and thereafter washed with phosphate-buffered saline containing 2 mg/ml of albumin 2 × 5 min on ice. Then serum-free Ham F-12 medium was added to the dishes, with or without high density lipoprotein (HDL_3). The efflux of [^{3}H]cholesterol was interrupted after 4 h by chilling dishes on ice, and the medium was aspirated. The [^{3}H]cholesterol content of the medium was determined by scintillation counting and cellular neutral lipids were extracted and separated as described below.

Assay procedures

The content of phosphatidylcholine and sphingomyelin in untreated, PC-PLC- and sphingomyelinase-treated cells was determined from the hexane–2-propanol 3:2 (v/v) extract of cells. Phospholipids were separated on silica gel thin-layer chromatography plates (Merck) using chloroform–methanol–acetic acid–water 25:15:4:2 (v/v) (15). [^{3}H]choline phospholipid spots were visualized by staining with iodine and identified from standards run in parallel. [^{3}H]sphingomyelin and phosphatidyl[^{3}H]choline spots were scraped into scintillation vials together with 3 ml of scintillation cocktail, and radioactivity was counted. Phospholipids from unlabeled cells were visualized by staining with cupric acetate (3%, w/v) in phosphoric acid (8%, v/v), and the color was developed by heating the plates for 30 min at 150°C. The absorbances of the three main phospholipid spots (sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine) were then determined with a scanning densitometer (E-C Apparatus Corp., St. Petersburg, FL). The mass of these phospholipids was calculated from standards run in parallel.

Neutral lipids from [^{3}H]cholesterol-labeled cells were separated by TLC sheets (Kodak) using hexane–diethyl ether–acetic acid 130:30:2 (v/v) as solvent. Sheets were stained with iodine and spots of [^{3}H]cholesterol, [^{3}H]cholestenone, and [^{3}H]cholesteryl ester were cut into scintillation vials for counting of radioactivity. Cell sterol mass in the lipid extract was determined by gas–liquid chromatography. 5β-Coprostanol was added as internal standard to each sample during the extraction. Before GLC analysis, sterols in the total lipid extract (dissolved in 50 μl of hexane) were silylated with 50 μl of bis(trimethylsilyl)trifluoroacetamide and 15 μl of dimethylchlorosilane (at ambient temperature). Sterols were separated on an Rtx-1 capillary column (Restek Corp., Bellefont, PA) in a temperature gradient from 240 to 280°C at 10°C/min, with helium as carrier gas.

Cell protein mass in unfixed cells was determined by the method of Lowry et al. (16), using bovine serum albumin as a standard.

Trypan blue exclusion was measured in cells which had been exposed to enzymes or serum-free medium only and subsequently detached from dishes by trypsin treatment. Cells were resuspended in buffer and trypan blue was added (0.2%), and the suspension was examined microscopically for uptake of the dye.

RESULTS

Degradation of plasma membrane phosphatidylcholine

Phosphatidylcholine-specific phospholipase C was used to degrade plasma membrane phosphatidylcholine. In human skin fibroblasts, as well as in BHK-21 cells, treatment with 1.0 U/ml of PC-PLC resulted in a degradation of approximately 15% of the total cellular phosphatidyl[^{3}H]choline within 45 min (**Fig. 1**). This roughly cor-

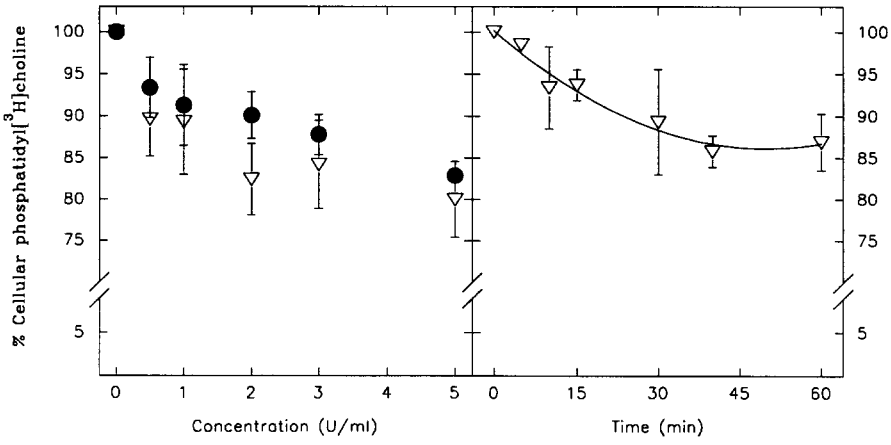

Fig. 1. PC-PLC-stimulated degradation of cellular phosphatidyl[^{3}H]choline. Confluent human skin fibroblasts (▽) and BHK-21 cells (●) with ^{3}H-labeled choline phospholipids were exposed to indicated amounts of PC-PLC for 30 min (left panel) or received 1.0 U/ml for indicated times (right panel). Data are mean values ± SD from six separate experiments (n = 3–9 for each point).

responds to the previously reported value for plasma membrane phosphatidylcholine content in BHK-21 cells (17). For determination of the total amount of phosphatidylcholine that was degraded during the PC-PLC treatment of human skin fibroblasts, and to compare this to the amount of sphingomyelin degraded with sphingomyelinase, the density of phospholipid spots was determined by scanning of the TLC plates (**Fig. 2**). Approximately 40 nmol of phosphatidylcholine was degraded per mg cell protein by the standard PC-PLC treatment used throughout this study, compared to approximately 20 nmol of sphingomyelin degraded per mg with the sphingomyelinase treatment. Of cellular sphingomyelin, 5–10% was degraded upon the PC-PLC treatment, whereas no decrease in cellular phosphatidylethanolamine mass was observed (data not shown).

Effect of phosphatidylcholine degradation on distribution of cellular [^{3}H]cholesterol

The degradation of plasma membrane sphingomyelin in cultured cells results in a massive redistribution of cellular cholesterol into intracellular organelles as evidenced by the decrease in cholesterol oxidase susceptibility (8). To investigate whether the degradation of plasma membrane phosphatidylcholine would result in a similar cholesterol mobilization, confluent fibroblasts with a "normal" level of cholesterol (i.e., cells grown in 12% FCS) were exposed to PC-PLC before fixation and exposure to cholesterol oxidase. As shown in **Fig. 3,** the susceptibility of cellular [^{3}H]cholesterol to cholesterol oxidase was not decreased after PC-PLC treatment. In fact, there seemed to be a slight and transient increase in oxidizability of cell surface cholesterol after PC-PLC treatment, which is in agreement with a report by Patzer, Wagner, and Barenholz (18), on phospholipase C-facilitated oxidation of cholesterol.

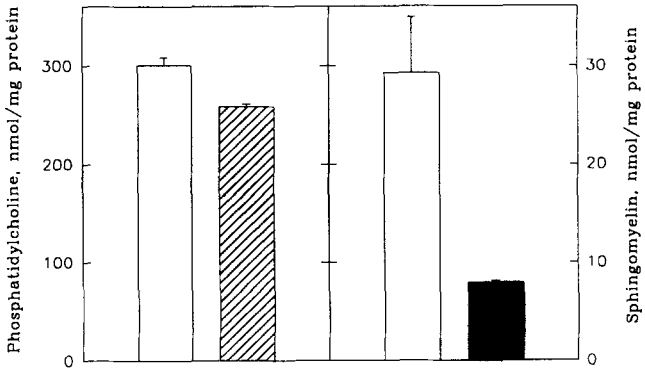

Fig. 2. Enzymatic degradation of choline phospholipid mass. Confluent human skin fibroblasts were exposed to 2.0 U of PC-PLC/ml (hatched bar) or 0.1 U of sphingomyelinase/ml (filled bar) for 45 min and choline phospholipid masses were determined as described under Materials and Methods. Cells exposed to Ham F-12 medium only were used as controls (empty bars). Results are presented as cellular mass of phospholipid per mg protein. Data are means ± range from one representative experiment (n = 2).

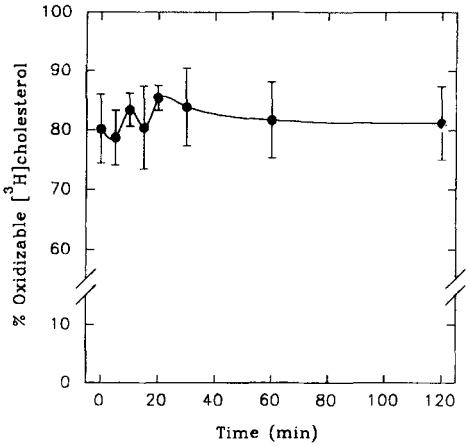

Fig. 3. Distribution of cellular [^{3}H]cholesterol after PC-PLC treatment. Human skin fibroblasts with [^{3}H]cholesterol were treated with 1.0 U of PC-PLC/ml for the times indicated. The fraction of cell cholesterol associated with the plasma membrane was then determined by the cholesterol oxidase method, as described under Materials and Methods. Data are means ± SD from four separate experiments (n = 7–11).

Effect of phosphatidylcholine degradation on distribution of cell cholesterol in cholesterol-depleted and cholesterol-loaded cells

Although the degradation of phosphatidylcholine did not result in any redistribution of cholesterol in cells with a normal cholesterol level, one could speculate that the cholesterol molecules in a plasma membrane loaded with cholesterol perhaps would be more dependent on the association with phosphatidylcholine. Therefore we performed the cholesterol oxidase experiment on cells which were either depleted of cholesterol by LPDS treatment or loaded with cholesterol by LDL treatment, as described in the Materials and Methods section. However, neither of the two treatments caused any change in the cholesterol oxidase susceptibility after PC-PLC treatment (**Fig. 4** and **Table 1**). A comparison of the results in Fig. 4 and Table 1 indicates that mass analysis of the cellular cholesterol content shows a less dramatic sphingomyelinase-induced cholesterol translocation, than the one observed in [^{3}H]cholesterol-labeled cells.

Esterification of [^{3}H]oleic acid with cholesterol in PC-PLC-treated cells

The sphingomyelinase-induced cholesterol translocation in cultured cells results in an activation of the esterification enzyme ACAT, as evidenced by an increased incorporation of [^{3}H]oleic acid into cholesteryl [^{3}H]oleate (7, 9). As shown in **Fig. 5,** exposure of cells to PC-PLC for up to 2 h did not increase the rate of cholesterol esterification, whereas sphingomyelinase caused a marked increase. The results in Figs. 3–5 suggest that no redistri-

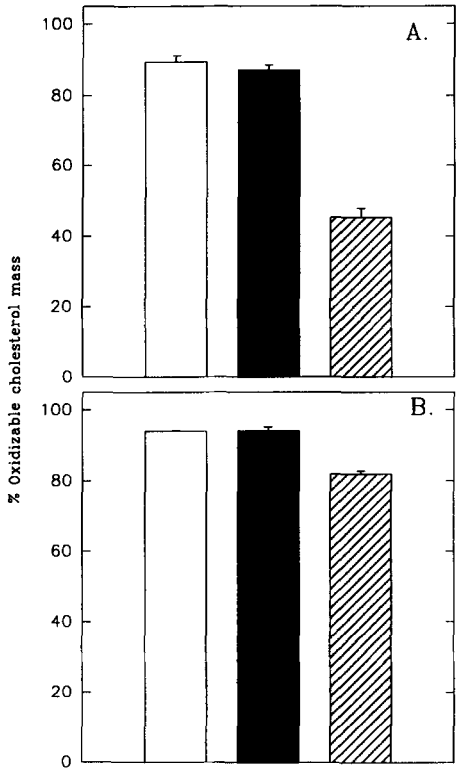

Fig. 4. Effects of PC-PLC and sphingomyelinase on the distribution of cell cholesterol determined by mass analysis. Human skin fibroblasts which were either cholesterol-depleted by LPDS treatment (panel A) or cholesterol-loaded by LDL treatment (panel B) were exposed to 1.0 U of PC-PLC/ml (filled bars) or 50 mU of sphingomyelinase/ml (hatched bars) for 60 min. Control cells received serum-free medium only (empty bars). The cell surface cholesterol was then oxidized by cholesterol oxidase and sterol masses were determined by GLC analysis. The unesterified cholesterol content of LPDS-treated cells was 70 ± 15 nmol/mg protein and for LDL-treated cells 180 ± 20 nmol/mg. Data are means ± SD from two separate experiments (n = 4).

TABLE 1. Relationship between cholesterol level and effects of PC-PLC and sphingomyelinase on distribution of cellular [^{3}H]cholesterol

Pretreatment	Enzyme	% Trypan Blue Permeability	n	% Oxidizable [^{3}H]Cholesterol
LPDS	None	5.0 ± 1.8	18	84.0 ± 4.2
LPDS	PC-PLC	7.0 ± 1.0	12	83.3 ± 5.5
LPDS	Sphingomyelinase	9.9 ± 2.6	12	24.6 ± 4.5
LDL	None		18	85.9 ± 4.3
LDL	PC-PLC		9	88.7 ± 2.7
LDL	Sphingomyelinase		9	56.6 ± 4.6

Human skin fibroblasts that had been pretreated with LPDS for cholesterol depletion or with LDL for cholesterol loading as described under Materials and Methods, and incubated with [^{3}H]cholesterol for 48 h, were exposed to 1.0 U of PC-PLC/ml or 50 mU of sphingomyelinase/ml for 45 min. Control dishes received serum-free medium only. Some dishes of cells were then exposed to trypsin and tested for trypan blue permeability. The fraction of cellular [^{3}H]cholesterol associated with the plasma membrane was determined by use of cholesterol oxidase. Data are means ± SD from six separate experiments.

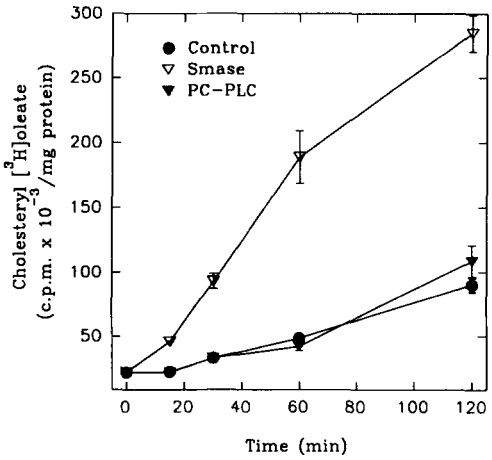

Fig. 5. Esterification of cellular cholesterol with [^{3}H]oleic acid in fibroblasts treated with PC-PLC or sphingomyelinase. Fibroblasts were pulsed with [^{3}H]oleic acid for 60 min and thereafter exposed to 2.0 U of PC-PLC/ml, 0.1 U of sphingomyelinase/ml (Smase), or serum-free medium only for the times indicated. Results are presented as cpm of cholesteryl [^{3}H]oleate formed per mg of cell protein. Data are means ± SD of triplicates. Similar results were obtained in four separate experiments.

bution of cell cholesterol occurred upon phosphatidylcholine degradation. Plasma membrane phosphatidylcholine thus appeared to be less important than sphingomyelin for the intracellular cholesterol distribution.

Efflux of [^{3}H]cholesterol to HDL_3 after treatment with PC-PLC or sphingomyelinase

The degradation of plasma membrane phosphatidylcholine failed to cause any influx of cell surface cholesterol. In order to test whether some plasma membrane cholesterol would be directed for efflux to an extracellular cholesterol acceptor after phosphatidylcholine or sphingomyelin degradation, fibroblasts were exposed to PC-PLC or sphingomyelinase, and the efflux of cell [^{3}H]cholesterol to HDL_3 was measured. As shown in **Fig. 6,** the efflux of [^{3}H]cholesterol from fibroblasts to HDL_3 remained unchanged after degradation of plasma membrane phosphatidylcholine, whereas an increase (approximately 30%) was observed after sphingomyelin degradation. In previous studies, sphingomyelinase has been reported not to affect the rate of cholesterol efflux from cells (11, 19), probably due to a different experimental protocol, as discussed below.

DISCUSSION

The profound importance of plasma membrane sphingomyelin for the maintenance of cellular cholesterol homeostasis has become evident in the past decade. The degradation of plasma membrane sphingomyelin by exogenous sphingomyelinase results in a modified activity

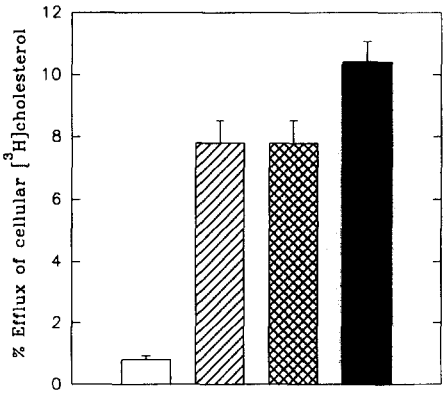

Fig. 6. Efflux of cellular [^{3}H]cholesterol to high density lipoprotein after degradation of cell surface phosphatidylcholine and sphingomyelin. Human skin fibroblasts with ^{3}H-labeled cholesterol pools were exposed to 2.0 U of PC-PLC/ml or 0.1 U of sphingomyelinase/ml for 30 min. Control cells received serum-free medium only. Cells were then exposed to HDL_3 (300 μg protein/ml) or serum-free medium for 4 h. Total [^{3}H]cholesterol efflux in control cells was 940 ± 140 dpm. Data are means ± SD from three separate experiments (n = 9). Empty bar, control; hatched bar, HDL_3; cross-hatched bar, PC-PLC + HDL_3; filled bar, sphingomyelinase + HDL_3.

of the important regulatory enzymes of cellular cholesterol metabolism; cholesterol esterification (ACAT activity) is increased (7, 9), cholesterol synthesis (HMG-CoA reductase activity) is decreased (7, 11), and in steroidogenic cells, the rate of conversion of cholesterol to steroid hormones is enhanced (10). A dramatic redistribution of cell cholesterol is evidenced by the decrease in oxidizable cell cholesterol (8, 9). Sphingolipid metabolites like ceramide and sphingosine are evidently not the cause of these events (11, and A-S. Härmälä, M. I. Pörn, and J. P. Slotte, unpublished observations). It is assumed that, when sphingomyelin is degraded, molecular interactions between sphingomyelin and cholesterol are destroyed, so that cholesterol is liberated from the plasma membrane and subsequently translocated to a putative regulatory pool of free sterol.

In contrast to the interest received by sphingomyelin, the possible role of the most abundant choline phospholipid of mammalian cells, phosphatidylcholine, in cellular cholesterol homeostasis has remained elusive. In the present study we have used PC-PLC from *B. cereus* to degrade cell surface phosphatidylcholine. The enzyme proved to be a useful tool for selective degradation of phosphatidylcholine, as less than 10% of cellular sphingomyelin was degraded and no decrease in phosphatidylethanolamine content was observed during standard procedures, where approximately 15% of cellular phosphatidylcholine was degraded. Van Meer (17) has calculated that 11% of the phosphatidylcholine in baby hamster kidney (BHK) cells would be present in the plasma membrane. The PC-PLC used in this study is likely to have access only to the exoleaflet of the plasma membrane, but it is possible that the rate of phospholipid flip-flop within the plasma membrane would increase as the exoleaflet is depleted of phosphatidylcholine, and this could result in a more complete degradation of plasma membrane phosphatidylcholine. For every molecule of sphingomyelin degraded during the PC-PLC treatment, at least 15 molecules of phosphatidylcholine were degraded. Moreover, since the sphingomyelinase treatment of cells resulted in the degradation of approximately 20 nmol of sphingomyelin/mg protein, and this is assumed to correspond to the plasma membrane pool of sphingomyelin (9), the degradation of 40 nmol of phosphatidylcholine should be more than sufficient for the breaking of possible interactions between cholesterol and phosphatidylcholine in the plasma membrane. In spite of this, the PC-PLC treatment did not cause any decrease in the oxidizability of cell [^{3}H]cholesterol. When cells are loaded with cholesterol from LDL the cholesterol/phospholipid ratio in the plasma membrane should increase, unless the phospholipid synthesis is up-regulated at the same time. This could result in a shortage of sphingomyelin molecules available to form bonds with excess cholesterol, and phosphatidylcholine would be likely to interact with this excess cholesterol. Nevertheless, cholesterol loading of fibroblasts did not result in any effect of PC-PLC on the distribution of [^{3}H]cholesterol or cholesterol mass. The sphingomyelinase-induced decrease in oxidizability of [^{3}H]cholesterol appeared to be more dramatic than the decrease in cholesterol mass oxidizability. This effect was not observed in an earlier study (8) and could perhaps be explained by differences in the cholesterol loading protocol ([^{3}H]cholesterol-fed cells vs. unlabeled cells). Also, the measurements of cholesterol esterification rate indicated that degradation of plasma membrane phosphatidylcholine had no effect on cellular cholesterol homeostasis; the formation of cholesteryl [^{3}H]oleate did not increase after PC-PLC treatment.

In the present study, the asymmetric distribution of cholesterol between plasma membrane and intracellular organelles appeared not to depend on interactions between cholesterol and phosphatidylcholine. Only sphingomyelin depletion results in a translocation of plasma membrane cholesterol to intracellular compartments (7-11). Theories about lateral lipid microdomains in plasma membranes have been discussed in the literature, although no hard evidence for their existence has been presented. Such domains could perhaps differ in the type of phospholipid associated with cholesterol, and moreover, the cholesterol of some domains might be directed for influx to intracellular membranes, whereas the cholesterol of other domains could be directed for efflux to lipoproteins. However, the efflux of [^{3}H]cholesterol from fibroblasts to HDL_3 appeared to be stimulated by sphingomyelinase only, and not by PC-PLC. These results agree with published reports on a correlation between a

high sphingomyelin content of donor membranes and a slow cholesterol exchange rate (20). It appears that in previous studies (11, 19) sphingomyelinase failed to increase the efflux of cell cholesterol because HDL_3 was added to the cells before sphingomyelinase, whereas in this study the enzyme was washed away before the exposure of cells to HDL_3. In the experiments on cholesterol distribution and esterification rate, PC-PLC was present in the medium during the entire experiment, and thus there should be no risk of any substantial replenishment of plasma membrane phosphatidylcholine by resynthesis or redistribution of intracellular phosphatidylcholine. In the cholesterol efflux experiments, however, the PC-PLC was removed from the cells before addition of HDL_3, in order to avoid lipoprotein degradation. Therefore, it cannot be excluded that some replenishment of the plasma membrane phosphatidylcholine occurred during this type of experiment.

The results of this study give a consistent picture of the role of choline phospholipids in cellular cholesterol homeostasis. When the molecular interactions between sphingomyelin and cholesterol are broken, the steady-state gradient of membrane cholesterol content collapses; cholesterol flows into the cell and, in the presence of an extracellular acceptor, cholesterol efflux occurs. The depletion of plasma membrane phosphatidylcholine did not result in any such changes in the cellular cholesterol homeostasis. Thus, it appears that cholesterol either does not interact with phosphatidylcholine in the plasma membrane or these interactions can be replaced with other interactions upon depletion of the phosphatidylcholine. This preferential interaction of cholesterol with sphingomyelin can be at least partly explained by the greater van der Waals attractive forces existing between sphingomyelin and cholesterol, compared to those between phosphatidylcholine and cholesterol (21).

The immediate degradation product of phosphatidylcholine degradation with PC-PLC is diacylglycerol, a well-known activator of protein kinase C (22). Mendez, Oram, and Bierman (23) have recently shown that addition of diacylglycerol to cultured fibroblasts results in an increased translocation of intracellular sterols to the plasma membrane. Hence, the activation of protein kinase C by diacylglycerol might be able to reverse the flow of cholesterol into the cell which is induced by sphingomyelinase (and possibly PC-PLC). However, the sphingomyelinase-induced cholesterol translocation appears to be unaffected by short-term treatment with PMA, another activator of protein kinase C (unpublished observations, A-S. Härmälä, M. I. Pörn, and J. P. Slotte). Although plasma membrane phosphatidylcholine in this study appeared not to have a direct role in cellular cholesterol homeostasis, it may influence the cellular cholesterol metabolism indirectly through the second messengers formed upon phosphatidylcholine degradation. It has been shown that not only phosphatidyl-inositol 4,5-bisphosphate but also phosphatidylcholine is an important precursor of the diacylglycerol which is formed during agonist-induced cellular signal transduction (24). Protein kinase C has been shown to induce transcription of the genes for the LDL receptor and the HMG-CoA reductase (25). These particular genes are induced by platelet-derived growth factor, apparently through phosphatidylcholine breakdown and subsequent diacylglycerol formation (24, 26). Thus, agonist-induced degradation of a small portion of plasma membrane phosphatidylcholine may indirectly have effects on cellular cholesterol metabolism.

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