Exchange of C_{16}-Ceramide between Phospholipid Vesicles†

Carl G. Simon Jr.,‡ Peter W. Holloway,§ and Adrian R. L. Gear*,§

National Institute of Standards and Technology, Polymers Division, 100 Bureau Drive, Gaithersburg, Maryland 20899, and University of Virginia, School of Medicine, Department of Biochemistry and Molecular Genetics, Box 440, Jordan Hall, Charlottesville, Virginia 22908

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ABSTRACT: Ceramide is considered to be an important signaling molecule in cellular processes such as cell growth, secretion, differentiation, and apoptosis. This implies that the molecule is able to move between cellular membranes. However, the ability of the molecule to undergo such exchange has been largely ignored despite the profound impact that this ability would have on its mechanism of action in signal-transduction cascades. With this in mind, the ability of a long-chain, radioactive ceramide, $^{14}C-C_{16}$-ceramide, to exchange between populations of lipid vesicles was evaluated. The rate of exchange of $^{14}C-C_{16}$-ceramide between lipid vesicles at lipid concentrations commonly found in cells (10−110 mM) was on the order of days ($t_{1/2}$ of 45−109 h). Simultaneous observations revealed negligible exchange of ^{3}H-cholesteryl oleate, which was included as a nontransferable marker to control for artifacts such as vesicle fusion and aggregation. In addition, all of the ceramide was exchangeable, and the exchange followed monoexponential kinetics, indicating that the ceramide underwent transbilayer movement at a rate faster than or equal to its rate of intervesicle exchange. Two conclusions can be drawn from these observations: (i) the spontaneous transfer of ceramide between cellular membranes is too slow to play a role in rapid, inter-membrane signaling phenomena and can only be a factor in cell functions that take place over days; and (ii) without the aid of an exchange protein, ceramide can only interact with target molecules that are located at the membrane where the ceramide is formed.

Ceramide is believed to function as a second messenger in a range of cellular functions from growth and differentiation to apoptosis and secretion (*1*, *2*). Several enzymes have been proposed as regulators of ceramide levels during signaling. The enzyme most widely accepted as being responsible for ceramide production, the magnesium-dependent neutral sphingomyelinase, catalyzes the hydrolysis of sphingomyelin, liberating ceramide and phosphocholine (*1*). The amino acid sequence, fractionation studies, and protease treatment reveal that the neutral sphingomyelinase is most likely an externally oriented, integral, plasma membrane protein (*3*, *4*). However, activity has also been detected in the nuclear membrane, in chromatin (*5*), and on the membrane of the endoplasmic reticulum (ER)[1] (*6*). Other isoforms have been implicated, a magnesium-*in*dependent neutral sphingomyelinase (*7*) and an acid sphingomyelinase (*8*, *9*), and they can be found in the cytosol (*7*) and lysosomes (*10*), respectively. Recent studies have identified extracellular sphingomyelinases in blood that are secreted by platelets and differentiating monocytes, and these enzymes are suspected to transduce signals during development (*11*, *12*).

Ceramide synthase (sphinganine N-acyl transferase) may also play a role in ceramide production and can be found in the mitochondria and on the ER (*13*−*15*). A series of experiments by Bourteele et al. (*14*) has connected glucosylceramide synthase, which catalyzes transfer of glucose from UDP-glucose to the headgroup of ceramide, and sphingomyelin synthase, which catalyzes transfer of phosphocholine from phosphatidylcholine to the headgroup of ceramide, to the ceramide pathway. The authors have shown that the activity of these ceramide-metabolizing enzymes appears to be regulated during cell stimulation by TNF (tumor necrosis factor) (*14*). Metabolic studies suggest that both of these enzymes are found in the trans-Golgi and endosomal compartments with sphingomyelin synthase facing the lumen and glucosylceramide synthase facing the cytosol (*16*). However, an independent study implicates a sphingomyelin synthase located at the plasma membrane as being involved in signaling (*17*). Thus, the enzymes proposed to regulate levels of cellular ceramide during signaling cascades can be found at several subcellular, and even extracellular, locations.

Ceramide has been postulated to exert its effects through regulation of a number of signaling proteins widely distributed throughout the cell. Those include the protein kinases "ceramide-activated protein kinase" (CAPK), c-raf, Src, protein kinase C-α, and -ζ (PKC-α and PKC-ζ) (*18*−*21*), and the protein phosphatases "ceramide-activated protein

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* Corresponding author. Tel: (804) 924-2373. Fax: (804) 924-5069. E-mail: alg4p@virginia.edu.

‡ National Institute of Standards and Technology.

§ University of Virginia.

[1] Abbreviations: $^{14}C-C_{16}$-ceramide, *N*-[palmitoyl-1-^{14}C]-D-sphingosine; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; DAG, diacylglycerol; ER, endoplasmic reticulum; ^{3}H-cholesteryl oleate, [1α,2α(n)-^{3}H]cholesteryl oleate; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PKC-α, protein kinase C-α; PKC-ζ, protein kinase C-ζ; PLA_2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PP2A, protein phosphatase 2A; SUV, small unilamellar vesicles; TNF, tumor necrosis factor.

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phosphatase" (CAPP) and protein phosphatase 2A (PP2A) (*22*−*24*). CAPP can be purified from the cytosol (*22*, *25*), whereas PP2A has been immunocytochemically localized in both the cytosol and the nucleus (*26*). CAPK is primarily bound to microsomal membranes (*19*, *27*). PKC-α (*28*−*30*) and c-Raf (*31*) can be found in both the cytosol and membrane fractions, while PKC-ζ is not associated with membrane but is cytosolic (*20*). Immunocytochemical and cell fractionation studies find Src on secretory granules, on the surface-connected canalicular system membrane, at the plasma membrane, in cytoskeletal fractions, associated with endosomal membranes, and in large adhesion plaques known as podosomes (*32*, *33*).

Finally, distinct pools of sphingomyelin found on the internal leaflet of the plasma membrane (*34*−*36*) and in caveolae (*37*, *38*) have been suggested to serve as "signaling pools" for the ceramide pathway. Thus, the enzymes that regulate ceramide levels, the enzymes regulated by ceramide, and the site of ceramide production can be found in a number of cellular locations.

The possibilities that ceramide can exchange between the various membrane systems within cells, or between cells, are of particular interest. Ceramide may readily diffuse between intra- and intercellular membrane compartments to find its target molecules, or it may be confined to the same membrane where it is produced, requiring its effectors to come to it or an exchange protein for its transfer to another membrane. These considerations have broad implications for the ceramide pathway, and we have therefore addressed the question of ceramide exchange between membranes.

By using artificial membranes composed of long-chain ceramide in a matrix of phosphatidylcholine, we have measured the ability of ceramide to move from small unilamellar vesicles (SUV) to large unilamellar vesicles (LUV). These vesicles can be separated from one another by gel-filtration chromatography. Further, we have used this approach to determine whether ceramide can undergo transbilayer movement or flip between the opposing leaflets of a membrane. Exchange of cholesteryl oleate, which is unable to move between vesicles, has also been examined and serves as an important control that validates the specific exchange of ceramide.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Polar-Lipids Inc. (Alabaster, AL). Cholesteryl oleate and non-hydroxy ceramide (bovine) were from Matreya, Inc. (Pleasant Gap, PA). $^{14}C-C_{16}$-ceramide (*N*-[palmitoyl-1-^{14}C]-D-sphingosine) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and had a specific activity of 55 mCi/mmol. [1α,2α(n)-^{3}H]cholesteryl oleate (44 Ci/mmol) was from Amersham Life Science, Inc. (Arlington Heights, IL). All other materials were from Sigma (St. Louis, MO) unless noted otherwise.

Preparation of Vesicles. Donor, small unilamellar vesicles (SUV) were made by sonication in Buffer A (140 mM NaCl, 25 mM HEPES, 0.25 mM EDTA, pH 7.4) as described (*39*). SUV were composed of POPC/ceramide/cholesteryl oleate (98:1.9:0.1, mole %, respectively) containing 800 000 dpm of $^{14}C-C_{16}$-ceramide and 2 220 000 dpm of ^{3}H-cholesteryl oleate. Briefly, lipids in organic solvents were added to a glass vial and vortexed, and solvents were evaporated under a gentle stream of nitrogen. The lipids were redissolved in choloroform, vortexed, dried down under nitrogen, and left overnight in a desiccator under vacuum. The dry lipid film was hydrated in Buffer A, vortexed, and sonicated on ice under a nitrogen atmosphere 10 times for 30 s until the solution became clear but opalescent. The vesicle suspension was then centrifuged for 1 h at 4 °C at 100000*g* (Ti-90 fixed-angle rotor), and the supernatant containing the SUV was removed and stored at 4 °C until use (*40*). Phospholipid concentration was determined by the phosphate assay described by Bartlett (*41*). Recovery of phosphate from preparation of the SUV was 90% and phosphate concentration in the SUV suspension averaged ∼8.5 mM over several preparations (∼8.8 mM total lipid, including ceramide and cholesteryl oleate).

Acceptor, large unilamellar POPC vesicles (LUV) were made as described (*42*, *43*). POPC was added to a glass vial, the solvent was evaporated under a gentle stream of nitrogen, and the dried lipid was left overnight in a desiccator under vacuum. Buffer A was added to the dry lipid, and the solution was vortexed. The lipid suspension was frozen and thawed 10 times to ensure complete hydration of the vesicles and passed through a hand-held extruder with a 100 μm pore size Costar filter (Cambridge, MA) 31 times. LUV were stored at 4 °C until use. The concentration of POPC in the LUV was determined by phosphate assay (*41*), recovery of POPC in the preparations was greater than 90%, and concentrations ranged from 25 to 172 mM depending on the preparation and the experiment.

Exchange Experiments and Gel-Filtration Chromatography. Exchange of radioactive ceramide from SUV to LUV was determined by gel-filtration chromatography (*44*). "Donor" POPC SUV containing the $^{14}C-C_{16}$-ceramide and ^{3}H-cholesteryl oleate were incubated with an excess of "acceptor" POPC LUV for various times (up to 18 days) at 37 °C. Samples were not stirred during the incubations since it has been previously reported that stirring does not affect the outcome of exchange experiments using SUV or LUV (*45*). Intervesicle transfer of ceramide was the major interest in this study, and cholesteryl oleate was included as a nontransferable marker to control for transfer artifacts such as vesicle fusion and aggregation (*44*). Incubation volumes during incubations ranged from 200 to 360 μL and were contained in small, covered borosilicate glass tubes to minimize changes in volume due to evaporation.

Transfer of ceramide from SUV to LUV after incubations was determined by passing the mixtures over a column of Sepharose 4B-CL (column volume 12.5 mL and length 25 cm). Radioactivity due to $^{14}C-C_{16}$-ceramide or ^{3}H-cholesteryl oleate eluting in the LUV and SUV fractions was determined after mixing each fraction with scintillation fluid (Pico-Flow IV, Packard Instrument Company, Inc., Meriden, CT). Occasionally, fractions from the column were also analyzed by phosphate assay (*41*) to ensure separation of LUV from SUV and to determine recovery. Recovery of lipid after incubation and column separation averaged ∼80% as determined by scintillation counting as well as by phosphate assay.

The radioactivity in SUV and LUV was determined by using a nonlinear least-squares analysis to fit 2 Gaussian shaped curves to the elution profile (Microcal Origin 5.0,

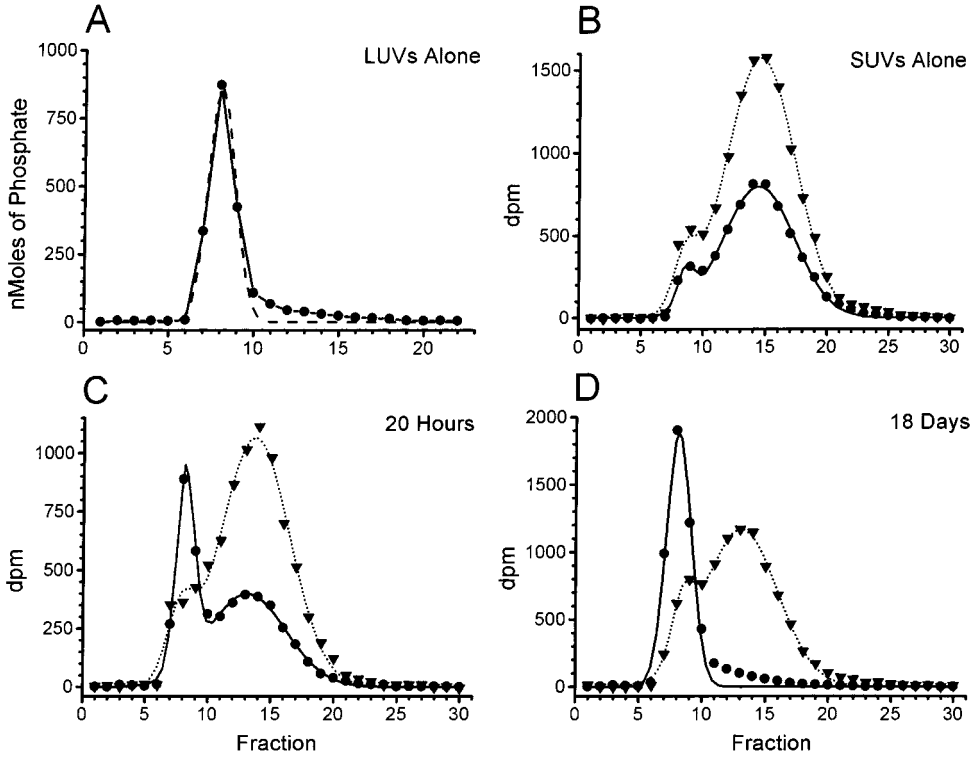

FIGURE 1: Characterization of LUV and SUV used to study ceramide exchange. (A) LUV were passed over Sepharose 4B−CL and fractions analyzed by phosphate assay (circles connected by solid line). Note that there is a small error associated with fitting a Gaussian peak (dashed line) to the data that was accounted for during our analysis as described in the Materials and Methods. (B) SUV were passed over the column and fractions analyzed for $^{14}C-C_{16}$-ceramide (circles) and ^{3}H-cholesteryl oleate (down-triangles) in a scintillation counter. Each profile was fit to two Gaussian peaks (one for LUV and one for SUV), and the sums of these two peaks are shown (solid line for $^{14}C-C_{16}$-ceramide and dotted line for ^{3}H-cholesteryl oleate). (C) LUV (107 mM) and SUV (3 mM) were mixed, incubated for 20 h at 37 °C, and separated on the column, and the fractions were analyzed for $^{14}C-C_{16}$-ceramide (circles) and ^{3}H-cholesteryl oleate (down-triangles) in a scintillation counter. Each profile was fit with two Gaussian peaks, and the sums of these two peaks are shown (solid line for $^{14}C-C_{16}$-ceramide and dotted line for ^{3}H-cholesteryl oleate). (D) LUV (107 mM) and SUV (3 mM) were mixed, incubated 18 days at 37 °C, and analyzed as in C. Note that all of the $^{14}C-C_{16}$-ceramide has moved to the LUV fractions (circles) by 18 days and that the ^{14}C profile is nearly identical to the "phosphate" profile of LUV alone (A). The data presented are from a representative experiment of three that were performed for each condition.

Northhampton, MA). One curve was fit to the LUV fractions while another was fit to the SUV fractions. The area under the LUV curve versus the sum of the area under both curves was used to determine the percent of counts transferred from SUV to LUV during the incubations. Column profiles of the original SUV or LUV preparations were similar to those previously described (*39*). SUV preparations were 94% SUV and 6% LUV/MLV (MLV, multilamellar vesicles) (Figure 1B), while LUV preparations contained no detectable SUV (Figure 1A).

As previously described (*39*), the profile of LUV eluted from Sepharose 4B-CL is not a perfect Gaussian distribution but is somewhat asymmetrical. This asymmetry caused a significant underestimation of exchange. The area under the curve of a Gaussian fit to the phosphate assay profile of LUV was only 84.6% of the sum of the actual data points (Figure 1A). Thus, we corrected the value for "percent in LUV" in each experiment by multiplying this value by 1.182.

Thin-Layer Chromatography. Purity of lipids was routinely checked by thin-layer chromatography. Lipids were separated on Whatman (Clifton, NJ) 60 Å silica gel TLC plates. For ceramide analysis, lipids were separated in chloroform/methanol/25% ammonia (90:10:1, v/v.). For cholesteryl oleate and POPC analysis, lipids were separated in cyclohexane/ethyl acetate (80:20, v/v) and chloroform/methanol/water (65:25:4, v/v), respectively. Location of ceramide, cholesteryl oleate, and POPC on the plates after separation was determined by running standards. Total lipids were visualized by staining the plates with iodine vapor, while radiolabeled lipids were detected by spraying plates with EN^3-HANCE (New England Nuclear, Boston, MA) and exposing the plates to radiography film at −70 °C. Densitometry of the iodine stain or autoradiographs was used to quantitate the relative amounts of lipids on the plates. All preparations were greater than 97% pure, even after the longest incubations of 18 days at 37 °C or storage at 4 °C for several months.

Kinetic Analysis. For determination of halftimes of exchange, the data were adjusted for initial contamination of the SUV by larger vesicles. For example, when SUV alone were passed over Sepharose 4B-CL, 6.9% of the $^{14}C-C_{16}$-ceramide counts and 7.4% of the ^{3}H-cholesteryl oleate counts eluted in the LUV fractions. The contaminating LUV/MLV are seen as a small shoulder on the SUV peak (Figure 1B), and this is typical for sonicated lipid dispersions (*39*, *46*).

The data were also normalized for the theoretical maximum amount of exchange that could be observed. The molar ratio of acceptor to donor vesicles in the experiments with 10 or 20 mM total lipid was 5:1. Thus, the theoretical maximum amount of exchange that could be observed was 83.3%. The molar ratio of acceptors to donors in the experiments with 110 mM total lipid was 34:1, and the

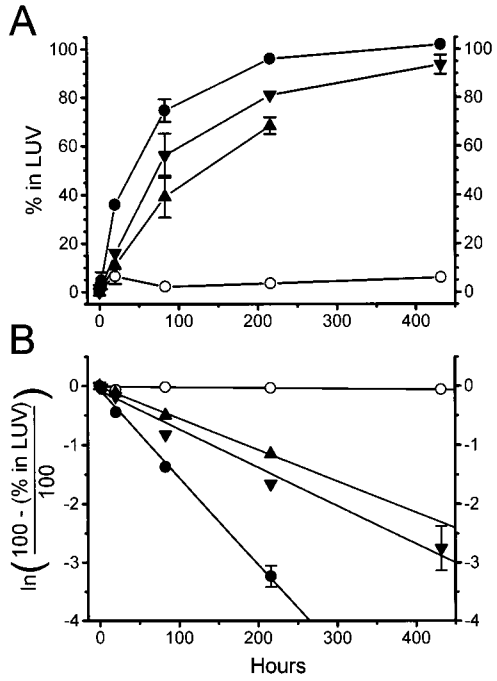

FIGURE 2: Transfer of $^{14}C-C_{16}$-ceramide from SUV to LUV. (A) A plot of the percent $^{14}C-C_{16}$-ceramide (solid symbols) or ^{3}H-cholesteryl oleate (open symbols) that was found in the LUV fractions after mixtures of LUV and SUV were incubated at 37 °C for various times is shown. Total lipid concentration for the experiments was 10 mM (up-triangles, 8.3 mM LUV and 1.7 mM SUV), 20 mM (down-triangles, 16.7 mM LUV and 3.3 mM SUV), or 110 mM (circles, 107 mM LUV and 3 mM SUV). The data have been corrected for initial contamination of SUV with large vesicles and theoretical maximum exchange as described in the Materials and Methods. The data points have been connected by solid lines. (B) The data from (A) have been expressed as "fraction remaining in donors" (X = [100 − (% in LUV)]/100), and the ln(X) has been plotted against time. Symbols for the data points correspond to the same conditions as delineated in (A). The solid lines represent fits derived from linear regression analysis ($r \geq 0.99$). In both (A) and (B), each data point represents the average ±SEM of triplicate experiments. When error bars are not shown, they were smaller than the height of the data points themselves.

theoretical maximum amount of exchange that could be observed was 97.1%.

After normalizing the data as outlined above (Figure 2A), we determined apparent first-order halftimes using the analysis described by Jones and Thompson (*47*). Their analysis is based on the generally accepted theory that exchange of lipid between bilayers occurs through and is limited by desorption of lipid monomers from the bilayer into the aqueous phase (*47*−*49*). The "percent in LUV" (or percent transfer) was converted to the "fraction of lipid remaining in the donors" given by X = [100 − (% in LUV)]/100. The slope of ln(X) versus time (Figure 2B) was determined by linear regression, and an apparent first-order rate constant, k_1 (h^{-1}), was calculated by $k_1 = (-\text{slope}[([D] + [A])/[A]])$, where [D] is the concentration of donor lipid (SUV) and [A] is the concentration of acceptor lipid (LUV). The term ([D] + [A])/[A] is included to correct for back transfer from the aqueous phase to the donors. The apparent first-order halftime, $t_{1/2}$, was calculated from $t_{1/2} = (\ln 2)/k_1$.

RESULTS

We have examined the ability of ceramide to move from "donor" POPC SUV to "acceptor" POPC LUV. The donors contained $^{14}C-C_{16}$-ceramide as well as ^{3}H-cholesteryl oleate, and the latter served as a nontransferable marker to control for vesicle fusion and aggregation. Excess acceptors contained only POPC and were incubated with small amounts of donors for various times at 37 °C. At the end of the incubations, the reaction mixtures were passed over a column of Sepharose 4B-CL to separate LUV from SUV (described in Materials and Methods), and fractions were counted in a scintillation counter to determine how much of the $^{14}C-C_{16}$-ceramide or ^{3}H-cholesteryl oleate had transferred from the SUV to the LUV.

In Figure 1A, LUV alone were passed over the column and fractions were analyzed for phosphate (circles connected by solid line) to characterize the preparation. The preparation is nearly homogeneous, although the peak is not a symmetrical Gaussian distribution (dashed line) as would be predicted from theory. However, this result is typical for such preparations (*39*, *43*). Our analysis of these peaks has included a correction factor (1.182) for this discrepancy, as discussed in Materials and Methods. In Figure 1B, SUV alone have been passed over the column and fractions counted for $^{14}C-C_{16}$-ceramide (circles) and ^{3}H-cholesteryl oleate (down-triangles). Both profiles are nearly symmetrical Gaussian distributions that have a small shoulder on the left. Such shoulders arise from contamination by larger-sized vesicles and are typical for sonicated lipid dispersions (*39*), amounting to ~7% of the $^{14}C-C_{16}$-ceramide or ^{3}H-cholesteryl oleate in the system. This contamination was taken into account in our analyses, as described in Materials and Methods.

Figure 1 panels C and D illustrate profiles of mixtures of SUV (3 mM) and LUV (107 mM) that have been incubated for 20 h or for 18 days (432 h), respectively. The amount of $^{14}C-C_{16}$-ceramide that eluted with the LUV (circles) increased progressively with time as the $^{14}C-C_{16}$-ceramide transferred from SUV to LUV, reaching 100% transfer by 18 days. In contrast, the amount of ^{3}H-cholesteryl oleate (down-triangles) that eluted with the LUV increased only slightly with time, attaining only 6% by 18 days.

Similar experiments were performed for various times and at 3 different lipid concentrations (Figure 2A). The rate of transfer of $^{14}C-C_{16}$-ceramide was dependent on the total concentration of lipid in the system, as can be seen from a comparison of experiments with 10 mM (up-triangles), 20 mM (down-triangles), or 110 mM (solid circles) lipid. Transfer of ^{3}H-cholesteryl oleate during the incubations with 110 mM lipid (open circles) was negligible (6%) during the incubation, indicating that transfer of $^{14}C-C_{16}$-ceramide was not a result of vesicle fusion or aggregation.

Transfer of $^{14}C-C_{16}$-ceramide approached 100% in experiments with 10 or 20 mM lipid and reached 100% in experiments with 110 mM lipid (Figure 2A). Thus, ceramide from both leaflets of the donor SUV was able to exchange to the LUV during the incubations. Since 67 mol % of the lipid in SUV resides in the outer leaflet of these vesicles (*50*), the extent of $^{14}C-C_{16}$-ceramide transfer would be expected to approach only 67% if ceramide located in the inner leaflet of the donor SUV was unable to undergo transbilayer movement and exchange to the LUV (*51*, *52*).

Apparent first-order halftimes for $^{14}C-C_{16}$-ceramide exchange can be derived from a kinetic analysis of the data as described in the Materials and Methods (*47*). When the data

Table 1: Apparent First-Order Halftimes for Intervesicle Exchange of C_{16}-Ceramide and Cholesteryl Oleate

	lipid (mM)[a]	A/D[b]	$t_{1/2}$ (h) ± SEM[c]
C_{16}-ceramide	110	34	45 ± 1
	20	5	89 ± 5
	10	5	109 ± 4
cholesteryl oleate	110	34	>5000
	20	5	>5000
	10	5	>5000

[a] Total lipid concentration during the experiment. [b] Ratio of acceptors to donors. [c] Apparent first-order halftime. Means and errors are from 3 separate experiments.

in Figure 2A are plotted as the natural log of "the fraction remaining in donor vesicles" (X = [100 − (% in LUV)]/100) versus time (Figure 2B), the slope of the plot can be used to calculate an apparent first-order rate constant by k_1 = (−slope[([D] + [A])/[A]]). [D] is the concentration of donor lipid (SUV), [A] is the concentration of acceptor lipid (LUV), and the halftime for exchange relates to k_1 by $t_{1/2}$ = ln 2/k_1. The linear regression analyses depicted in Figure 2B each yielded a correlation coefficient (r) of 0.99 or better, and the apparent halftimes calculated from the slopes produced by these fits are given in Table 1. The apparent halftimes (Table 1) for $^{14}C-C_{16}$-ceramide exchange at different lipid concentrations differed significantly from one another ($p < 0.05$). Thus, ceramide exchange between lipid vesicles at the concentrations examined depended on lipid concentration. This observation suggests that ceramide exchanges by a combination of events, and as described by Jones & Thompson (*47*) for POPC, by a first-order and by a second-order process.

The first-order process involves lipid exchange via monomers through the aqueous phase and is independent of concentration. At higher concentrations of lipid, exchange can also occur by a second-order process involving collisions of vesicles in a manner dependent on lipid concentration. Thus, at high lipid concentrations (10−110 mM), ceramide exchanges both as a soluble monomer and by vesicle collisions (*47*). The observations that (i) ceramide exchange can be described by a monoexponential (Figure 2B) and that (ii) all of the $^{14}C-C_{16}$-ceramide is exchangeable (Figure 2A) imply that transbilayer movement of ceramide occurs at a rate faster than or equal to intervesicle exchange. If the rate of transbilayer movement of $^{14}C-C_{16}$-ceramide were slower but comparable to its rate of intervesicle exchange, then nonexponential kinetics would be expected and the plots in Figure 2B would not be linear (*53*).

DISCUSSION

Recent research has prompted re-evaluation of ceramide's role as a second messenger (*54*). This re-evaluation has focused on several areas including concern about assays for quantifying ceramide and the effects of ceramide analogues often used for in vitro studies. Thus, the common method to measure changes in levels of intracellular ceramide during a signaling event, the diacylglycerol-kinase assay (DAG-kinase assay), can be misleading since DAG-kinase may be regulated by second messengers produced by stimulated cells. Watts et al. (*55*) observed an increase in cellular ceramide in stimulated cells using the DAG-kinase assay but not when using a mass spectrometric analysis. In addition, C_2-ceramide, a short-chain ceramide frequently employed as an analogue of ceramides of natural origin, may not adequately mimic long-chain, endogenous ceramides. Short-chain ceramides (C_2-, C_6-, and C_8-ceramide) and C_{16}-ceramide have opposite effects on the ordering of the acyl chains in phospholipid vesicles, while C_{16}-ceramide, the more natural ceramide, induces lipid phase separation as well (*56*). The ceramide-induced membrane perturbations also exerted opposing effects on phospholipase A_2 (PLA_2) activity. The short-chain ceramides inhibit PLA_2 activity, while C_{16}-ceramide causes activation (*56*). Moreover, the effects of C_2-ceramide on platelets reflect those of detergents by inhibiting aggregation, increasing membrane permeability, and causing cell lysis (*57*). These inconsistencies in the ceramide literature deserve consideration and require further study.

Our present studies have evaluated the intervesicle exchange of a radiolabeled, natural-length ceramide molecule ($^{14}C-C_{16}$-ceramide) with implications for intercellular and inter-membrane ceramide signaling. Previous studies of ceramide exchange in a cell-free system have used fluorescently labeled, short-chain ceramide analogues (*58*, *59*). Shortening ceramide's acyl chain can affect its physical properties, and addition of a fluorophore may confound this issue. In the current study, the rate of exchange of $^{14}C-C_{16}$-ceramide has not been influenced by the attachment of a fluorophore or by the decreased hydrophobicity that results from using an analogue containing a shortened acyl chain.

Our results show that ceramide exchange between lipid vesicles required days, implying that this process is too slow to be a significant factor in rapid, inter-membrane signal transduction events. The observations that all of the ceramide was exchangeable (Figure 2A) and that the kinetics of its exchange followed monoexponential kinetics show that ceramide was able to flip between the opposing leaflets of the bilayer at a rate faster than or equal to intervesicle exchange. In addition, the SUV to LUV exchange assay used herein is well established and has been used to determine the rates of ^{3}H-sphingomyelin (*44*) and ^{3}H-galactosyl-ceramide (*60*) exchange between vesicles. Thus, our experimental approach is proven and should provide an accurate measurement of intervesicle $^{14}C-C_{16}$-ceramide exchange that is relevant to movement of endogenous ceramides.

Application of our observations to ceramide-signaling in vivo must take into account several factors; ionic strength, bilayer charge, bilayer curvature, and lipid concentration. First, increasing salt concentration above 200 mM can inhibit lipid exchange (*45*). However, our experiments were conducted at a physiological salt concentration (140 mM) where the rates of lipid exchange are not sensitive to ionic strength. Second, charged vesicles repel one another, inhibiting collision-mediated lipid exchange (*47*). Thus, charge only affects exchange at high lipid concentrations where collisional exchange becomes prominent. Our assay used neutral vesicles at high concentration, suggesting that the rates of exchange we have observed could be somewhat faster than what might be expected to occur in cells, since biological membranes typically possess a net negative charge.

Third, lipid exchange can be enhanced by vesicle collisions, which become more frequent at high lipid concentrations. In the case of POPC exchange between SUV, collision-mediated exchange becomes significant at concentrations greater than 3 mM lipid (*45*, *47*). Similarly, the rate of

ceramide exchange increased with lipid concentration over the range of concentrations we have studied (10−110 mM). We chose to examine ceramide exchange at high lipid concentrations (10−110 mM), where vesicle collisions are more frequent, to assess what might occur at physiological lipid concentrations. For instance, the lipid concentration in red blood cells, which have a relatively modest amount of lipid membrane per cell, is ∼10 mM (*61*). In contrast, blood platelets, which have an extensive membrane system consisting of caveolae, an open canalicular system, and numerous secretory granules, have a lipid concentration of ∼100 mM in each cell (*62*, *63*). Nevertheless, it is possible that the "collisional" contribution to the rates of ceramide exchange we have observed in vitro may be lessened in vivo by the charge repulsion expected to occur between typical biological membranes.

Fourth, in the case of POPC exchange between vesicles, the rate of exchange between LUV is slightly slower than that between SUV (*45*). This implies that desorption of lipid monomers from SUV, which are highly curved and have strained molecular packing conditions, is more favorable than desorption from relatively "relaxed" LUV. In the current study, we determined the rate of ceramide exchange from SUV to LUV. If exchange between LUV is more applicable to an in-vivo situation, then the rates of ceramide exchange that we have observed could be somewhat faster than ceramide exchange in vivo, further reinforcing our conclusion that spontaneous ceramide exchange is unlikely to be important with regard to its role as a signaling molecule.

Our in vitro results support and extend observations made in vivo by Chatelut et al. (*64*) that natural ceramide formed in lysosomes is unable to escape this cellular compartment. When cells derived from patients with Farber's disease, who lack ceramidase activity, are labeled with radioactive sphingomyelin (radiolabeled in the ceramide moiety), the sphingomyelin is degraded into radioactive ceramide, which accumulates in lysosomes. Even after 24 h, cellular fractionation studies revealed that the ceramide was still trapped in the lysosomes. In contrast, radioactive ceramide produced in lysosomes of control cells is metabolized and translocates to other cellular compartments (*64*). Similar to the present study, these results suggest that exchange of natural ceramides between lipid membranes requires days.

In sharp contrast, it takes less than a minute for short-chain, fluorescent ceramide analogues (C_6−NBD-ceramide and C_5-DMB-ceramide) to exchange between lipid vesicles (*58*, *59*). These analogues also undergo transbilayer movement, C_5-DMB-ceramide with a halftime of 20 min. As discussed by the authors, these analogues were intended for exploration of the general principles that govern the exchange process, and the transfer rates derived from their study cannot be directly applied to the endogenous molecules that they represent. Our studies did not provide an exact halftime for transbilayer movement (flip-flop) of ceramide, although they support the conclusion that transbilayer movement occurs at a rate faster than or equal to the rate of intervesicle exchange. Thus, it could be that endogenous long-chain ceramides rapidly equilibrate across lipid bilayers as does C_5-DMB-ceramide. This possibility seems likely since the reduced hydrophobicity resulting from the absence of a polar headgroup on diacylglycerol allows it to traverse the bilayer in 15 s, while one of its parent molecules, which contains a polar headgroup, phosphatidylglycerol, takes 8 days (*65*).

Taken together, our results suggest that spontaneous ceramide exchange between lipid membranes is too slow to participate in a majority of signaling events, which take place in seconds, minutes, or hours. It could be more likely that ceramide's potential target molecules diffuse to the site of its production to interact with this second messenger, an appealing possibility when considering that many of its targets are soluble. Ceramide might also act locally by changing the physical properties of the membrane where it is synthesized, an equally attractive prospect since formation of C_{16}-ceramide microdomains in phosphatidylcholine bilayers correlates with changes in PLA_2 activity (a potential ceramide target) (*56*). Another option involves delivery of ceramide to its targets by a lipid-transfer protein. Indeed, movement of ceramide from the ER to the Golgi is not mediated by vesicle trafficking (*66*), implicating either diffusion or a transfer protein as a means of transport. Our study suggests that diffusion is unlikely to contribute to ceramide transport, but it is consistent with the existence of a ceramide transfer protein (*66*). The hypothesis that ceramide transport is mediated by a transfer protein during signaling events is not unfounded since transfer proteins are a large and diverse family of intracellular, lipid-binding proteins that can be both specific and nonspecific (*67*).

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