The capacity of ceramides to modify the permeability barrier of cell membranes has been explored. Membrane efflux induced either by in situ generated ceramides (through enzymatic cleavage of sphingomyelin) or by addition of ceramides to preformed membranes has been studied. Large unilamellar vesicles composed of different phospholipids and cholesterol, and containing entrapped fluorescent molecules, have been used as a system to assay ceramide-dependent efflux. Small proportions of ceramide (10 mol % of total lipid) that may exist under physiological conditions of ceramide-dependent signaling have been used in most experiments. When long chain (egg-derived) ceramides are used, both externally added or enzymatically produced ceramides induce release of vesicle contents. However, the same proportion of ceramides generated by sphingomyelinase induce faster and more extensive efflux than when added in organic solution to the preformed vesicles. Under our conditions 10 mol % of N-acetyl-sphingosine (C2-ceramide) did not induce any efflux. On the other hand, sphingomyelinase treatment of bilayers containing 50 mol % sphingomyelin gave rise to release of fluorescein-derivatised dextrans of molecular mass $\approx 20$ kDa, i.e. larger than cytochrome c. These results have been discussed in the light of our own previous data (Ruiz-Argüello, M. B., Basañez, G., Goñi, F. M., and Alonso, A. (1996) J. Biol. Chem. 271, 26616–26621) and of the observations by Siskind and Colombini (Siskind, L. J., and Colombini, M. (2000) J. Biol. Chem. 275, 38640–38644). Our spectroscopic observations appear to be in good agreement with the electrophysiological studies of the latter authors. Furthermore, some experiments in this paper have been designed to explore the mechanism of ceramide-induced efflux. Two properties of ceramide, namely its capacity to induce negative monolayer curvature and its tendency to segregate into ceramide-rich domains, appear to be important in the membrane restructuring process.

Ceramides have emerged in the last decade as important messengers in cell signaling involved among others, in processes of cell differentiation, growth suppression, and apoptosis. Mechanisms of ceramide-mediated signal transduction are now starting to be understood (see Refs. 1 and 2, for reviews). One striking property of ceramides, that may be linked to their physiological effect, is their capacity to restructure the permeability barrier of model and cell membranes, thus giving rise to vesicle or cell efflux. Ceramide-induced release of aqueous contents from liposomes and resealed erythrocyte ghosts was first observed by Ruiz-Argüello et al. (3), who induced in situ generation of ceramide by treating the sphingomyelin-containing model or cell membranes with bacterial sphingomyelinase. Ceramide-induced membrane efflux may be important physiologically, e.g. in generating local ion fluxes, or even in the release of large molecules, like cytochrome c, whose efflux from mitochondria is crucial for the activation of apoptosis.

In more recent years, a number of studies have dealt with the issue of membrane rearrangement by ceramides. Simon and Gear (4) found that N-acetyl-sphingosine (C2-ceramide), a short chain ceramide, caused release of $^{3}$H]adenine from platelets at a ceramide:l lipid ratio of 0.2. Ghafourifar et al. (5) showed that C2- and C6-ceramide induced cytochrome c release from isolated mitochondria. Di Paola et al. (6) showed cytochrome c release from isolated mitochondria induced by C2- and C16-ceramide. However, Di Paola et al. (6) observed that C2-ceramide, but not N-palmitoylsphingosine (C16-ceramide, an abundant natural ceramide) was able to induce efflux from the inner mitochondrial membrane in isolated mitochondrial suspensions as evidenced from its ability to dissipate the inner mitochondrial membrane potential. Also recently, Siskind and Colombini (7) used electrophysiological methods to demonstrate the formation of stable pores by short and long chain ceramides in planar lipid bilayers. In their studies ceramides in organic solvent were added to preformed bilayers or admixed with the component lipids at total ceramide:l lipid ratios of 0.05. Pore formation was observed after a few minutes.

In the present study, we have attempted to answer a number of questions that remain open after publication of the above data, in particular the possibility of inducing the release of large molecules (the size of cytochrome c) by ceramide, the existence of common aspects in the efflux induced by in situ enzyme-generated and by externally added ceramide, and the correlation between electrophysiological observations of channel formation and biochemical/biophysical detection of release of vesicle or cell contents. For that purpose we have prepared

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1 The abbreviations used are: C2-ceramide, N-acetyl-sphingosine; C6-ceramide, N-hexanoylsphingosine; LUV, large unilamellar vesicles; ANTS, 8-amino-1-naphthalene-1,3,6-trisulfonic acid; DPX, p-xylenebis (pyridinium bromide); PTTC, fluorescein isothiocyanate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; Ch, cholesterol; DPPC, dipalmitoylphosphatidylcholine; DEPE, dielaidoylphosphatidylethanolamine.
large unilamellar vesicles with varying lipid compositions, sphingomyelin ranging between 0 and 50 mol % of the total lipid and loaded them with water-soluble fluorescent molecules. Ceramides have been generated \textit{in situ} by sphingomyelinase, or added to the preformed vesicles, or mixed with the other lipids in the process of liposome preparation. Our results show that the presence of ceramides can indeed allow the efflux of large molecules (molecular mass $\sim 20$ kDa) through membranes, that both \textit{in situ} generation and external addition of ceramides can induce release, and that electrophysiological detection of pore formation correlates with release of vesicular contents (although with significant differences in the time scale of detection of the phenomena). Moreover, the role of the molecular geometry of ceramides, and of their immiscibility with other lipids in the mechanism of membrane permeabilization are supported by the experimental data.

### MATERIALS AND METHODS

Sphingomyelinase (EC 3.1.4.12) from \textit{Bacillus cereus} was supplied by Sigma. Egg PC and egg PE were purchased from Lipid Products (South Nutfield, UK). Egg SM, egg ceramide, lyso-PC, and plant asolectin were from Avanti Polar Lipids (Alabaster, AL). Plant asolectin (soya bean phospholipids) contained 46% phosphatidylcholine, 22% phosphatidylethanolamine, 18% phosphatidylinositol, 7% phosphatidic acid, 7% others, according to the manufacturer. ANTS and DPX were supplied by Molecular Probes, Inc. (Eugene, OR). Ch was from Sigma, and FITC-dextran was from Serva (Frankfurt, Germany).

Large unilamellar vesicles (LUV) of diameters 100–150 nm were prepared by the extrusion method (8) using Nucleopore filters of 0.1 μm pore diameter at room temperature, in 10 mM HEPES, 200 mM NaCl, 10 mM CaCl$_2$, 2 mM MgCl$_2$, pH 7.0. Quantitative analysis of the LUV preparations, as described by Ruiz-Aranguillo \textit{et al.} (3), showed that their composition did not differ significantly from the initial lipid mixture. All experiments were performed at 37 °C, except when asolectin vesicles were involved, in which case temperature was 20 °C, because of the large spontaneous permeability of asolectin vesicles at 37 °C. Lipid concentration was 0.3 mM, and sphingomyelinase was used at 1.6 units/ml.

Sphingomyelinase activity was assayed by determining phosphorus contents in the aqueous phase of an extraction mixture (chloroform: methanol, 2:1) after addition of aliquots from the reaction mixture at different times. Because of the 1.1 ceramide:phosphate stoichiometry of the enzyme products, enzyme activities could be given either as ceramide production or as phosphate production. Since commercial sphingomyelinase preparations may contain phospholipase C impurities, 2 mM o-phenanthroline (9) was routinely added in all our enzyme assays. In the absence of this specific inhibitor of phospholipase C, thin layer chromatography experiments demonstrated that only SM, and not PC or PE, were degraded by the enzyme.

Vesicle efflux was usually assayed with the ANTS:DPX fluorescent system (10). Alternatively, FITC-derived dextran of molecular mass 20,000 was entrapped in the vesicles. Details on the use of these fluorescent probes, including assay calibration, have been given elsewhere (11, 12). Fluorescence measurements were performed in an Aminco Bowman Series 2 luminescence spectrometer.

Ceramide was introduced in the vesicle membranes by one of these four procedures: (i) sphingomyelinase (0.8 unit) was added to 0.5 ml of vesicle suspension, 0.3 mM in lipid (3); (ii) ceramide was dissolved (3.75 mM) in dodecane/ethanol (2:98 by volume) (13) and a small volume (2–4 μl) added to 0.5 ml of vesicle suspension (0.3 mM in lipid) in the cuvette; (iii) ceramide was dissolved (3.75 mM) in ethanol at 40 °C and a small volume (2–4 μl) added to 0.5 ml of vesicle suspension; and (iv) ceramide was mixed in chloroform-methanol with the other lipids at the beginning of the process of vesicle preparation, then solvent was evaporated and the lipid mixture hydrated in buffer.

### RESULTS

#### In Situ Generated Ceramides

When large unilamellar vesicles composed of SM:PE:Ch (2:1 mole ratio) were treated with sphingomyelinase under the conditions described under “Materials and Methods,” ceramide was generated within the lipid bilayers as a result of sphingomyelin cleavage (Fig. 1A). When the vesicles were loaded, under isotonic conditions, with water-soluble fluorescent probes, efflux could be observed concomitantly with ceramide production (Fig. 1B). In a previous study (3) we had described the release of low molecular weight markers, i.e. ANTS, DPX, from the vesicles. We have now extended these observations to include the release of larger molecules, of molecular masses up to 20 kDa. As seen in Fig. 1B, fluorescent dextrans the size of small proteins, e.g. cytochrome c, could be released through the activity of sphingomyelinase on SM-containing bilayers.

In another series of experiments, the proportion of sphingomyelin in the bilayer, thus of ceramide formed by enzyme action, was varied. For this purpose a number of LUV preparations were made, of general composition SM:PC:PE:Ch (X:Y:Z:W, mole ratio), such that X + Y = 0.5, and X varied from 0 (control) to 50. Fig. 2 shows the kinetics of ceramide production and ANTS release from LUV composed of SM:PC:PE:Ch (10:40:25:25, mole ratio). Under these conditions release was slow, note the time scale in the \textit{abscissa}, as compared with Fig. 1. Virtually no release was observed in the first 10 min after enzyme addition. When the proportion of SM in the bilayer was increased, at least in the 0–50% range, both the initial rates and extents of probe liberation increased accordingly (Fig. 3). This result shows that efflux is very sensitive to the concentration of ceramide in the bilayer.

#### Externally Added Ceramides

The above results, and those published by Siskind and Colombini (7) (see Introduction), prompted us to study in our system the effect of adding ceramides to lipid bilayers. This was done in either of two forms: (i) co-dissolving ceramide with phospholipids in organic solvent, then drying and preparing the liposomes in the usual way or (ii) adding a small volume of ceramide in organic solvent to a suspension of vesicles. In the latter case, ceramides were dissolved either in dodecane:ethanol (2:98, by volume) at room temperature or in absolute ethanol at ca. 40 °C. The bilayer lipid composition was either SM:PE:Ch (2:1:1, mole ratio), or...
Fig. 2. Ceramide-induced efflux of ANTS-DPX. Ceramide was generated by sphingomyelinase action on SM:PC:PE-Ch (10:40:25:25) LUV. A, ceramide production, expressed as mol % of total lipid. B, release of entrapped ANTS-DPX. —, control, in the absence of enzyme; ○, in the presence of sphingomyelinase, 1.6 units/ml.

The effect of short chain ceramides (i.e. N-acylsphingosine with C₂ or C₆ acyl chains) on membrane restructuring was also tested in a similar way. Only ethanol was used as solvent, since both C₂- and C₆-ceramides were highly soluble in it. The results are also summarized in Table I (bottom lines). C₂-ceramide induced some efflux on SM:PE-Ch but not on asolectin bilayers, while C₆-ceramide was inactive on both systems. While C2-ceramide was inactive on both systems, induced some efflux on SM:PE:Ch but not on asolectin bilayers, LUV, generated by sphingomyelinase action on SM:PC:PE:Ch (10:40:25:25) (2, 3, 14) that the tendency of ceramides to induce a "negative" curvature properties (for the convention of negative and positive curvatures see (15)). To test this hypothesis, we prepared LUV whose bilayers contained lysophosphatidylethanol, a lipid with the same headgroup as SM, but inducing a positive curvature because of its peculiar geometry. Fig. 5A shows the effect of lyso-PC on the passive efflux of ANTS induced by 5% ceramide (added in dodecane/ethanol) in SM:PC:Ch (5:1, mole ratio), or plant asolectin. The first composition was also used by Ruiz-Argüello et al. (3), and the last two were used by Siskind and Colombini (7). With asolectin vesicles measurements were performed at 20 °C, because the liposomes had a very high spontaneous permeability at 37 °C, the temperature at which our experiments were routinely conducted. In all cases, a control experiment ("0% ceramide") was performed in which only the organic solvent was added.

The results of ceramide-induced efflux are summarized in Table I, and some selected observations are shown in Fig. 4. Ceramides added to bilayers, the latter either in the process of preparation or already formed in water, did elicit a certain degree of release of ANTS, although at a slower rate and to smaller extent than enzymatically generated ceramides (see, e.g. Figs. 2B and 4A). Bilayer lipid composition was important in the process, PC:Ch being more stable than SM:PE:Ch or asolectin (see Table I) (more on this subject below). Ceramide solvent was also significant when ceramides were added to preformed liposomes, dodecane/ethanol being consistently more efficient than warm ethanol (Table I).

The effect of short chain ceramides (i.e. N-acylsphingosine with C₂ or C₆ acyl chains) on membrane restructuring was also tested in a similar way. Only ethanol was used as solvent, since both C₂- and C₆-ceramides were highly soluble in it. The results are also summarized in Table I (bottom lines). C₂-ceramide induced some efflux on SM:PE-Ch but not on asolectin bilayers, while C₆-ceramide was inactive on both systems.

The Role of Lipid Geometry—We have suggested elsewhere (2, 3, 14) that the tendency of ceramides to induce a "negative" curvature in the bilayer could be related to its membrane-restructuring properties (for the convention of negative and positive curvatures see (15)). To test this hypothesis, we prepared LUV whose bilayers contained lysophosphatidylcholine, a lipid with the same headgroup as SM, but inducing a positive curvature because of its peculiar geometry. Fig. 5A shows the effect of lyso-PC on the passive efflux of ANTS induced by 5% ceramide (added in dodecane/ethanol) in SM:PC:Ch (2:1:1) bilayers. Lyso-PC caused a clear inhibition of ceramide-induced release that was particularly evident at longer incubation times (after 6 h). The effect when ceramide was enzymatically generated in the bilayer is interesting (Fig. 5B). Lyso-PC shifted the efflux curve toward longer times, i.e. a lag time of ~200 s was observed. The simplest explanation is that while the number of ceramide molecules was smaller than that of lyso-PC molecules the geometric effects of each other were compensated, release starting only when the ceramide molecules "outnumbered" or overcame the opposite influence of lyso-PC.

**DISCUSSION**

The main results in this paper concur in demonstrating that ceramides induce the rearrangement of lipid bilayers, irrespective of the method followed to bring them into the membrane. However, important differences in the kinetics and extent of efflux exist, according to the experimental procedures used. Thus there are two main topics for discussion in this context, the mechanism of bilayer restructuring, that may allow even the passage of macromolecules, and the concordance of the different experimental methods.

The Mechanism of Bilayer Restructuring and Efflux—In our previous studies (2, 3, 14) we have suggested that two properties of ceramides may be related to the release effect, namely the ceramide geometry, that promotes the negative curvature of a lipid monolayer and its tendency to form ceramide-rich domains segregated in the plane of the membrane. As a result of its geometrical constraints ceramide facilitates the lamellar-
to-hexagonal transitions in lipid bilayers (3, 14), and such transitions, even if localized at certain points in the membrane, are likely to allow extensive communication between the inner and outer compartments. Perhaps not even the actual transition is required to take place, but the “propensity” (16) of the bilayer to adopt the hexagonal form is enough to destabilize transiently the bilayer. This would explain our observations (3) of efflux in the absence of nonlamellar signals in $^{31}$P NMR spectra.

The role of ceramide geometry in promoting efflux is clearly supported by the data in Fig. 5, in which the presence of lyso-PC in the bilayers counteracts the effects of ceramide. In fact, the geometry of lyso-PC opposes that of ceramide, the former favoring positive curvature of monolayers, or, in other words, micelle formation (15, 17). PE, a lipid that favors negative curvature, has the opposite effect than lyso-PC, i.e., it enhances the effect of ceramide. This is demonstrated by the higher efflux from SM:PE:Ch bilayers as compared with PC:Ch bilayers (other conditions being the same) (Table I). Note that SM forms more stable bilayers than PC, despite which SM:PE:Ch membranes are more easily reorganized by ceramide than PC:Ch ones.

The lateral segregation of ceramide-rich domains in the plane of the membrane is another important mechanism that explains efflux. The phenomenon was first observed by Huang et al. (18) and then reported, on the basis of different techniques, by Holopainen et al. (19, 20), Carrer and Maggio (21), and Veiga et al. (14). Ceramides have much higher melting points than SM (110 °C versus 40 °C) (22). Consequently, at 37 °C the ceramide-rich domains constitute rigid islands in a sea of fluid lipid. Co-existing ordered and disordered domains is a well known condition that allows efflux to occur (23). The interfaces between ceramide-rich and -poor regions could give rise to the observed release of vesicle contents. It is interesting in this context that C$_2$-ceramides did not elicit efflux under our conditions (Table I, bottom lines). Huang et al. (18) observed that C$_{16}$-ceramide, but not C$_2$-ceramide, gave rise to in-plane phase separations according to their NMR measurements.

The fact that enzyme-derived ceramide induces very fast efflux is also in agreement with the hypothesis that lateral separation of ceramide-rich domains is at the origin of release (see below). In summary, the data in this paper are in agreement with the idea that ceramide lateral segregation and ceramide tendency to induce nonlamellar lipid phases can jointly

| Bilayer composition, mol % ceramide in bilayer | Solvent | Initial rate (% fluorescence/min) | Extent (% fluorescence) |
|-----------------------------------------------|---------|----------------------------------|-------------------------|
| SM:PC:PE:Ch (10:40:25:25) In situ$^a$         |         | 0.32                             | 62                      |
| SM:PC:Ch (2:1:1) In situ$^a$                  |         | 18.0                             | 94                      |
| SM:PC:Ch (2:1:1) CHCl$_2$/MeOH$^c$            |         | 0.040                            | 6.0                     |
| 0 %                                           |         | 0.10                             | 8.3                     |
| 5 %                                           |         | 0.17                             | 21                      |
| 10 %                                          |         | 0.027                            | 8.0                     |
| SM:PC:Ch (2:1:1) Dodecane/EtOH$^c$            |         | 0.11                             | 28                      |
| 0 %                                           |         | 0.016                            | 4.0                     |
| 10 %                                          |         | 0.087                            | 9.3                     |
| PC:Ch (5:1) CHCl$_2$/MeOH$^c$                 |         | 0.032                            | 4.0                     |
| 0 %                                           |         | 0.035                            | 4.0                     |
| 5 %                                           |         | 0.038                            | 4.0                     |
| 10 %                                          |         | 0.017                            | 5.0                     |
| PC:Ch (5:1) Dodecane/EtOH$^c$                 |         | 0.040                            | 13                      |
| 0 %                                           |         | 0.027                            | 5.2                     |
| 10 %                                          |         | 0.086                            | 6.7                     |
| Asolectin CHCl$_2$/MeOH$^c$                    |         | 0.007                            | 3.0                     |
| 0 %                                           |         | 0.015                            | 5.0                     |
| 5 %                                           |         | 0.022                            | 4.5                     |
| 10 %                                          |         | 0.026                            | 4.0                     |
| Asolectin Dodecane/EtOH$^c$                   |         | 0.060                            | 39                      |
| 0 %                                           |         | 0.076                            | 33                      |
| 10 %                                          |         | 0.035                            | 4.0                     |
| Asolectin EtOH$^c$                            |         | 0.15                             | 9.3                     |
| 0 %                                           |         | 0.29                             | 14                      |
| 5 %                                           |         | 0.088                            | 6.7                     |
| 10 %                                          |         | 0.007                            | 3.0                     |
| Asolectin CHCl$_2$/MeOH$^c$                    |         | 0.015                            | 5.0                     |
| 0 %                                           |         | 0.022                            | 4.5                     |
| 5 %                                           |         | 0.026                            | 4.0                     |
| 10 %                                          |         | 0.076                            | 33                      |
| Asolectin Dodecane/EtOH$^c$                   |         | 0.035                            | 4.0                     |
| 0 %                                           |         | 0.087                            | 9.3                     |
| 5 %                                           |         | 0.15                             | 9.3                     |
| 10 %                                          |         | 0.086                            | 6.7                     |
| Asolectin EtOH$^c$                            |         | 0.007                            | 3.0                     |
| 0 %                                           |         | 0.015                            | 5.0                     |
| 5 %                                           |         | 0.022                            | 4.5                     |
| 10 %                                          |         | 0.076                            | 33                      |

$^a$ Percent release after 360 min.
$^b$ Ceramide generated from SM by 1.6 units/ml sphingomyelinase. See “Materials and Methods.”
$^c$ Ceramide mixed with other lipids prior to liposome preparation.
$^d$ Ceramide added in solvent to vesicles in suspension.
explain bilayer restructuring by ceramides. Note that, in our view, the most likely mode for solute efflux from vesicles is through transient, irregular interfaces between ceramide-rich and -poor domains or may be through local destabilization of short-lived nonlamellar structures, rather than through well structured channels.

A Comparison of Methods and Results—Of the various reports describing ceramide-induced restructuring of membranes (see Introduction), those involving C2-ceramide, notably the work by Di Paola et al. (6) and by Simon and Gear (4), should be analyzed separately. Di Paola et al. (6) showed that C2-ceramide can dissipate the inner mitochondrial membrane potential. Simon and Gear (4) observed actual lysis of platelets at ceramide:lipid ratios of 0.5. This, together with the structure of C2-ceramide, very similar to lyso-PC or to palmitoylcarnitine (24), suggests that C2 may have detergent properties, so that its efflux-inducing and lytic properties could occur, particularly at high ceramide:lipid ratios, through a mechanism different from that of the long chain ceramides. It is noteworthy that, under our conditions, low proportions (10 mol %) of C2 did not induce efflux, while the longer chain C6-ceramide was more active in this respect. Work from this and other laboratories (25–27) has shown that the transmembrane asymmetry of ceramide distribution is very important in the induction of membrane reorganization in liposomes. Bai and Pagano (28) have estimated the $t_{1/2}$ for the transbilayer movement of a fluorescent ceramide derivative at $\sim 22$ min, and additional data support the idea that natural ceramides should have flip-flop $t_{1/2}$ values of the same order of magnitude. However, short chain (e.g. C2) ceramides are expected to have significantly faster transbilayer movements. In our experiments, ceramides are added to, or generated in, the outer monolayer of the liposomal membrane, thus a clearly asymmetric distribution of the natural ceramides may be expected at least for the initial stages of our observations, when efflux is faster. With C2 ceramide, transbilayer equilibration may be much faster, and distribution consequently symmetric, contributing to the observed lack of effect. Nevertheless, a specific investigation of the surfactant properties of C2-ceramide and of its dehydro derivative is required to clarify this matter.

Release induced by long chain ceramides has been explicitly shown by Ruiz-Arguello et al. (3) and by Siskind and Colombini (7). In the former case ceramide was generated in situ by enzymatic cleavage of SM, in the latter case it was mixed in organic solvent with the other bilayer components prior to membrane formation. Ruiz-Arguello et al. (3) used LUV with entrapped fluorescent probes to detect efflux, while Siskind and Colombini (7) detected pore formation in planar bilayers through electrophysiological methods. The "large, stable channels" observed by Siskind and Colombini (7) in the presence of 5% C16-ceramide pose a problem. Theoretical calculations predict (29) that a vesicle of our size ($\sim 100$ nm) containing one of those channels would become empty in less than $10^{-2}$ s. But the fact is that, as just mentioned, efflux takes place slowly for many hours. The answer to the paradox...
must rely on intrinsic aspects of the respective techniques. We may suggest, among others, that electric measurements detect individual events, while fluorescence is reporting on the overall effect. The surface area of a typical planar membrane compared with that of one lipidome shows a difference of roughly 6 orders of magnitude. The observation of one permeability pathway in a planar membrane experiment would then correspond to one permeability pathway in $10^6$ liposomes. The formation of one permeability pathway in $10^6$ liposomes would not be detected by the fluorescence method, but one pathway in one planar membrane would elicit an electric signal. It should also be considered that the precise electric signal given by a channel whose size will allow passage of ANTS/DPX cannot be accurately predicted. Moreover the results from planar lipid membranes may be influenced by residual solvent. Finally, LUV may display curvature effects that are lost in the planar membranes.

Other methodological aspects are also relevant when measuring release, e.g., dodecane–ethanol leads to more efflux than warm ethanol, for reasons that are probably related to ceramide solubility. Also significant is the fact that 10% warm ethanol, for reasons that are probably related to ceramide fractionation and modulated, both positively and negatively, by proteins.

Physiological Relevance—The above observations in model membranes may be relevant in understanding ceramide-mediated processes at the cellular level. However, a degree of caution must be exerted when comparing experimental data from cell and model membranes. One important issue is the actual concentration of ceramide in living mammalian cells and the change in ceramide concentrations upon activation. The overall concentration of ceramide in cells is smaller, by 2–3 orders of magnitude, than the one used in our experiments, and activation of ceramide-dependent pathways leads only to a modest increase in overall concentration (31), but average figures of weight compounds, (ii) both in situ generated and externally added ceramide induce an increased efflux in membranes, and (iii) in situ generation of ceramides by enzymatic cleavage of sphingomyelin leads to a faster and more extensive efflux than addition of the same amount of ceramide to a pre-existing membrane.

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