Membrane-Destabilizing Properties of C_2-Ceramide May Be Responsible for Its Ability To Inhibit Platelet Aggregation†

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ABSTRACT: We have studied the effects of short-chain ceramides on platelet structure and function. *N*-Acetylsphingosine (C_2-ceramide), a cell-permeable short-chain analogue, and *N*-acetyldihydrosphingosine (C_2-dihydroceramide), which lacks the 4−5 double bond, have been investigated. C_2-Ceramide (15 μM) inhibited ADP-induced aggregation by 50% at a platelet concentration of 1.25×10^8/mL, while it took twice that concentration to inhibit aggregation by 50% when the platelet concentration was doubled. This indicates that the effect of C_2-ceramide on ADP-induced platelet aggregation depends on the ratio of ceramide to total platelet lipid, with a ratio of 0.2 giving significant inhibition. C_2-Ceramide at a ceramide:lipid ratio of 0.2 caused platelets to form fenestrations and pseudopodia which were longer and thinner than those caused by agonists such as ADP or thrombin. C_2-Dihydroceramide had no effect on ADP-induced aggregation or platelet morphology at any ceramide:lipid ratio. Platelet lysis was induced by C_2-ceramide at higher ceramide:lipid ratios (0.5), whereas C_2-dihydroceramide did not induce lysis, suggesting that C_2-ceramide is able to destabilize membranes. This was tested directly by assessing whether the ceramides induced leakage of 6-carboxyfluorescein from lipid vesicles. C_2-Ceramide caused nearly total leakage of dye from the vesicles at a ceramide:lipid ratio of 10. The leakage caused by C_2-dihydroceramide at a ceramide:lipid ratio of 10 was equal to that induced by C_2-ceramide at a ratio of 0.2 (∼3%). The ability of the ceramides to destabilize membranes was also examined by measuring changes in fluorescence anisotropy of the fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into lipid vesicles. C_2-Ceramide induced a larger decrease in anisotropy than a detergent (Triton X-100) which is known to lyse membranes. C_2-Dihydroceramide did not alter membrane fluidity. The ability of C_2-ceramide to cause platelet fenestrations, formation of irregular platelet pseudopodia, platelet lysis, lipid vesicle leakage, and increases in the fluidity of lipid vesicles all suggest that C_2-ceramide inhibits platelet aggregation because it destabilizes the platelet membrane. C_2-Dihydroceramide did not inhibit platelet aggregation and lacked the nonspecific effects on membranes that C_2-ceramide possessed, suggesting that C_2-dihydroceramide is not an appropriate control for the nonspecific effects of C_2-ceramide.

Ceramide has recently been identified as an intracellular signaling molecule which may mediate diverse functions such as cell growth, differentiation, secretion, and apoptosis (*1*). Initially it was observed that differentiation of HL-60 cells (a human promyelocytic leukemia cell line) induced by vitamin D_3 caused sphingomyelin turnover and a concomitant increase in ceramide levels. This observation lead to the suggestion that a signaling pathway exists in which ceramide formed by activation of a sphingomyelinase serves as a second messenger (*2*). Additional studies have identified other inducers of sphingomyelin turnover: interleukin-1 in human dermal fibroblasts, γ-interferon in HL-60 cells, and TNFα in Jurkat T cells among others (*1*). A search for candidate signaling targets of ceramide has implicated ceramide-activated protein phosphatase (*3*), ceramide-activated protein kinase (*4*), protein phosphatase 2A (PP2A)[1] (*5*, *6*), PKCζ (*7*), and c-raf (*8*).

The role of protein phosphatases in signal transduction pathways is well documented, stemming from original work done 30 years ago on the regulation of glycogen metabolism (*9*). Recently, their involvement in platelet function has come to light (*10*−*18*). Two highly specific inhibitors of protein phosphatase 1 (PP1) and PP2A, okadaic acid and calyculin A, inhibit platelet aggregation and secretion (*10*−*12*) as well as alter platelet morphology (*13*). Additional platelet studies using these inhibitors have implicated PP1 and/or PP2A in several aspects of platelet function. Watson's group has shown that okadaic acid inhibits formation of inositol phosphates during thrombin-induced platelet activation, suggesting that phospholipase C is inhibited (*14*). Murata et al. (*15*) and Sakon et al. (*16*) have evidence that

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[1] Abbreviations: BSA, bovine serum albumin; C_2-ceramide, *N*-acetylsphingosine; C_2-dihydroceramide, *N*-acetyldihydrosphingosine; ACD, acid−citrate−dextrose; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; PKC, protein kinase C; DPH, 1,6-diphenyl-1,3,5-hexatriene; 6-CF, 6-carboxyfluorescein; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; SUV's, small unilamellar vesicles; LUV's, large unilamellar vesicles; PAF, platelet activating factor; TXA_2, thromboxane A_2; LPA, lysophosphatidic acid; NMR, nuclear magnetic resonance.

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calyculin A inhibits platelet aggregation by blocking Ca^{2+} influx, and by blocking the exposure of $\alpha_{IIb}\beta_3$ on the surface of thrombin-stimulated platelets. This inhibition of platelet aggregation was correlated with increased phosphorylation of the cytoskeletal protein talin (*17*) which has been shown to interact with the $\alpha_{IIb}\beta_3$ integrin (*19, 20*), an interaction that may be regulated by talin phosphorylation (*21, 22*). Indications that platelet proteins are actually dephosphorylated come from Turini et al. (*23*) and Wallace and Bensusan (*24*), who found that a 68 kDa protein band is dephosphorylated during platelet activation. Our own laboratory has recently shown that this 68−67 kDa band contains the 70 kDa cognate heat-shock protein (hsc70), which is dephosphorylated following platelet adhesion to collagen, and which exists in a large phosphoprotein complex in resting platelets (*18*). These studies taken together suggest that protein phosphatases are involved in platelet function (*10*−*18, 23, 24*), with the possibility that PP2A is regulated by ceramide (*3, 5, 6*).

Evidence that sphingolipid signaling may be involved in platelet function comes from two studies. Hisano et al. (*25*) observed that the platelet sphingosine mass increases during aggregation, and Yatomi et al. (*26*) reported that aggregation is potentiated by sphingosine 1-phosphate which is released by platelets during activation (*26*). In addition, some preliminary work on the role of ceramide in platelet function has been published. Hannun et al. reported in 1987 (*27*) that C_2-ceramide caused slight inhibition of ADP-induced platelet aggregation and had no effect on thrombin-induced aggregation. In a more recent study, Yatomi et al. (*26*) studied the effects of sphingolipids on platelets and reported that bovine ceramide (type III), C_8-ceramide, or C_2-ceramide did not by themselves (in the absence of agonists such as ADP or thrombin) cause platelet aggregation or shape change as detected by a luminometer (aggregometer). Wong and Li (*28*) examined the effects of C_6-ceramide, C_2-ceramide, and C_2-dihydroceramide on aggregation and found that C_2-ceramide at a ceramide:lipid ratio of 0.38 inhibited thrombin-induced aggregation (0.1 unit/mL), while C_6-ceramide or C_2-dihydroceramide had no effect. In light of these reports, it seems plausible that platelets might utilize ceramide as a second messenger.

To test the hypothesis that ceramide is involved in platelet function, we evaluated the effects of exogenous ceramide on platelets. Since naturally occurring ceramides are essentially insoluble in aqueous systems, we employed the widely used, soluble short-chain analogue C_2-ceramide, which has an acetyl group substituted for the amide-linked acyl chain (*29*−*31*). C_2-Dihydroceramide, which lacks the 4−5 double bond in the long chain base, was used as a negative control to account for possible nonspecific effects of C_2-ceramide. Similar to previous reports (*27, 28*), we have found that C_2-ceramide inhibited ADP-induced platelet aggregation. We also make the new observation that C_2-ceramide causes morphological changes similar to those caused by single-chain amphiphiles. Since single-chain amphiphiles also inhibit aggregation, we have considered the possibility that the effect of C_2-ceramide on platelets results from its ability to destabilize the platelet membrane. To this end, we have also studied the effects of C_2-ceramide on model membranes and found that it perturbs model membranes as well.

EXPERIMENTAL PROCEDURES

Materials. *D-erythro*-C_2-Ceramide and *D-erythro*-C_2-dihydroceramide were purchased from Biomol (Plymouth Meeting, PA). Stock solutions for both were made at the maximum solubility of C_2-dihydroceramide, 14.6 mM in ethanol, and were stored at −20 °C. [3-^{3}H]C_2-Ceramide and [3-^{3}H]C_2-dihydroceramide were a gift from Neale D. Ridgway (Dalhousie University, Canada), and each had a specific activity of 30.8 dpm/pmol (*32*). The purity of normal and radioactive ceramides was routinely checked by TLC using two different solvent systems (methylene chloride/methanol, 9:1 v/v, and diethyl ether/methanol, 9:1 v/v) and iodine staining. Both ceramides were found to be stable under these storage conditions for up to 6 months. [^{3}H]Adenine (30 Ci/mmol) and 6-carboxyfluorescein were from ICN Radiochemicals (Irvine, CA) and Eastman Kodak (Rochester, NY), respectively. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was from Molecular Probes (Eugene, OR). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were from Avanti Polar-Lipids, Inc. (Alabaster, AL). Cholesterol, fatty acid-free BSA, and other specialty chemicals used were obtained from Sigma (St. Louis, MO).

Preparation of Human Platelets. Human venous blood was anticoagulated with ACD to a final citrate concentration of 11.5 mM in whole blood and centrifuged at 350*g*, twice for 3 min and once for 5 min, all at room temperature. The supernatant was gently removed from the centrifuged whole blood, and the resultant platelet-rich plasma was mixed with 0.05 volume of ACD, apyrase (grade VII, 5 units/mL ADPase activity), indomethacin (1 μg/mL), and PGI_2 (0.3 μg/mL) and centrifuged at 620*g* for 20 min. For ceramide uptake experiments, the platelet pellet was washed in ACD with apyrase, centrifuged, and suspended in a modified BSA-free Tyrode's−Hepes buffer (140 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 10 mM Hepes, 12 mM $NaHCO_3$, 5 mM glucose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4). For electron microscopy and aggregation experiments, the first 620*g* pellet was washed in ACD with apyrase, centrifuged, and suspended in an Eagle's−Hepes medium (*33*) containing 0.5 mg/mL fibrinogen. Final platelet concentrations were (1−3) × 10^8/mL. For [^{3}H]adenine labeling, the first 620*g* platelet pellet was suspended in 6 mL of the modified BSA-free Tyrode's−Hepes buffer (100 μM $MgCl_2$, 50 μM $CaCl_2$) with apyrase, left for 20 min at 37 °C, and incubated with 5 μCi/mL [^{3}H]adenine for 1 h. ACD (0.05 volume) was then added, and the platelets were centrifuged and suspended in the modified BSA-free Tyrode's−Hepes buffer (2 mM $MgCl_2$, 1 mM $CaCl_2$).

Platelet Aggregation Assay. Prewarmed platelets were incubated with C_2-ceramide, C_2-dihydroceramide, ethanol vehicle, or 154 mM NaCl (saline) for 10 min at 37 °C and then reacted with 10 μM ADP or saline for 10 s at a shear stress of 20 dyn/cm^2 in a quenched-flow system under physiological flow conditions (*34*). Glutaraldehyde (0.17% final concentration) was used as the quenching agents, and percent aggregation was determined by counting the percentage loss of single platelets measured by resistive-particle counting (*34*).

Scanning Electron Microscopy. Platelets were incubated at 37 °C with C_2-ceramide, C_2-dihydroceramide, ethanol

vehicle, or with saline for the times indicated, fixed with 1% glutaraldehyde, subjected to graded alcohol dehydrations as described previously (*35*), and observed in a scanning electron microscope (JSM-6400, JEOL).

[^{3}H]Adenine Release from Platelets. Five hundred microliters of [^{3}H]adenine-labeled platelets at 1×10^8/mL was incubated at 37 °C with C_2-ceramide, C_2-dihydroceramide, Triton X-100, or with ethanol (as vehicle control). After 15 min, the suspensions were centrifuged at 15000*g* for 5 min in a microfuge, and a 125 mL aliquot of the supernatant was removed for liquid scintillation counting. Percent lysis was determined by the following equation: $[(S_C - S_S)/(T_S - S_S)] \times 100$, where S_C is the counts in an aliquot of supernatant after treatment with test agent, S_S is the counts in an aliquot of supernatant after ethanol treatment, and T_S is the counts in an aliquot of the total platelet suspension after treatment with ethanol.

Leakage of 6-Carboxyfluorescein from POPC Vesicles. Small unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC SUV's) were made by sonication as described (*36*) in the presence of 200 mM 6-carboxyfluorescein (6-CF), pH 7.4 (*37*). Vesicles were stored at 4 °C until use, at which time an aliquot was passed over a gel filtration column of Sephadex G-75 to remove free 6-CF. Phospholipid concentration was determined by a modification of the procedure described by Bartlett (*38*). 6-CF at 200 mM inside the POPC vesicles is mostly self-quenched, but when released into the media, such as by detergent solubilization of the vesicles, the diluted 6-CF fluoresces intensely (*37*). The increase in fluorescence of 6-CF released from the vesicles was measured in an SLM-8000c spectrofluorometer. Excitation was at 490 nm (4 nm slit width), and emission intensity was monitored at 520 nm (8 nm slit width). Measurements were made at 37 °C in a thermostated 2.5 mL quartz cuvette. Small volumes of vesicles were diluted into the modified BSA-free Tyrode's−Hepes buffer (2 mM $MgCl_2$, 1 mM $CaCl_2$) to yield 2 mL total volume. A base line measurement of fluorescence intensity was made of vesicles alone, and then C_2-ceramide, C_2-dihydroceramide, or ethanol vehicle was added. Fluorescence was measured for 15 min, and then a small volume of concentrated Triton X-100 (1.7 mM final) was added in order to determine the maximum fluorescence attainable under conditions of totally solubilized vesicles. Percent lysis was determined by the following equation: $[(F_{15} - F_{Ves})/(F_{Max} - F_{Ves})] \times 100$, where F_{15} is the maximum intensity approximately 15 min after addition of test agent, F_{Ves} is the intensity of vesicles alone at time zero, and F_{Max} is the intensity after addition of Triton X-100.

Fluorescence Anisotropy. DMPC/cholesterol (3:1, mole/mole) large unilamellar vesicles (LUV's) were made as described (*39*, *40*). DMPC and cholesterol were dissolved together in chloroform, lyophilized overnight, rehydrated in buffer (25 mM Hepes, 0.25 mM EDTA, 10 mM NaCl, pH 7.5), and passed through a hand-held extruder with a 100 nm pore size filter 30 times. The concentration of the DMPC component of the lipid vesicles was determined by phosphate assay (*38*). Measurements of steady-state fluorescence anisotropy were made using an SLM-8000c spectrofluorometer using one detector (L-format) with excitation at 365 nm (4 nm slit width), and emission intensity was monitored at 460 nm (8 nm slit width). The LUV's were labeled with the fluorescent probe DPH by using 2 μL of a 3 mM DPH stock solution suspended in tetrahydrofuran added to 2 mL of lipid vesicles (1.5 mol % DPH) and were incubated for 30 min at 37 °C. DPH-labeled LUV's were suspended in a thermostated 500 μL quartz cuvette at 37 °C for 10 min before fluorescence measurements were commenced. After a base line anisotropy measurement was performed, additions of the appropriate compounds were made, and the sample was allowed to equilibrate for 10 min before taking further measurements. The following equation (*41*) was used to calculate fluorescence anisotropy (r): $r = [I_{VV} - (I_{HV}/I_{HH})I_{VH}]/[I_{VV} + 2(I_{HV}/I_{HH})I_{VH}]$, where I refers to measured fluorescence intensity and the V (vertical) and H (horizontal) subscripts refer to the orientation of the polarizers for the excitation beam (first subscript) and the emission beam (second subscript), respectively.

Ceramide Uptake. Five hundred microliters of platelets (2.5×10^8/mL) was incubated at 37 °C with [3-^{3}H]C_2-ceramide or [3-^{3}H]C_2-dihydroceramide. After 10 min, a 150 μL aliquot of the total suspension was taken for liquid scintillation counting. The remaining sample was centrifuged at 5000 rpm for 2 min in a microfuge and a 150 μL aliquot of the supernatant taken for counting. The short, low-speed spin was the mildest found that would sediment all the platelets, and this mild spin was used instead of a more forceful spin to minimize the amount of possible insoluble aggregates of ceramides that were pelleted during centrifugation. Controls using buffer alone were performed to determine the amount of ceramide that would pellet during the mild spin. Percent uptake was determined using the following equation: $[[(T_P - S_P)/T_P] - [(T_B - S_B)/T_B]] \times 100$, where T_P is the counts in an aliquot of total platelet suspension, S_P is the counts in an aliquot of platelet supernatant, T_B is the counts in an aliquot of buffer, and S_B is the counts in an aliquot of buffer supernatant. Radioactive ceramides were used to spike stocks of regular ceramides, and ~1000 dpm were added to each platelet sample for determining uptake.

Statistics. Student's *t*-test for paired and/or unpaired data was used to determine the statistical significance of the data where indicated. Results are expressed as means ± SE.

RESULTS

Preincubation of washed platelets for 10 min at 37 °C with C_2-ceramide inhibited platelet aggregation stimulated by 10 μM ADP (Figure 1A), while preincubation with C_2-dihydroceramide (Figure 1A) or ethanol vehicle (data not shown) had no effect. The aggregation assay was performed using a quenched-flow system, and platelets were reacted with 10 μM ADP for 10 s at a shear stress of 20 dyn/cm^2 (*34*). The experiment in Figure 1A was performed at two concentrations of platelets to determine if the inhibition was dependent on absolute ceramide concentration, or on the ratio of ceramide to total platelet lipid. When the data are plotted against the ceramide:lipid ratio (assuming the 'total platelet lipid concentration' is 67 μM for 1×10^8 platelets/mL; *42*, *43*), the aggregation plots essentially overlap at the two differing platelet concentrations (Figure 1B). This suggests that the inhibition was dependent on the ratio of ceramide to total platelet lipid. The difference in inhibition of aggregation between C_2-ceramide and C_2-dihydroceramide

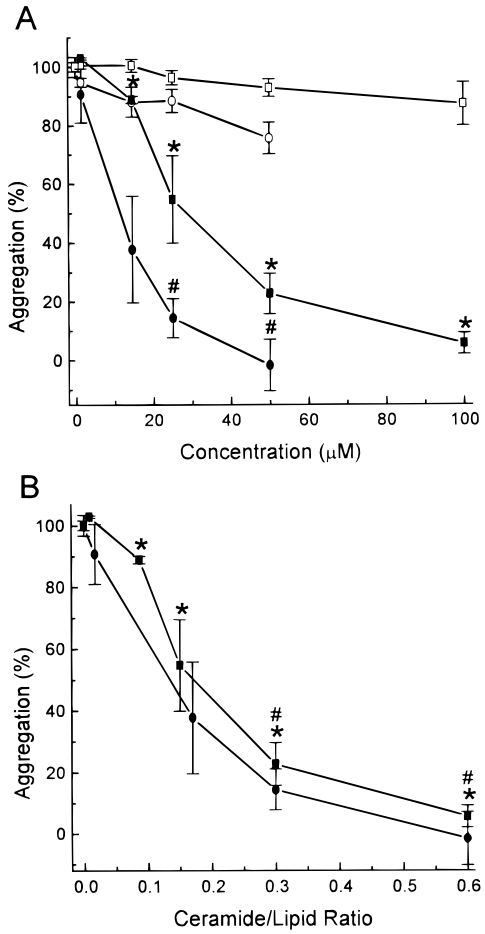

FIGURE 1: C_2-Ceramide inhibits platelet aggregation. Washed, human platelets, prewarmed at 37 °C for 10 min, were incubated with the indicated concentrations of C_2-ceramide (closed symbols) or C_2-dihydroceramide (open symbols) for 10 min at 37 °C and then reacted with 10 μM ADP in the quenched-flow system for 10 s at a shear stress of 20 dyn/cm^2 (*34*). Glutaraldehyde (0.17% final concentration) was used as the quenching agent, and percent aggregation was determined from loss of single platelets measured by resistive-particle counting as described under Experimental Procedures. Experiments were performed at two platelet concentrations: 1.25×10^8/mL (circles) and 2.5×10^8/mL (squares). The data were normalized such that aggregation stimulated by 10 μM ADP in the absence of ceramides was set to 100%. Controls using platelets preincubated with saline and then "reacted" with saline gave a background of ~3% loss of singlets (aggregation) due to activation by shear stresses. Platelets preincubated with saline and reacted with ADP for 10 s in the quenched-flow apparatus typically gave ~75% loss of singlets (no C_2-ceramides) at both platelet concentrations. There was no difference in the total amount of aggregation at the two different platelet concentrations because the assay conditions yielded maximal aggregation. Ethanol vehicle had no effect on aggregation. Aggregation is plotted against ceramide concentration in (A) while in (B) the C_2-ceramide data are plotted against the ceramide:lipid ratio. Significant differences ($p < 0.05$) between paired C_2-ceramide and C_2-dihydroceramide data points for platelet concentrations of 2.5×10^8/mL are indicated by an asterisk (*) while those for 1.25×10^8/mL are indicated by a number sign (#). The data are the mean of four separate experiments, and the error bars represent ± SE.

(Figure 1B) became significant at a ratio of approximately 0.15, which corresponds to 13 mol % C_2-ceramide.

Scanning electron micrographs showed that C_2-ceramide at a ceramide:lipid ratio of 0.24 (19 mol %) caused a dramatic shape change of platelets within 1 min (Figure 2C), which was sustained over 1 h (Figure 2D). Nearly all of the C_2-ceramide-treated platelets formed fenestrations and multiple pseudopodia that are longer and thinner than those induced by platelet agonists such as thrombin or ADP (*35*, *44*). The morphological effects depended on the ceramide:lipid ratio since C_2-ceramide caused formation of fenestrations and irregularly shaped pseudopodia at a constant ceramide:lipid ratio of 0.24, at two different concentrations of platelets (Figure 2C,D,G,H). C_2-Dihydroceramide-treated platelets resembled untreated platelets at both high (Figure 2E,F) and low (data not shown) platelet concentrations. Ethanol vehicle at concentrations equal to those used in the ceramide exposures had no effect on platelet morphology in any of the experiments (data not shown). The effects of C_2-ceramide seen at a lower ceramide:lipid ratio (0.024) were transient since fenestrations and irregularly shaped pseudopodia were visible after a 1 min exposure (Figure 2I) but had disappeared within an hour (Figure 2J). These data indicate that C_2-ceramide induced changes in platelet morphology while C_2-dihydroceramide did not.

It is significant that C_2-ceramide caused formation of fenestrations and long, thin, irregularly shaped pseudopodia (Figure 2) because amphiphiles and detergents generally have similar effects on platelets as C_2-ceramide (*44*−*51*). Amphiphiles such as Triton X-100, octyl glucoside, sodium dodecyl sulfate (SDS), deoxycholate (*45*), other bile salts (*46*), Brij 58 (polyoxyethylene detergent) (*47*), lysophosphatidylcholine (*44*, *48*), ganodermic acids (*49*), and 13-methylmyristate (*50*), as well as other free fatty acids (*51*), inhibit ADP-induced aggregation, cause fenestrations, and cause formation of long, thin, irregular pseudopodia in the absence of increases in intracellular calcium (*45*), and/or cause formation of transient pseudopodia at lower ratios of amphiphile (*45*). The authors of these studies conclude that the effects of such amphiphiles are due to membrane perturbations. In addition, we noted that incubations of resting platelets with ratios of C_2-ceramide higher than those which inhibited ADP-induced aggregation caused platelets to lyse as indicated by a total loss of detectable single platelets in the resistive-particle counter (data not shown).

Since the effects of C_2-ceramide on platelets generally mimicked those of nonspecific amphiphiles, relatively high ratios (0.2) of C_2-ceramide to platelet lipid were required to induce effects in platelets, and C_2-ceramide is a single-chain amphiphile which structurally resembles a detergent, it seemed plausible that our observed effects of C_2-ceramide on platelets were due to membrane perturbations. To test this 'membrane perturbation' hypothesis, we examined directly whether C_2-ceramide actually lyses platelets.

Platelets were labeled with [^{3}H]adenine and incubated with ceramides for 15 min at 37 °C, and the amount of [^{3}H]-adenine released to the supernatant was determined as a measure of platelet lysis (Figure 3). C_2-Ceramide caused detectable [^{3}H]adenine release at a ceramide:lipid ratio of 0.2 (17 mol %), and this release (62%) became significant at a ceramide:lipid ratio of 0.75 (43 mol%). C_2-Dihydroceramide or ethanol vehicle did not cause any leakage of [^{3}H]adenine at any ratio (Figure 3). When an excess of a common detergent that solubilizes membranes (Triton X-100 at 6.8 mM) was added to the platelets (Figure 3), 85% release of [^{3}H]adenine was observed, and C_2-ceramide was able to approach this amount of release at a ratio of 1 (50 mol %). These data indicate that C_2-ceramide can destabilize the membrane of platelets, while C_2-dihydroceramide cannot.

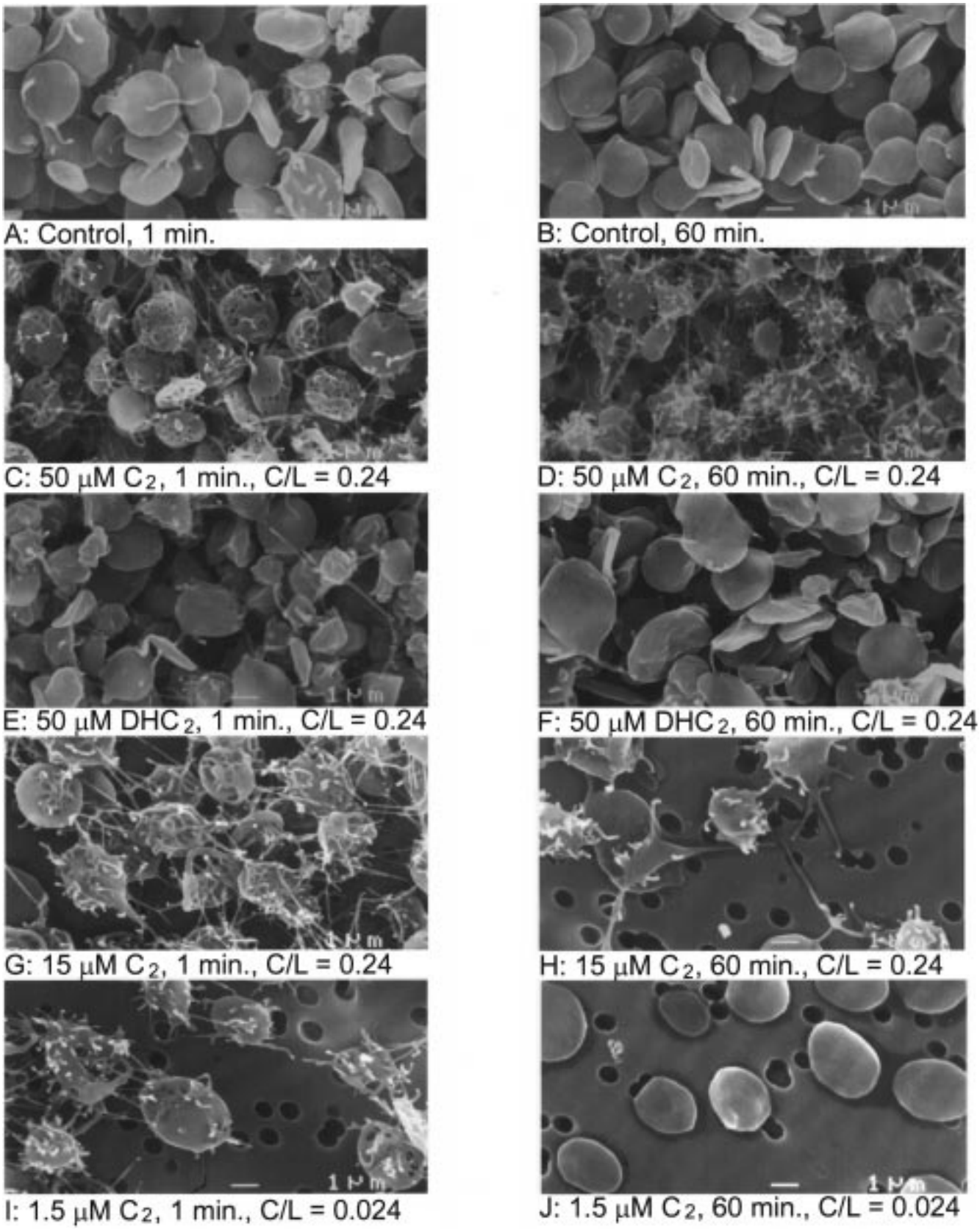

FIGURE 2: C_2-Ceramide causes formation of irregular pseudopodia. Washed, human platelets were incubated with the indicated reagents at 37 °C for 1 or 60 min, fixed in 1% glutaraldehyde, and prepared for scanning electron microscopy. The platelet concentrations in panels A-F were 3×10^8/mL, while in panels G−J they were 1×10^8/mL. Ceramide:lipid ratio (C/L) is given in panels C−J. The micrographs presented are representative of four separate experiments. The gray bars at the bottom of each micrograph indicate 1 μm.

To determine if the destabilizing effect of C_2-ceramide on platelet membranes was general and not peculiar to whole cells, a similar experiment was performed using synthetic lipid vesicles. POPC SUV's were made by sonication in the presence of 200 mM 6-CF such that 200 mM 6-CF was trapped inside the lipid vesicles. 6-CF is a fluorescent dye

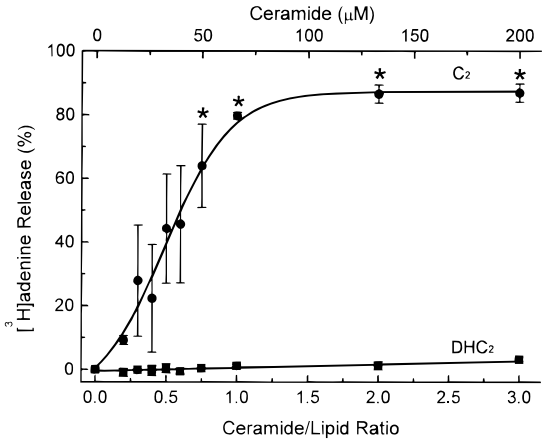

FIGURE 3: C_2-Ceramide can lyse platelets. 500 μL of [^{3}H]adenine-labeled platelets at 1×10^8/mL was incubated at 37 °C with C_2-ceramide (circles) or C_2-dihydroceramide (squares). After 15 min, the suspensions were centrifuged at 15 000*g* for 5 min, and a 125 μL aliquot of the supernatant was removed for liquid scintillation counting. The counts in the supernatants with respect to the total counts in control samples were used to calculate percent lysis as described under Experimental Procedures. Aliquots of total suspensions of control platelets were typically 25 000 dpm while their supernatants were typically 5000 dpm. Similar experiments were done adding Triton X-100 (6.8 mM final) at a detergent:lipid ratio of 100 which caused ~85% adenine release. Experiments using ethanol at the same concentrations used to deliver the ceramides caused less than 1% [^{3}H]adenine release. The curves shown were drawn using the sigmoidal curve-fitting tool provided by Origin software (Microcal Software, Inc., Norhthampton, MA) using the Boltzmann equation. The bottom *x*-axis is the ratio of the ceramide to platelet lipid, and the top *x*-axis is the actual concentration of the ceramides. The data represent the means of three separate experiments, and error bars represent ± SE. Significant differences ($p < 0.05$) between paired C_2-ceramide and C_2-dihydroceramide data points are indicated by an asterisk (*).

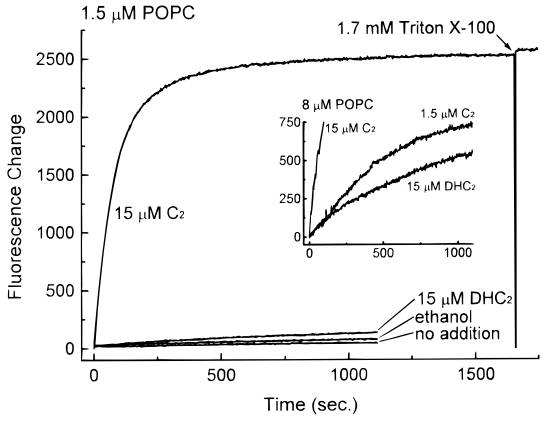

FIGURE 4: C_2-Ceramide can cause POPC vesicles to leak. Release of 6-CF from POPC vesicles containing 200 mM 6-CF was monitored by using a spectrofluorometer to measure the increase in fluorescence intensity over time. 6-CF self-quenches at 200 mM inside the vesicles, but when released to the external media, it is diluted and its fluorescence increases. In the main figure, vesicles were incubated at 37 °C for 15 min either alone ('no addition'), or with C_2-ceramide ('15 μM C_2'), C_2-dihydroceramide ('15 μM DHC_2'), or 0.1% ethanol vehicle ('ethanol'). The concentration of POPC was 1.5 μM, giving a ceramide:lipid ratio of 10. Triton X-100 (1.7 mM final) was added at the end of each run to determine the maximum fluorescence attainable if all the 6-CF were to be released from the vesicles. The different runs were then normalized with one another such that the maximum fluorescence intensity after addition of Triton X-100 was equal. An addition of Triton X-100 is shown only for the C_2-ceramide run. A typical run of vesicles alone gave a base line fluorescence intensity of 695 fluorescence units, which increased to 715 after 15 min at 37 °C, which further increased to 3000 after addition of Triton X-100. The experiments shown in the inset used 7.7 μM POPC with additions of C_2-ceramide ('15 μM C_2' or '1.5 μM C_2'; ceramide:lipid ratios of 2 or 0.2, respectively) or C_2-dihydroceramide ('1.5 μM DHC_2'; ceramide:lipid ratio of 2). The data are from a representative experiment of three that were performed and are plotted as arbitrary fluorescence units versus time (seconds).

which is self-quenched at these high concentrations inside the vesicles, but when released from the vesicles and diluted in the medium, its fluorescence intensity increases. Fifteen micromolar C_2-ceramide (ceramide:lipid ratio of 10) induced near total leakage of 6-CF from POPC vesicles in 10 min, since addition of excess Triton X-100 (1.7 mM) to the C_2-ceramide-treated sample caused minimal (<3%) increase in fluorescence intensity (Figure 4). Fifteen micromolar C_2-dihydroceramide (ceramide:lipid ratio of 10) caused little leakage beyond that caused by ethanol vehicle (Figure 4). The same concentration of C_2-ceramide (15 μM) at a 5-fold lower ceramide:lipid ratio of 2 caused a 13% increase in fluorescence by 10 min. (data partially shown in the inset to Figure 4). C_2-Ceramide (1.5 μM) at a ceramide:lipid ratio of 0.2 caused a greater increase in fluorescence than a 10-fold higher ratio of C_2-dihydroceramide (15 μM) (Figure 4, inset). These data indicate that C_2-ceramide can destabilize synthetic membranes as well as platelet membranes and that C_2-dihydroceramide was unable to do so.

As a further test of the membrane-perturbing influence of C_2-ceramide, we determined its effect on membrane fluidity as sensed by the fluorescence anisotropy of DPH incorporated into lipid vesicles. DMPC/cholesterol LUV's were used as opposed to POPC SUV's since the fluorescence anisotropy of DPH in POPC vesicles was not significantly affected by lytic concentrations of Triton X-100 (10 mM), indicating that DPH was insensitive to membrane perturbations in this environment (data not shown). At 37 °C in DPH-labeled DMPC/cholesterol vesicles, C_2-ceramide caused a significant decrease in fluorescence anisotropy at a ceramide:lipid ratio of 0.3, and its effect on the change in this parameter was roughly twice that of Triton X-100 (Figure 5). C_2-Dihydroceramide or ethanol vehicle had no significant effects on membrane anisotropy. These data suggest that C_2-ceramide influences acyl chain packing in lipid bilayers and that C_2-dihydroceramide does not.

It was important to determine if the differences between C_2-ceramide and C_2-dihydroceramide were due to differences in their uptake by membranes. Platelets were therefore incubated with radioactive ceramides for 10 min at 37 °C, and the counts removed from the supernatant by pelleting the platelets by centrifugation were determined. Figure 6 shows that platelet uptake of C_2-ceramide and C_2-dihydroceramide was essentially equal at ceramide concentrations ranging from 1.5 to 100 μM (ceramide:lipid ratios ranging from 0.08 to 0.6). There appeared to be a difference in uptake at 50 μM, but even if this observation is significant; it represented a difference of 30% and could not account for the functional and physical differences between the two compounds, especially since most of the data obtained in this study were done at concentrations below 50 μM. These data indicate that the different effects of C_2-ceramide and C_2-dihydroceramide are unlikely to result from differences in their membrane uptake.

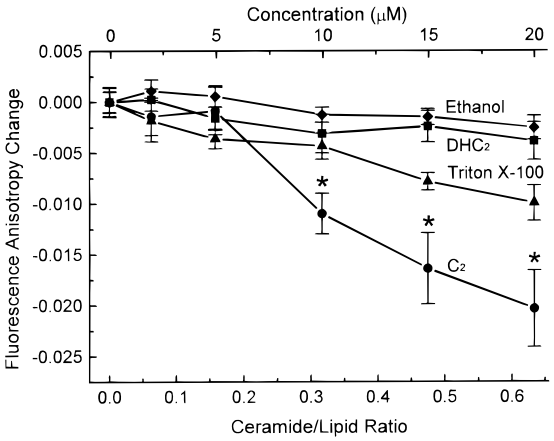

FIGURE 5: C_2-Ceramide can affect the fluidity of lipid vesicles. The effects of C_2-ceramide (circles), C_2-dihydroceramide (squares), ethanol vehicle (diamonds), or Triton X-100 (up-triangles) on the fluorescence anisotropy of DPH incorporated into DMPC/cholesterol (3:1, mole/mole) LUV's were determined. Lipid concentration was 32 μM, and measurements were made at 37 °C as described under Experimental Procedures. The bottom *x*-axis is the ratio of the ceramide or Triton X-100 to platelet lipid, and the top *x*-axis is the actual concentration of the ceramides or Triton X-100. The ethanol concentrations were equal to those used as vehicle for the ceramides. Typical fluorescence anisotropy values before ceramide treatment were 0.24. The data are the mean of three separate experiments, and error bars represent ± SE.

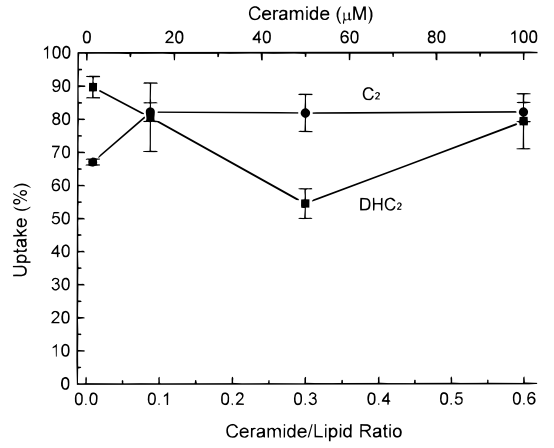

FIGURE 6: Uptake of C_2-ceramide and C_2-dihydroceramide by platelets. Platelets at 2.5×10^8/mL were incubated at 37 °C with [3-^{3}H]C_2-ceramide (circles) or [3-^{3}H]C_2-dihydroceramide (squares) as described under Experimental Procedures. The bottom *x*-axis is the ratio of the ceramide to platelet lipid, and the top *x*-axis is the actual concentration of the ceramides. Data are plotted as the percent of ceramides added to samples that were then taken up by the platelets. They are means of four separate experiments, and error bars represent ± SE.

DISCUSSION

C_2-Ceramide inhibited ADP-induced aggregation (Figure 1) and induced formation of fenestrations and pseudopodia (Figure 2) that were longer and thinner than those induced by platelet agonists such as ADP and thrombin (*35*, *44*). In addition, C_2-ceramide induced release of [^{3}H]adenine from platelets (Figure 3). A large body of work has shown that the effects of many amphiphiles and detergents on platelets are similar to C_2-ceramide (*44*−*51*). Thus, amphiphiles such as common detergents (Triton X-100, SDS, octyl glucoside, Brij 58), bile salts (deoxycholate), free fatty acids, or lysophosphatidylcholine have all been shown to inhibit ADP-induced aggregation, induce fenestrations in platelets, and/or cause platelets to form irregularly shaped pseudopodia which are transient at lower amphiphile:lipid ratios. C_2-Ceramide also caused leakage of fluorescent dye from lipid vesicles (Figure 4) and caused greater increases in the fluidity of lipid vesicles than did a common detergent, Triton X-100 (Figure 5). Although we cannot categorically exclude the possibility that C_2-ceramide may have specific biochemical interactions, our observations strongly suggest that the ability of C_2-ceramide to inhibit platelet aggregation is the result of membrane perturbations.

C_2-Dihydroceramide exhibited none of the effects displayed by C_2-ceramide in this study, having no influence on platelet aggregation, platelet morphology, release of [^{3}H]-adenine from platelets, leakage of 6-CF from lipid vesicles, or membrane fluidity of lipid vesicles. The different effects of C_2-ceramide and C_2-dihydroceramide were not due to variations in uptake of the two compounds, since both radiolabeled ceramides were taken up in similar amounts by platelets (Figure 6). Our results show that C_2-dihydroceramide did not destabilize membranes as did C_2-ceramide. Since the physical effects of C_2-dihydroceramide on membranes were very different from C_2-ceramide, C_2-dihydroceramide cannot serve as an interpretable control for nonspecific effects of C_2-ceramide.

Comparison of the molecular structures of C_2-ceramide, C_2-dihydroceramide, and the most common ceramide found in sphingomyelin from platelets (*43*, *52*), *N*-behenylsphingosine (C_{22}-ceramide), is relevant in regard to our observations. C_2-Ceramide and C_{22}-ceramide differ greatly in the length of their N-linked acyl chains (Figure 7A,B), such that the two molecules have significantly different physical properties. Thus, C_{22}-ceramide is essentially insoluble in aqueous solutions, while C_2-ceramide is soluble to about 50 μM (*3*, *30*). C_2-Ceramide is a single-chain amphiphile resembling a detergent, in contrast to C_{22}-ceramide which has two long acyl chains. Double-chain amphiphiles are generally stable in lipid bilayers while single-chain amphiphiles and detergents are well-known to destabilize bilayers (*53*). Therefore, it is unclear whether C_{22}-ceramide would destabilize bilayers to the same extent as does C_2-ceramide. This raises the possibility that if C_{22}-ceramide were generated endogenously from sphingomyelin in platelets, its effects could occur via different mechanisms than those of exogenously added C_2-ceramide.

A possible mechanistic explanation for the difference in behavior of C_2-ceramide versus C_2-dihydroceramide is depicted in Figure 7B−E. C_2-Ceramide differs structurally from C_2-dihydroceramide only by the presence of the 4−5 *trans* double bond (Figure 7B,C). Notably, the secondary hydroxyl group is in an allyl position relative to the double bond, and allyl alcohols are invariably more reactive than their saturated counterparts (*54*). The location of the double bond allows the formation of resonance structures (Figure 7D) that could increase the electronegativity of the alcoholic oxygen atom, stabilizing its ionization and making it more prone to be an acceptor in a hydrogen bond (Figure 7E) (*55*). Since C_2-ceramide possesses the double bond, the headgroup of C_2-ceramide may therefore be more hydrophilic than that of C_2-dihydroceramide, giving C_2-ceramide different physical properties than C_2-dihydroceramide. These different physical properties could contribute to the different effects exerted

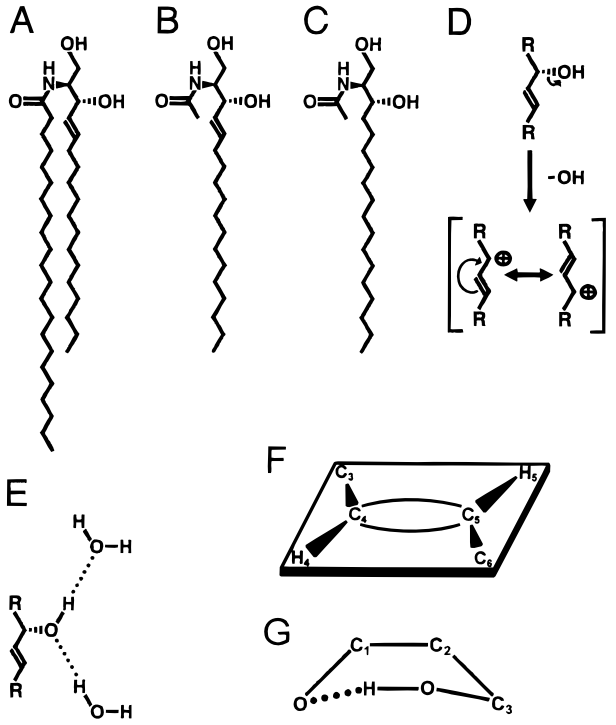

FIGURE 7: Molecular structures of ceramides: possible explanations for the different properties of the C_2-ceramides. (A) The structure of the most prevalent ceramide found in sphingomyelin from human platelets (C_{22}-ceramide): C_{22}-ceramide or *N*-behenylsphingosine (*43*, *52*). (B) The soluble analogue commonly used to study ceramide signaling: C_2-ceramide or *N*-acetylsphingosine. (C) The analogue used as a control for C_2-ceramide and which lacks the 4−5 *trans* double bond: C_2-dihydroceramide or *N*-acetyldihydrosphingosine. (D) C_2-Ceramide has a double bond not found in C_2-dihydroceramide, which is allyl relative to its secondary hydroxyl group and which may allow formation of the depicted resonance structures (*54*). (E) The increased electronegativity of the oxygen of the allyl hydroxyl group of C_2-ceramide may make it more prone to serve as an acceptor in hydrogen bonds than that of C_2-dihydroceramide (*55*). The hydrogen from the hydroxyl group of both C_2-ceramide and C_2-dihydroceramide could serve equally well as a donor in hydrogen bonding, but the oxygen of C_2-ceramide may be a stronger acceptor. (F) The double bond of C_2-ceramide will form a plane, adding rigidity and bulk to its headgroup. (G) The rigidity of the double bond of C_2-ceramide may make the formation of a hydrogen bond between the hydroxyl groups more likely. This hydrogen bond would complete a six-membered ring, forming a second plane in C_2-ceramide's headgroup (*56*).

by the two molecules in this study. Evidence that the allyl hydroxyl of C_2-ceramide makes its headgroup more hydrophilic than that of C_2-dihydroceramide may also be seen in the fact that C_2-ceramide is 5 times more soluble in ethanol than C_2-dihydroceramide (Biomol).

Another possible explanation for the differences between the two short-chain ceramides is also based on the presence of the 4−5 *trans* double bond. The double bond found in C_2-ceramide may add two intramolecular planes to its headgroup, causing it to be more rigid and bulky than that of C_2-dihydroceramide. The double bond between carbon-4 and carbon-5 will induce one plane to form that includes carbon-3, -4, -5, and -6 (Figure 7F). The plane's reduced mobility may induce an intramolecular hydrogen bond to form between the primary and secondary hydroxyl groups, completing a six-membered ring (*56*, *57*). Completion of the six-membered ring would require a second plane to form consisting of carbon-1, carbon-2, and both the hydrogen and oxygen atoms of the secondary hydroxyl (Figure 7G). The existence of these two planes would add rigidity and bulk to the headgroup of C_2-ceramide. C_2-Dihydroceramide lacks the 4−5 double bond and will not form the first plane. Absence of the planar double bond will make the headgroup of C_2-dihydroceramide more flexible and less likely to form the second plane. Without these two planar structures, the headgroup of C_2-dihydroceramide may be less bulky and rigid than the headgroup of C_2-ceramide. In sum, the potential formation of two rigid planes in the headgroup of C_2-ceramide provides an additional mechanistic interpretation for the differences observed between the effects of these molecules on bilayers.

We have not studied the detergent properties of the short-chain ceramides by determining whether C_2-ceramide was actually forming micelles because this was not required for our conclusions. We have investigated the physical properties of C_2-ceramide and C_2-dihydroceramide to the extent of determining their effects on platelet-membrane leakiness (Figure 3), model-membrane leakiness (Figure 4), and model-membrane fluidity (Figure 5). In each of these experiments, C_2-ceramide destabilized the membrane while C_2-dihydroceramide had no effect. Aqueous solutions of C_2-ceramide above 10 μM become cloudy, implying that micelles are not present, since micellar solutions are generally clear. C_2-Ceramide may prefer structures other than a micelle in aqueous solutions that also destabilize bilayers, such as inverted micelles, a hexagonal phase, or large disorganized aggregates. In addition, C_2-ceramide at 1.5 μM, a concentration where the solution is clear, increased the leakiness of model membranes (Figure 4). Regardless of the mechanism of destabilization, C_2-ceramide destabilized membranes while C_2-dihydroceramide did not.

It is possible that the differences between C_2-ceramide and C_2-dihydroceramide that we observe are due to differences in their stereochemistry. However, both compounds are in the D-*erythro* configuration, since they were made from stereochemically pure D-*erythro*-sphingosine (*58*), and the short-chain ceramides retain its stereochemistry.

It is also possible that the effects of C_2-ceramide on platelets represent an isolated situation, but we do not support this hypothesis for the following reasons. Many laboratories (including our own) have used C_2-ceramide as an experimental probe to evaluate potential functional roles for ceramide. Based on our results, we believe that the nonspecific, membrane-destabilizing effects of C_2-ceramide should be considered when interpreting these experiments. Second, human platelets are a well-studied cell for investigating signal transduction. They are primary human cells which respond in characteristic ways to a range of agonists from ADP to thrombin or collagen. Distinct phases of function such as aggregation, shape change, adhesion, and secretion are easily quantitated. Third, human platelets respond positively to many lipids: lysophosphatidic acid (LPA), platelet activating factor (PAF), arachidonic acid, thromboxane A_2 (TXA_2), and sphingosine 1-phosphate (*59*). Thus, platelet function is not generally inhibited by lipophilic molecules. In fact, TXA_2, LPA, PAF, diacylglycerol, and sphingosine 1-phosphate have been reported to cause aggregation or potentiate aggregation or are used as signaling molecules by platelets during aggregation. Fourth, LPA, PAF, TXA_2, and sphingosine 1-phosphate structurally re-

semble detergents. Yet, they stimulate or potentiate aggregation, instead of inhibiting aggregation as did C_2-ceramide (Figure 1). Fifth, there is evidence that platelets use sphingosine and sphingosine 1-phosphate as signaling molecules (*25*, *26*), making it plausible that platelets might utilize ceramide in a signaling role. Sixth, the effects of detergents on human platelet function have been well-characterized in an extensive literature (comments under Results, Figure 2). This 'detergent' literature allowed us to evaluate the similarity between the effects of C_2-ceramide and detergents on platelet function. Last, and importantly, the destabilizing effect of C_2-ceramide on model membranes represents a general effect that the molecule should exert on any membrane. Our experiments with synthetic lipid vesicles are directly relevant in this regard. Therefore, we feel our conclusions are not limited to platelets.

Three other studies are relevant to our results. Yatomi et al. (*26*) reported that bovine ceramide (type III), C_8-ceramide, or C_2-ceramide (ceramide:lipid ratio <0.2) did not by themselves (in the absence of agonists such as ADP or thrombin) stimulate platelet aggregation or shape change as detected by a luminometer (aggregometer). However, they did not assay the effects on ADP-induced aggregation or check morphology by scanning electron microscopy as we have done here.

In 1987, Hannun et al. (*27*) reported that C_2-ceramide (ceramide:lipid ratio of $\sim$0.4) caused slight inhibition of ADP-induced platelet aggregation with no effect on thrombin-induced aggregation. Wong and Li (*28*) found that C_2-ceramide (ceramide:lipid ratio of 0.38) weakly inhibited thrombin-induced aggregation (0.1 unit/mL), while C_6-ceramide or C_2-dihydroceramide did not. We observed a similar inhibition of aggregation by C_2-ceramide in this study, but have performed many additional experiments aimed at determining the specificity and potential mechanisms responsible for C_2-ceramide's effects. These additional studies suggest that inhibition of platelet aggregation by C_2-ceramide is a consequence of membrane destabilization.

An explanation for the lack of effect of C_6-ceramide on aggregation observed by Wong and Li (*28*) mentioned above could be that its N-linked acyl chain is four carbons longer than that of C_2-ceramide and this increased length influences its bilayer-perturbing capabilities. The weak effects of C_2-ceramide on thrombin-induced aggregation also mentioned above (*27*, *28*) are paralleled by the effects of detergents on thrombin-induced aggregation. Thus, Shiao et al. (*45*) observed that detergents (Triton X-100, SDS, octyl glucoside, and deoxycholate) are generally much stronger inhibitors of ADP-induced aggregation than of thrombin-induced aggregation, agreeing with our hypothesis that the effects of C_2-ceramide on platelets are a result of membrane destabilization.

The mechanism of action of two other sphingoid bases, sphingosine and sphinganine, on cells has been questioned due to their ability to destabilize cell membranes. Sphingosine and sphinganine can inhibit PKC in vitro and have been used as tools to evaluate PKC function in cells (*60*). Sphingosine slows platelet aggregation (*27*), while sphingosine and sphinganine have been reported to block superoxide formation by human neutrophils (*61*). However, the specific biochemical mechanisms responsible for these observations have been questioned, since both drugs can also cause cell leakiness (*62*, *63*). Thus, there is precedence for sphingosine and sphinganine, which are structurally quite similar to C_2-ceramide, being able to destabilize membranes.

Similar to our study, others have observed differences between a sphingolipid and its dihydro form. For instance, Corver et al. (*64*) showed that Semliki Forest virus was able to fuse with target membranes containing C_8-ceramide but not C_8-dihydroceramide. The authors attribute this to a specific interaction between the virus and the C_8-ceramide, but it is possible that the lack of the double bond in C_8-dihydroceramide affects the physical properties of the target membrane such that it is no longer able to fuse with the virus.

In addition, the chemical shift observed by ^{31}P-nuclear magnetic resonance (NMR) spectroscopy for the resonance of the phosphorus nucleus in dihydrosphingomyelin (*65*) is different and well resolved from that of sphingomyelin ($\delta = -0.09$ ppm). This suggests that the loss of the double bond significantly affects the environment of the phosphorus. Similarly, the resonances corresponding to the proton associated with the third carbon of the sphingoid bases are different for the two sphingolipids. This proton is adjacent to the 4−5 double bond and demonstrates local effects resulting from the loss of the double bond (*65*). The phase transition temperature of dihydrosphingomyelin is 9 °C higher than sphingomyelin, indicative that the former forms a more ordered, rigid structure than the latter (*66*). Finally, infrared and NMR spectral data acquired by Yappert et al. suggest that there is stronger intermolecular H-bonding between dihydrosphingomyelin molecules than between sphingomyelin molecules (M. C. Yappert, personal communication). These H-bonds may involve the secondary hydroxyl and the amide moieties of adjacent molecules. Thus, these studies support our conclusion that the loss of the double bond from the backbone of a sphingolipid affects its physical and chemical properties.

Our data raise interesting questions for further research, but they also address three important issues. C_2-Ceramide and detergents have similar effects on platelets. C_2-Ceramide destabilized bilayers at the concentrations (5−20 μM) commonly used in cell-signaling studies. C_2-Dihydroceramide does not control for the nonspecific effects of C_2-ceramide. These observations therefore call into question the use of C_2-ceramide and C_2-dihydroceramide as 'mimics' of endogenous ceramide.

Although delivery of short-chain ceramides to cells is easier than delivery of endogenous long-chain ceramides, the short-chain ceramides should be used cautiously. It is necessary to demonstrate that the short-chain analogues are able to represent or 'mimic' the physical properties of endogenous ceramides. Based on our current results, we think it would be productive to develop methods of delivering endogenous or 'natural' ceramides to cells in order to evaluate their contributions as second messengers. Successful delivery of C_{18}-ceramide to cells has been reported using either pure sonicated dispersions or ceramide incorporated into phosphatidylcholine vesicles, although uptake was less than 1% even after overnight incubations (*67*−*69*). Complexing long-chain ceramide with BSA or incorporating ceramide into vesicles made of a variety of phospholipids or mixtures of phospholipids might be more efficient.

In this study, we significantly extend previous observations that C_2-ceramide can inhibit platelet aggregation (*27*, *28*) with new platelet-morphological studies and by examining the effect of C_2-ceramide on model membranes. Our results provide rational alternatives for interpreting data derived from experiments that employ the increasingly popular short-chain ceramides. Many studies have used and continue to use C_2-ceramide under the assumption that it mimics effects of endogenous ceramide. C_2-Dihydroceramide also frequently serves as a control for nonspecific effects of C_2-ceramide. We have shown for human platelets, a well-characterized system for studying signal transduction initiated by a wide range of agonists, that use of these short-chain ceramides may be misleading. In summary, our study shows the ability of C_2-ceramide to inhibit ADP-induced platelet aggregation could be the result of membrane destabilization. Further, C_2-ceramide and C_2-dihydroceramide exerted different 'nonspecific' effects on membranes, suggesting that C_2-dihydroceramide cannot serve as an appropriate control for the nonspecific effects of C_2-ceramide.

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