Positively Charged Ceramide Is a Potent Inducer of Mitochondrial Permeabilization*

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Ceramide-induced cell death is thought to be mediated by change in mitochondrial function, although the precise mechanism is unclear. Proposed models suggest that ceramide induces cell death through interaction with latent binding sites on the outer or inner mitochondrial membranes, followed by an increase in membrane permeability, as an intermediate step in ceramide signal propagation. To investigate these models, we developed a new generation of positively charged ceramides that readily accumulate in isolated and in situ mitochondria. Accumulated, positively charged ceramides increased inner membrane permeability and triggered release of mitochondrial cytochrome c. Furthermore, the positively charged ceramide-induced permeability increase was suppressed by cyclosporin A (60%) and 1,3-dicyclohexylcarbodiimide (90%). These observations suggest that the inner membrane permeability increase is due to activation of specific ion transporters, not the generalized loss of lipid bilayer barrier functions. The difference in sensitivity of ceramide-induced ion fluxes to inhibitors of mitochondrial transporters suggests activation of at least two transport systems: the permeability transition pore and the electrogenic H+ channel. Our results indicate the presence of specific ceramide targets in the mitochondrial matrix, the occupation of which triggers permeability alterations of the inner and outer mitochondrial membranes. These findings also suggest a novel therapeutic role for positively charged ceramides.

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1 The abbreviations used are: PTP, permeability transition pore; TRMM, tetr methylrhodamine methyl ester; D-erythro-C6 pyridinium-DMAS-ceramide, D-erythro-2-N-[6-[(1'-4'-2,3-benzoxazol-4-y1amino)hexanoyl]-D-erythro-sphingosine; TPP1, tetraphenylphosphonium; FCCP, carbonyl cyanide p-trifluromethoxyphenylhydrazone; CSA, cyclosporin A; DCCD, 1,3-dicyclohexylcarbodiimide.

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mechanism by which ceramides alter mitochondrial membrane permeabilization are varied, and the localization of functionally significant ceramide targets (outer mitochondrial membrane, inner membrane, or matrix space) is also unclear.

Given these intimate and direct connections between ceramide and mitochondria, we sought to develop a strategy by which ceramide can be selectively delivered to the mitochondrial matrix to probe its mechanisms of action. In this study, we report on the development of derivatives of ceramide with a fixed positive charge (Fig. 1). These molecules are expected to accumulate in the mitochondrial matrix based on their electrochemical potential and thus would serve as direct probes of ceramide functions on mitochondria. Our results show that these novel agents do localize selectively in mitochondria in living cells. We next investigated the effects of these ceramides on permeability of the inner and outer mitochondrial membranes. Our study shows that these positively charged ceramides increase permeability of the inner membrane (increase in ΔΨ and large amplitude swelling), which, in turn, results in release of cytochrome c. In addition, we provide evidence that ceramide-induced permeabilization of the inner mitochondrial membrane is mediated by specific ion transport systems, viz. the PTP and electrogenic H^+ transporter.

**EXPERIMENTAL PROCEDURES**

**Materials—** RPMI 1640 medium, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were from Invitrogen. TMRM was from Molecular Probes, Inc. C2-NBD-ceramide was from Matreya. Ceramides and their derivatives were from the Lipidomics Core of the Medical University of South Carolina. All other reagents were from Sigma.

**Preparation of Mitochondria from Rat Liver—**Mitochondria were prepared from livers of male Sprague-Dawley rats (220–250 g) fasted overnight. Livers from two rats were homogenized in 100 ml of isolation medium containing 230 mM mannitol, 70 mM sucrose, 2 mM EDTA, and 10 mM HEPES (pH 7.4 adjusted with KOH). The homogenate was centrifuged at 579 × gmax for 10 min to pellet the nucleus and unbroken cells. The supernatant from the previous step was centrifuged at 8000 × gmax for 10 min to pellet the mitochondria. The mitochondrial pellet was washed with 25 ml and then with 12.5 ml of isolation medium without EDTA. The final mitochondrial pellet was resuspended in the above medium to provide a protein concentration of 60 mg/ml. Mitochondrial protein concentration was determined by the BCA assay using bovine serum albumin as the standard (20).

**Mitochondrial Incubation Medium—**Unless otherwise specified, incubations of isolated mitochondria were conducted at 25 °C with 1 mg/ml protein in medium containing 250 mM sucrose, 10 mM HEPES (pH 7.4 adjusted with KOH), 10 mM succinate, 5 mM K2HPO4, and 2 μM rotenone. Deviations from this medium and other reagents employed are described in the figure legends.

**Mitochondrial Respiration—**Oxygen consumption by mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Instech Laboratories) under the conditions described under “Mitochondrial Incubation Medium.”

**Synthesis of Mitochondrially Targeted Ceramide Molecules—**The mitochondrial targeted compounds consisted of the lipophilic cation pyridinium covalently linked to ceramide. These pyridinium-ceramides were prepared by N-acetylation of d-erythro-sphingosine with α-bromo acid chlorides following quaternization of pyridine with the formed α-bromoceramides. The detailed synthesis of pyridinium-ceramides has been described.3

**Measurement of Mitochondrial Permeabilization—**Inner membrane permeabilization was assayed by measurements of ΔΨ and mitochondrial swelling and by changes in mitochondrial ultrastructure. ΔΨ was estimated from the accumulation of TFP – in the mitochondrial matrix as described by Kamo et al. (22). TPP+ (2 μM) was added to the incubation medium as indicated in the figure legends. Mitochondrial swelling was measured by changes in absorbance at 520 nm using a Brinkmann PC 900 probe colorimeter and fiberoptic probe.

Changes in mitochondrial ultrastructure were examined by electron microscopy. Mitochondria were fixed with 3% glutaraldehyde for 15 min, followed by sedimentation and additional fixation overnight. The fixed mitochondria were washed three times with 0.1 M sodium cacodylate (pH 7.4), treated with 2% osmium tetroxide for 1 h, dehydrated through a graded ethanol series, and embedded in Embed 812 resin. Thin sections (70 nm) were stained with uranyl acetate and lead citrate and subsequently examined using a Jeol/JEM1010 electron microscope.

**Cytochrome c Release from Mitochondria—**Aliquots of mitochondrial suspension were taken as indicated in the figure legends and centrifuged at 15,000 × g for 3 min. The supernatant and mitochondrial pellet were frozen and stored at −20 °C. Cytochrome c in the supernatants and pellets was quantified using the Quantikine cytochrome c enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN).

**Cell Culture—**HepG2 cells (obtained from American Type Culture Collection) were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 1.5 g/liter sodium bicarbonate in humidified air (5% CO2) at 37 °C. For confocal microscopy, cells were plated onto poly-D-lysine-coated 35-mm glass bottom microwell dishes at a density of 20,000–25,000/cm2 and were grown for 2 days. MCF7 cells (obtained from American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine in humidified air (5% CO2) at 37 °C. All media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

**Isolation of Mitochondria from HepG2 Cells—**For studies with mitochondria isolated from HepG2 cells, cells were cultured in the medium described under “Cell Culture” for 3 days in 75-cm2 flasks until 70% confluent. Cells were detached by treatment with 3 ml of 0.05% trypsin and 0.53 mM EDTA, diluted to 13 ml with incubation medium, and sedimented at 900 × g for 10 min. The pellet was washed with 1 ml of ice-cold phosphate-buffered saline, and cells were resuspended in 300 μl of isolation medium and then disrupted by 20 passages through a 28-gauge needle (½ inch). The homogenate was centrifuged at 900 × g for 10 min to pellet the nucleus and unbroken cells. The supernatant from the previous step was centrifuged at 10,000 × g for 10 min to pellet the mitochondria, which were then resuspended in incubation medium to provide a protein concentration of ~10 mg/ml.

**Measurement of Cell Viability—**HepG2 or MCF7 cells were plated at a density of 104 cells/well in 96-well plates in the medium described under “Cell Culture.” After 24 h of incubation, the cells were treated with ceramides for 46 h in 2% fetal bovine serum. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) following the manufacturer’s instructions.

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3 Szulc, Z. M., Gustilo, M., Mayroo, N. I. El-Zahwary, A., Bielawski, J., S.-Gracz, H., Hannun, Y. A., Obeid, L. M., and Bielawski, A. (2005) Bioorg.Med.Chem., manuscript in preparation.
DMAS-ceramide, or 2 LSM 510 META system equipped with a krypton/argon laser and a medium supplemented with 2% fetal bovine serum. After 30 min, un-coupling mixture (10 μM oligomycin A) for an additional 30 min to the discharge mitochondrial dria of HepG2 cells in an energy-dependent manner.

A and oligomycin, respectively) resulted in diffuse staining of the cytoplasm for both fluorophores, indicating that mitochondrial accumulation of C₀-pyridinium-DMAS-ceramide in intact cells is indeed energy-dependent. In the presence of uncouplers, the diffuse staining of C₀-pyridinium-DMAS-ceramide probably reflects equilibration of this molecule in cell membranes without specific concentration in any one compartment.

In contrast to C₀-pyridinium-DMAS-ceramide, cells treated with a fluorescent analog of neutral C₆-ceramide, viz. C₀-NBD-ceramide, developed prominent fluorescence in a perinuclear region (Fig. 2A), whereas mitochondrial staining was minimal. These results are consistent with several previous studies that have identified this compartment as the Golgi apparatus, and indeed, C₀-NBD-ceramide has been accepted as a specific marker of this compartment (23, 24). Also, in agreement with previous observations that accumulation of C₀-NBD-ceramide in the Golgi apparatus is energy-independent (24), Fig. 2B demonstrates that uncouplers of oxidative phosphorylation did not affect staining of the perinuclear compartment by C₀-NBD-ceramide. Taken together, these experiments provide evidence that exogenously added pyridinium-ceramide localizes preferentially to mitochondria and that its mitochondrial accumulation in situ is energy-dependent.

To demonstrate definitively that C₀-pyridinium-ceramide preferentially accumulates in mitochondria, HepG2 cells were treated with equal concentrations of C₀-ceramide and C₀-pyridinium-ceramide (3 μM) for 30 min; mitochondria were then isolated; and ceramide values were determined by mass spectroscopy. The amount of C₀-pyridinium-ceramide in mitochondria was ~7-fold higher compared with the amount of C₀-ceramide (985 and 142 pmol/mg of protein, respectively). C₀-pyridinium-ceramide Is a Potent Effector of Cell Viability—Next, we tested whether C₀-pyridinium-ceramide is a more potent cell-killing agent compared with its uncharged analog

**RESULTS**

C₀-pyridinium-ceramide Accumulates in Intact Cell Mitochondria in an Energy-dependent Manner—To determine whether whole cells will accumulate exogenous pyridinium-ceramides in the mitochondrial matrix, we used a fluorescent analog of C₆-pyridinium-ceramide, C₀-pyridinium-DMAS-ceramide. Fig. 2 shows the distribution pattern of C₀-pyridinium-DMAS-ceramide (panels C and D) and the specific mitochondrial reporter TMRM (panels E and F), which is known to accumulate inside the mitochondrial matrix. Both C₀-pyridinium-DMAS-ceramide- and TMRM-treated cells demonstrated a similar punctate pattern of staining, characteristic of mitochondria. Thus, C₀-pyridinium-DMAS-ceramide accumulates selectively in mitochondria in living cells.

Confluent Microscopy—Plated cells were washed once with serum-free medium and treated with 2 ml of 100 nM TMRM, 2 μM C₀-pyridinium-DMAS-ceramide, or 2 μM C₆-NBD-ceramide dissolved in the culture medium supplemented with 2% fetal bovine serum. After 30 min, un-bound dyes were washed out, and images were collected using a Zeiss LSM 510 META system equipped with a krypton/argon laser and a ×63 oil objective (numerical aperture of 1.4). In parallel experiments, after initial loading of cells with TMRM or ceramides, cells were treated with uncoupling mixture (10 μM FCCP, 5 μg/ml antimycin A, and 10 μg/ml oligomycin A) for an additional 30 min to the discharge mitochondrial inner membrane potential. The TMRM images were collected by excitation at 543 nm and emission at 560 nm with a long path emission filter. The C₀-pyridinium-DMAS-ceramide and C₀-NBD-ceramide images were collected by excitation at 488 nm and emission at 505 nm with a long path emission filter.

**Analysis of Ceramides by Mass Spectroscopy**—Accumulated ceramides in mitochondria were analyzed by mass spectroscopy using reverse-phase high performance liquid chromatography couple with an ionization in the multiple reaction monitoring mode. Mass separations were performed using a ThermoFinnigan TSQ 7000 mass spectrometer according to the methodology described by Bielawski et al. (45).

Statistical Analysis—Standard curves and the data for cytochrome c release were computed by generation of a four-parameter logistic curve fit. The values for ceramide accumulation and cytochrome c release are expressed as the means ± S.E. Differences between data were analyzed for significance by Student’s t test. The results were considered significant at p < 0.05.

**Fig. 2.** C₆-pyridinium-ceramide accumulates in the mitochondria of HepG2 cells in an energy-dependent manner. HepG2 cells were treated as described under “Experimental Procedures.” A, C, and E show fluorescent images of the cells stained for 30 min with C₆-NBD-ceramide (C-6-NBD cer), C₀-pyridinium-DMAS-ceramide (C-6-pyridinium-DMAS cer), and TMRM, respectively. In B, D, and F, cells were pre-treated with fluorophores for 30 min and washed, and ΔΨ was dissipated for additional 30 min by a combination of inhibitors of oxidative phosphorylation (see “Experimental Procedures”).

**Fig. 3.** Dose-response curves of the effect of ceramides on the viability of HepG2 and MCF7 cells. HepG2 (A) and MCF7 (B) cells were incubated under the conditions described under “Experimental Procedures.” C₀-ceramide (C-6; traces 1) and C₀-pyridinium-ceramide (C-6-pyr; traces 2) at the concentrations indicated were present from the beginning of the experiment. Cell viability was assessed 48 h after the addition of ceramides. Data are expressed as the means ± S.E. (n = 3).
Indeed, C₆-pyridinium-ceramide readily induced killing of hepatocarcinoma HepG2 cells (IC₅₀ ≈ 8 μM) (Fig. 3A, trace 2), whereas electroneutral C₆-ceramide was much less effective at the same range of concentrations (IC₅₀ ≈ 31 μM) (trace 1). The effect of C₆-pyridinium-ceramide is not unique for HepG2 cells. MCF7 breast cancer cells also responded to this compound (Fig. 3B). MCF7 cells appeared to be more sensitive to C₆-ceramide compared with HepG2 cells (IC₅₀ = 16 μM) and demonstrated a considerable increase in sensitivity to C₆-pyridinium-ceramide (IC₅₀ ≈ 2 μM).

**Accumulation of C₆-pyridinium-ceramide in Isolated Rat Liver Mitochondria Is Energy-dependent—**Next, we set out to determine whether the accumulation of C₆-pyridinium-ceramide by isolated mitochondria is energy-dependent. The addition of C₆-pyridinium-ceramide (10 μM) to mitochondria resulted in 95% association with mitochondria (Fig. 4). Dissipation of mitochondrial ∆Ψ by simultaneous addition of the complex III inhibitor antimycin A and the protonophore FCCP suppressed accumulation of C₆-pyridinium-ceramide by 66.8% (Fig. 4). The difference in the amount of ceramide bound in the absence and presence of uncouplers of oxidative phosphorylation provides the amount of ceramide accumulated by mitochondria in an energy-dependent manner, whereas the component resistant to uncouplers indicates the ceramide that may be partitioned into the lipid phase of mitochondrial membranes or associated with nonspecific binding sites. Calculating the approximate mitochondrial matrix volume as 1.6 μl/mg of protein (25) and the ∆Ψ-dependent uptake of pyridinium-ceramide as 6.28 nmol/mg of protein, the concentration of pyridinium-ceramide in the matrix space can reach 3.9 mM. On the other hand, the addition of uncharged C₆-ceramide (10 μM) also resulted in its considerable association (79.3%) with mitochondria. The striking difference between the association of positively charged C₆-pyridinium and electroneutral C₆-ceramides is that the association of the latter is insensitive to dissipation of ∆Ψ. Thus, the association of C₆-ceramide with mitochondria is exclusively related to its partitioning into the lipid phase of mitochondria and/or its association with nonspecific mitochondrial binding sites. Therefore, C₆-ceramide is evenly redistributed between the lipid phase of the inner and outer membranes with equal concentration of free ceramide in the intermembrane space and the matrix. In contrast, C₆-pyridinium-ceramide is highly enriched in the inner membrane of energized mitochondria, and its free concentration in the matrix space is considerably elevated compared with that in the intermembrane space.

**C₆-pyridinium-ceramide Is a Potent and Specific Inducer of Inner Mitochondrial Membrane Permeabilization—**The results shown above suggest that, because of its greater accumulation in the mitochondrial matrix, C₆-pyridinium-ceramide should affect mitochondrial function more potently compared with neutral ceramides. To this end, we compared the effects of C₆-pyridinium-ceramide and its neutral derivative on permeabilization and to determine the full extent of the potential changes in the parameters of interest. Where indicated, ceramides (40 μM) were present from the beginning of the experiment. Alamethicin (ALA; 7 μg/mg of protein), a pore-forming peptide, was added as indicated to induce permeabilization and to determine the full extent of the changes in the parameters of interest. Where indicated, ceramides (40 μM) were present from the beginning of the experiment. C₆-pyridinium-ceramide (C-6 pyrid); traces 3, C₆-ceramide (C-6); traces 4, C₆-pyridinium-ceramide (C-6 pyrid). RLM, rat liver mitochondria. C, dose-response curves of ceramide effects on mitochondrial swelling. The degree of mitochondrial swelling was determined 30 min after ceramide treatment. Trace 1, C₆-pyridinium-ceramide; trace 2, C₆-ceramide.
reflecting complete dissipation of $\Delta \Psi$. This later phase was accompanied by a rapid increase in absorbance, which indicates stimulation of large amplitude swelling caused by increased permeability of the inner membrane to the components of the incubation medium (Fig. 5B, trace 2). Indeed, the effects of C$_6$-pyridinium-ceramide were very similar to those of the pore-forming peptide alamethicin, the addition of which to the mitochondrial suspension produced essentially the same light-scattering response as C$_6$-pyridinium-ceramide (Fig. 5B, trace 3), suggesting that this ceramide enhances pore formation. Importantly, examination of mitochondrial ultrastructure by electron microscopy before (Fig. 6A) and after (Fig. 6B) the addition of C$_6$-pyridinium-ceramide revealed the typical picture of large amplitude mitochondrial swelling, whereas in the absence of C$_6$-pyridinium-ceramide, mitochondria remained in the aggregated configuration characterized by a shrunken matrix space and a large intracrystal space (Fig. 6A). Incubation of mitochondria with C$_6$-pyridinium-ceramide resulted in an extensive increase in matrix volume and unfolded cristae, characteristic of colloid/osmotic swelling (Fig. 6B). The inner membrane remained apparently intact, whereas the outer membrane was mostly ruptured and detached from the inner membrane. Thus, the results show that C$_6$-pyridinium-ceramide exerts significant effects on isolated mitochondria, which are characterized by a relatively specific increase in permeability of the inner membrane. In contrast to C$_6$-pyridinium-ceramide, which induced dissipation of $\Delta \Psi$ (Fig. 5A, trace 2) as well as mitochondrial swelling (Fig. 5C, trace 1) with IC$_{50}$ ~ 27.5 $\mu$M, neutral C$_6$-ceramide failed to induce dissipation of $\Delta \Psi$ (Fig. 5A, trace 2) or mitochondrial permeabilization (Fig. 5, B, trace 3; and C, trace 2) at concentrations up to 60 $\mu$M.

Structural Specificity of C$_6$-pyridinium-ceramide Action—To verify that the effect of C$_6$-pyridinium-ceramide is due to its acting as a ceramide analog, we compared its effect with the action of a number of structurally related and unrelated positively charged compounds. First, we determined the effect of the pyridinium moiety on mitochondrial permeabilization. To this end, the effects of short chain C$_2$-pyridinium-ceramide and cetylpyridinium were evaluated. Fig. 5 (A, trace 4; and B, trace 4) shows that, employed at the same concentration as C$_6$-pyridinium-ceramide (40 $\mu$M), C$_2$-pyridinium-ceramide caused only minor changes in the magnitude of mitochondrial swelling and the value of $\Delta \Psi$ compared with the control. Even employed at 60 $\mu$M (binding of 29.8 ± 1.4 nmol/mg of protein at 4 min) (Fig. 7A, trace 2), C$_2$-pyridinium-ceramide failed to induce the same degree of swelling that was observed with 30 $\mu$M C$_6$-pyridinium-ceramide (binding of 5 ± 0.8 nmol/mg of protein at 4 min). To the contrary, inhibition of the basal swelling rate was observed.

When used at 60 $\mu$M, cetylpyridinium provided only moderate mitochondrial swelling (Fig. 7B, trace 4). In line with this notion, two other hydrophobic cations (viz. TPP$^+$ and TMRM) that readily accumulated in the mitochondrial matrix driven by $\Delta \Psi$ (negative inside) failed to induce large amplitude swelling even at concentrations twice as high as C$_6$-pyridinium-ceramide (TMRM (Fig. 7, A, trace 3) and TPP$^+$ (Fig. 7B, trace 5), concentration of 60 $\mu$M, binding of 50 ± 2.6 nmol/mg of protein at 4 min; and C$_6$-pyridinium-ceramide (Fig. 8A, trace 2), concentration of 30 $\mu$M, binding of 5 ± 0.8 nmol/mg of protein at 4 min). To the contrary, inhibition of the basal swelling rate was observed.

To further confirm that the effect of C$_6$-pyridinium-ceramide is specific with respect to the structure of this molecule, we investigated the permeabilizing properties of its structural analog, viz. C$_6$-pyridinium-dihydroceramide, which differs only by the lack of a 4,5-trans-double bond in the sphingoid backbone. Fig. 8A shows a ~3-fold increase in the lag period of the induction of mitochondrial swelling in the presence of C$_6$-pyridinium-dihydroceramide (trace 3; concentration of 30 $\mu$M, binding of 9.8 ± 0.7 nmol/mg of protein at 4 min) compared with C$_6$-pyridinium-ceramide (trace 2; concentration of 30 $\mu$M, binding of 5 ± 0.8 nmol/mg of protein at 4 min). Moreover, the dose-response curves (Fig. 8B) demonstrate that increases in the different ceramide concentrations shortened the lag periods of C$_6$-pyridinium- and C$_6$-pyridinium-dihydroceramide.

Fig. 6. C$_6$-pyridinium-ceramide induces ultrastructural changes in isolated rat liver mitochondria characteristic of colloid/osmotic swelling. Mitochondria were incubated under the conditions described under “Experimental Procedures”, fixed, and examined using an electron microscope. Photographs show mitochondria images before (A) and 30 min after (B) the addition of 40 $\mu$M C$_6$-pyridinium-ceramide.
induced swelling. These data indicate that the unsaturated pyridinium-ceramide analog is somewhat more effective than the pyridinium-dihydroceramide analog. Overall, these results indicate that C₆-pyridinium-ceramide can be considered as an analog of the uncharged ceramide and that its action does not reflect nonspecific mitochondrial perturbation that could be expected with any cationic hydrophobic compound.

Inhibitors of Mitochondrial Ion Transporters (CSA and DCCD) Suppress the C₆-pyridinium-ceramide-induced Mitochondrial Permeability Increase—The permeability increase observed in the presence of C₆-pyridinium-ceramide could arise from the formation of lipid channels as a result of perturbation of the hydrophobic portion of the inner membrane, or alternatively, C₆-pyridinium-ceramide could regulate specific transport pathways, resulting in equilibration of small molecules and ions across the inner membrane, large amplitude swelling, and dissipation of $\Delta$$V$. To discriminate between these two possibilities and to address the mechanism by which C₆-pyridinium-ceramide induces mitochondrial permeability, we investigated the effects of the transmembrane PTP inhibitor CSA and the mitochondrial ion transporter nonselective inhibitor DCCD on C₆-pyridinium-ceramide-induced permeabilization of the inner membrane.

As shown in Fig. 9, CSA substantially suppressed (60%) and delayed the pyridinium-ceramide-induced decreases in $\Delta$$V$ and large amplitude swelling (A, trace 3; and B, trace 3, respectively). Chelation of the PTP activator Ca$^{2+}$ by EDTA as well as the use of another PTP inhibitor, bongkrekic acid, resulted in a similar degree of suppression of ceramide-induced mitochondrial alterations (data not shown). Although not as specific as CSA, the carboxylate group modifier DCCD is also known to be an inhibitor of PTP opening (26–28). DCCD also suppressed the permeability increase induced by C₆-pyridinium-ceramide by 90% (Fig. 10A, trace 2). This inhibition reached a maximum at a DCCD concentration of $\sim$40 nmol/mg of protein (Fig. 10, inset).

In contrast to the slow phase of $\Delta$$V$ discharge, the initial fast phase was insensitive to CSA (Fig. 9A, traces 1 and 3) and was accompanied by the shrinkage of mitochondria rather than by large amplitude swelling (Fig. 9B, traces 1 and 3). This rapid discharge of $\Delta$$V$ could be explained by ceramide-induced suppression of respiratory chain activity that could occur directly as described (12–14) or indirectly as a result of cytochrome c release from the intermembrane space (17, 29, 30). However, the addition of 10 $\mu$M cytochrome c (an amount exceeding that for maximum activation of respiratory chain activity (30)) to the incubation medium did not modify the mitochondrial response to C₆-pyridinium-ceramide (data not shown). Moreover, measurement of oxygen consumption of the mitochondrial suspension showed nearly maximum acceleration of respiration within the first minutes after C₆-pyridinium-ceramide addition (Fig. 10B). These data strongly suggest activation of an electrogenic H⁺ leak across the inner membrane as a cause of

**Fig. 7.** Effect of hydrophobic cations on the time course of mitochondrial large amplitude swelling. A and B, mitochondria were incubated under the conditions described under “Experimental Procedures.” Alamethicin (ALA; 7 $\mu$g/mg of protein), a pore-forming peptide, was added as indicated to induce permeabilization and to determine the full extent of mitochondrial swelling. Where indicated, cations (60 $\mu$M) were added to the incubation medium. Traces 1, no addition (A and B); trace 2 (A), C₂-pyridinium-ceramide (C-2 pyr); trace 3 (A), TMRM; trace 4 (B), cetylpyridinium (cetyl-pyr); trace 5, TPP⁺ (B). Trace 3 was corrected for the absorbance of TMRM. For determination of cation and C₆-pyridinium-ceramide binding to mitochondria, the mitochondria were incubated under essentially the same conditions, but 100 $\mu$M DCCD was present from the beginning of the experiment. Four minutes after the addition of ceramides, mitochondria were sedimented, and the amount of ceramides in the pellet was determined by mass spectroscopy. TPP⁺ binding was determined using a TPP⁺-selective electrode as described under “Experimental Procedures.” Because TMRM (similar to TPP⁺) rapidly equilibrates across the inner membrane according to its electrochemical potential, its accumulated amount was assumed to be equal to that of TPP⁺. RLM, rat liver mitochondria.

**Fig. 8.** Time course (A) and dose-response curves (B) of the effect of C₆-pyridinium-ceramide versus C₆-pyridinium-dihydroceramide on mitochondrial large amplitude swelling. Mitochondria were incubated under the conditions described under “Experimental Procedures.” Alamethicin (ALA; 7 $\mu$g/mg of protein), a pore-forming peptide, was added as indicated to induce permeabilization and to determine the full extent of mitochondrial swelling. Where indicated, ceramides (30 $\mu$M) were added to the incubation medium. A, time courses of ceramide effects on mitochondrial swelling. Trace 1, no addition; trace 2, C₆-pyridinium-ceramide (C-6 pyr); trace 3, C₆-pyridinium-dihydroceramide (C-6 Dh pyr). B, dose-response curves of ceramide effects on mitochondrial swelling. The degree of mitochondrial swelling was determined 15 min after ceramide treatment. Trace 1, C₆-pyridinium-dihydroceramide; trace 2, C₆-pyridinium-ceramide. Determination of C₆-pyridinium-ceramide and C₂-pyridinium-dihydroceramide binding to mitochondria was performed as described in the legend to Fig. 7 for C₂-pyridinium-ceramide. RLM, rat liver mitochondria.
 Increased mitochondrial volume, 3-fold decrease in cytochrome c release was observed (Fig. 11B). As shown in Fig. 11B, incubation of mitochondria with C_{6}-pyridinium-ceramide resulted in progressive large amplitude swelling. After 20 min of incubation with C_{6}-pyridinium-ceramide, ~40% of the cytochrome c was released from mitochondria (Fig. 11A). When C_{6}-pyridinium-ceramide-induced mitochondrial swelling was suppressed by the addition of FCCP plus antimycin A (Fig. 11B), an ~3-fold decrease in cytochrome c release was observed (Fig. 11A), which was comparable with the control value. C_{6}-ceramide exerted no effect on cytochrome c release in both the absence and presence of uncouplers of oxidative phosphorylation compared with the control (Fig. 11A). Under the same conditions, C_{6}-ceramide failed to increase large amplitude swelling (Fig. 11B). The addition of the pore-forming peptide alamethicin provided a 100% response in the parameters of interest that can be observed under the conditions employed. These results indicate that the preferential mechanism of cytochrome c release by C_{6}-pyridinium-ceramide is permeabilization of the inner membrane as an initial step, with subsequent swelling and rupture of the outer membrane.

**DISCUSSION**

In this study, we have shown that positively charged C_{6}-pyridinium-ceramide readily permeates the lipid bilayer and specifically targets the inner mitochondrial membrane and matrix space. Because of the large mitochondrial inner membrane potential (negative inside), these molecules accumulate inside isolated mitochondria and within mitochondria in cultured cells. Moreover, accumulation of these molecules is reversible and can be prevented by discharge of ΔΨ. In addition, the accumulation of these ceramides in the mitochondrial matrix space increases permeability of mitochondrial membranes by activating putative ion porters of the inner mitochondrial membrane: PTP and the electrogenic H^{+} channel.

Several observations are in favor of this conclusion. First, C_{6}-pyridinium-ceramide induced a light-scattering response (indicative of change in mitochondrial ultrastructure) that was similar in magnitude to that observed upon conventional Ca^{2+} treatment (data not shown) or in the presence of the pore-forming peptide alamethicin (Fig. 5B, trace 3). This suggests that the light-scattering response observed in the presence of C_{6}-pyridinium-ceramide reflects mitochondrial large amplitude swelling, which is colloid/osmotic in nature as opposed to nonspecific amphiphilic compound-mediated solubilization of mitochondrial membranes. Additional support for the relative specificity of the permeability defect created by C_{6}-pyridinium-ceramide in the inner membrane comes from examination of mitochondrial ultrastructure by electron microscopy (Fig. 6, A and B). Comparison of mitochondrial ultrastructure before and after ceramide treatment revealed all the features of classical permeability transition: increased mitochondrial volume, unfolded cristae, ruptured outer membranes, and apparent intactness of the inner membrane.

A second observation in favor of PTP opening came from the use of the PTP inhibitors CSA and DCCD. These inhibitors suppressed or delayed mitochondrial large amplitude swelling and discharge of ΔΨ by 60 and 90%, respectively. This indicates that the permeability transition observed in the presence of...
C6-pyridinium-ceramide is likely attributed to the activation of protein transporters of the inner mitochondrial membrane rather than the formation of lipid channels created by segregation of ceramides in a special domain, as was proposed previously for the outer membrane (16).

Our data also provide evidence that C6-pyridinium-ceramide activates additional ion transport pathways distinct from PTP. Indeed, the shrinkage phase observed during the first minutes after ceramide addition and the accompanying discharge of $\Delta$$\Psi$ indicate selective loss of cations from the mitochondrial matrix and activation of electrogenic ion fluxes without a simultaneous increase in permeability to sucrose, which is usually observed in classical models of permeability transition. Perhaps these relatively specific cation fluxes reflect operation of PTP in a low conductance (impermeable to sucrose) state, as has been demonstrated previously (31, 32). However, the lack of sensitivity of these fluxes to CSA does not support this notion. It also should be kept in mind that, although DCCD suppresses both these selective fluxes and the nonspecific permeability increase, this does not unequivocally indicate operation of PTP because, in contrast to CSA, this compound can modify other mitochondrial proteins such as the K$^+$/H$^+$ exchanger (33) of the inner mitochondrial membrane and the F$\text{O}$ channel of mitochondrial ATPase (34, 35).

The best explanation for the initial mitochondrial response to ceramide treatment seems to be simultaneous activation of selective electrogenic K$^+$ and H$^+$ fluxes. K$^+$ is known to be the most abundant ion in the mitochondrial matrix, playing a major role in regulation of mitochondrial volume (36). In this model, increased H$^+$ permeability across the inner membrane...
Alamethicin (7/H1106) from isolated mitochondria under the effect of C2-dihydroceramide (38) demonstrate a high degree of specificity for zymes (drial permeabilization. Some of the cellular responses and en-

tion of reactive oxygen species by mitochondria appears to lack

ment Procedures." C6-pyridinium-ceramide (17) demonstrates a

time, genera-

FIG. 11. C6-pyridinium-ceramide-induced large amplitude swelling (B) is accompanied by cytochrome c release (A). Mitochon-dria were incubated under the conditions described under “Experimental Procedures.” C6-pyridinium-ceramide (C-6 pyr) or C6-ceramide (C-6) at 40 µM was added at 2 min, and mitochondria were incubated for an additional 20 min, followed by the addition of CSA (1 µM) and EGTA (1 mM) to prevent further permeabilization. Two minutes after the addition of CSA and EGTA, samples were collected and treated for cytochrome c analysis as described under “Experimental Procedures.” Alamethicin (7 µg/mg of protein) was added as indicated to determine the full degree of permeabilization and maximum cytochrome c release. Where indicated, FCCP (1 µM) and antimycin A (0.5 µg/mg of protein) were present from the beginning of the experiment. The total amount of cytochrome c is 1.95 µg/mg of protein. Data are expressed as the means ± S.E. (n = 3). *, p < 0.05 versus the control.

dissipates Δψ, which allows K+ to be lost from the matrix according to its electrochemical potential, which, in turn, results in mitochondrial shrinkage.

One of the interesting questions is the structural specificity of C6-pyridinium-ceramide action in the induction of mitochondrial permeabilization. Some of the cellular responses and enzymes (e.g. apoptosis (37) and ceramide-activated protein phosphatase (38)) demonstrate a high degree of specificity for ceramide versus dihydroceramide. At the same time, gene-

eration of reactive oxygen species by mitochondria appears to lack this specificity (12). Also, a report from Richter and co-workers (17) demonstrates a >3-fold increase in cytochrome c release from isolated mitochondria under the effect of C2-dihydroceramide compared with the control. These considerations are important with regard to ceramide interactions with PTP, which has been implicated by this and previous studies to

mediate the ceramide effect on mitochondria. Previous work by Gudz et al. (39) and Walter et al. (40) postulated the presence of low and high affinity binding sites that can nonspecifically interact with a variety of hydrophobic compounds, resulting in PTP opening or closure. The natural effectors of these sites are unknown, but ubiquinones of the respiratory chain (40) or ceramides (18, 19) may be good candidates for this role. This could explain our observation that the selectivity for C6-pyridinium-ceramide versus C6-pyridinium-dihydroceramide is not absolute.

Our observation that suppression of C6-pyridinium-ceramide-induced mitochondrial swelling by FCCP also resulted in suppression of cytochrome c release indicates that mitochondrial swelling is a prerequisite for the outer membrane permeability alterations. Even at 40 nmol/mg of protein, a concentration twice that used by Siskind et al. (16), neutral C6-ceramide failed to induce considerable cytochrome c release compared with the control. It has been reported that loss of cytochrome c by mitochondria under the effect of C2-ceramide is highly dependent on the redox state of this protein, with the oxidized state favoring the release (17). However, we found no substantial release (compared with the control) of cytochrome c by C6-ceramide and C6-pyridinium-ceramide under conditions in which the respiratory chain downstream of complex III is completely oxidized by the presence of oxidative phosphorylation uncouplers. On the contrary, suppression of cytochrome c release was observed. This provides evidence that, in our experiments, the limiting step in cytochrome c release is not a redox state value, but the formation of a permeability pathway for cytochrome c across the outer membrane. This conclusion fits well with the observation of Kristal and Brown (41), who suggested that, under conditions in which C2-ceramide (100 nmol/mg of protein) is unable to induce the permeability transition of the inner membrane, no cytochrome c release is observed. In agreement with these data, in the experiments of Szalai et al. (19), conditions that resulted in C2-dependent increase in permeability of the inner membrane were also found to trigger cytochrome c release from mitochondria in a CSA-sensitive manner.

Notably, previous studies suggested that either Ca2+ at 100–150 µM or Bax is required in addition to ceramide to cause permeability change in the outer and inner membranes (18, 19). In contrast, in our experiments, C6-pyridinium-ceramide by itself induced permeabilization of the mitochondria, or the requirement for Ca2+ was extremely low. (The estimated endogenous Ca2+ concentration is ~10 nmol/mg of protein.) This effectiveness of C6-pyridinium-ceramide is best explained by its greater accumulation in the mitochondrial matrix. In addition, the low potency of C2-pyridinium compared with C6-pyridinium-ceramide likely excludes the possibility of a nonspecific effect of the pyridinium group on mitochondrial membranes and underscores the importance of the length of the N-fatty acylphosphoglycerol moiety in mitochondrial permeabilization.

Our results obtained by in vitro experiments indicate that mitochondria are the primary targets for C6-pyridinium-ceramide in cell death and that the mechanism of cell death involves disruption of mitochondrial function. Indeed, by confocal microscopy, we observed preferential accumulation of C6-pyridinium-ceramide in the mitochondrial compartment, and the relative potency of C6-pyridinium-ceramide to induce permeabilization of isolated mitochondria corresponds well with its ability to kill cells. One of the factors that should be kept in mind while considering the effect of ceramide treatment on cell viability is the concentration of ceramide in the vicinity of its target. Electroneutral ceramides redistribute preferentially in the Golgi apparatus (Fig. 2, A and B), which decreases their
effective concentration in mitochondria. In contrast, positively charged ceramides are specifically concentrated within their immediate target, the inner mitochondrial membrane, whereas redistribution to other compartments is relatively small (Fig. 2C). This specific redistribution of positively charged ceramide correlates well with its higher potency in cell killing compared with its neutral counterpart. In this way, our results support the hypothesis that the mechanism by which ceramides induce cell killing is permeabilization of the inner mitochondrial membrane with subsequent release of cytochrome c. With respect to the mechanism of pyridinium-ceramide-induced cell death, it should be noted that the permeability alterations of the inner mitochondrial membrane, whereas positively charged ceramides are specifically concentrated within their effective concentration in mitochondria. In contrast, positively charged ceramides to increase their accumulation in the mitochondrial matrix, the occ-