ABSTRACT

Title of Document: CERAMIDE PERMEABILIZATION OF MITOCHONDRIAL OUTER MEMBRANE: PHARMACOLOGICAL CHARACTERIZATION AND RELATION TO MAC AND BAX

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In apoptosis, the mitochondrial outer membrane (MOM) becomes permeable, releasing proteins. This permeability has been attributed to the action of various factors, including mitochondrial apoptosis-induced channel (MAC), Bax and ceramide channels. Amphiphilic cations that inhibited MAC and Bax-induced permeabilization were tested on ceramide-induced permeabilization of MOM of mammalian and yeast mitochondria, as well as liposomes. Both propranolol and dibucaine inhibited C_2- and C_{16}-ceramide-induced permeabilization of mammalian MOM with an IC_{50} for C_{16}-ceramide of 410 and 230 μM, respectively. In yeast mitochondria, propranolol and dibucaine inhibited C_2-ceramide-induced permeabilization, but potentiated the effect of C_{16}-ceramide. Similar results were obtained in liposome experiments. These results suggest that inhibition is via another factor found in mammalian cells but not the other systems. The pharmacology of

ceramide membrane permeabilization is inconsistent with that of MAC but is compatible to that of Bax.

CERAMIDE PERMEABILIZATION OF MITOCHONDRIAL OUTER MEMBRANE: PHARMACOLOGICAL CHARACTERIZATION AND RELATION TO MAC AND BAX

By

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CF:	carboxyfluorescein;
DPX:	*p*-xylene-bis-pyridinium bromide
C_2-ceramide:	*N*-acetyl-D-*erythro*-sphingosine
C_{16}-ceramide:	*N*-palmitoyl-D-*erythro*-sphingosine
MOM:	mitochondrial outer membrane
PTP:	permeability transition pore
MAC:	mitochondrial apoptosis-induced channel
TFP:	trifluoroperazine

Chapter 1: Introduction

Apoptosis is a necessary process required to maintain homeostasis in higher organisms. Various stimuli, including physiological factors as well as cytotoxic agents, can initiate apoptosis, whereby the cell undergoes a series of biochemical and physical changes, resulting in its termination. Apoptosis is an organized way for the cell to die, and is morphologically different from necrosis. Necrosis is characterized by a rupture of the plasma membrane, leading to a leakage of contents, resulting in an inflammatory response. Whereas in apoptosis, the cell contents are broken down and repackaged into apoptotic bodies. These apoptotic bodies are then engulfed by phagocytes, resulting in no inflammatory response (for review see 1-2).

In a healthy organism, the regulation of apoptosis is used to maintain the balance between multiplying and dying cells. For example, apoptosis eliminates damaged and differentiated cells that are no longer needed and would otherwise take up space and consume resources. However, apoptosis is also fundamental to many diseases. For example, the failure of cells to undergo apoptosis occurs in cancers and excessive apoptosis is central to neurodegenerative diseases, such as Parkinson's and Alzheimer's (for review see 3). In order to develop more effective therapy and drugs to treat these many diseases, it is essential to gain a better understanding of the apoptotic mechanism, more specifically, the initiation steps.

In addition to the naturally programmed cell death, the initiation of apoptosis can occur under a variety of conditions, such as serum deprivation, oxidative stress and treatment with cytotoxic drugs. The cell can undergo apoptosis via an extrinsic (receptor-mediated) or intrinsic (mitochondrial) pathway (for review see 1-2).

Briefly, the extrinsic pathway is triggered by receptors located on the cell that will undergo apoptosis. Once activated by extracellular ligands, a series of intracellular events occur ultimately leading to the activation of death caspases, which are responsible for degrading the cell. The intrinsic pathway is initiated from within the cell, causing a permeability increase of the mitochondrial outer membrane (MOM). This permeability increase results in the release of pro-apoptotic proteins, such as cytochrome c, AIF, endonuclease G, Smac/DIABLO and HtrA2/OMI from the intermembrane space (for review see 4). Once released into the cytosol, these death proteins can then activate caspase dependent and independent cell death pathways. The intrinsic and extrinsic pathways converge at the caspase level; however, the extrinsic pathway can also stimulate the intrinsic pathway.

One major goal of many apoptosis researchers is to gain a better understanding of how the mitochondria release the pro-apoptotic proteins. The mechanism which results in an increase in the permeability of the mitochondrial outer membrane to pro-apoptotic proteins continues to be an intense topic of debate. In one hypothesis, a permeability transition pore (PTP) complex is formed early in apoptosis.[5] PTP complex is believed to be made up of the inner mitochondrial membrane adenine nucleotide translocase in association with the outer mitochondrial membrane voltage-dependent anion channel (VDAC) and a matrix protein, cyclophilin D. This pore complex spans the outer and inner membranes dissipating the membrane potential and allowing low molecular weight solutes to permeate. As a result, the matrix swells, thereby causing rupture to the outer membrane, leading to the release of pro-apoptotic proteins into the cytosol.[6] While some experiments have

shown that the loss of potential is required for a complete loss of cytochrome c[7], others propose that cytochrome c release can occur prior to or in the absence of changes in the inner membrane potential.[8,9] Furthermore, the mitochondrial outer membrane remains intact after the release of cytochrome c in some cell types.[7] These findings suggest that a pore may be forming in the MOM in order to facilitate the release of the pro-apoptotic factors.

The nature and components of a channel formed to release pro-apoptotic proteins is unknown. Several hypotheses are based on channels formed by Bcl-2 family proteins. Bcl-2 family proteins are known regulators of apoptosis, and are classified as pro- or anti-apoptotic.[1,10-12] There are three main groups of Bcl-2 proteins. Group I contains anti-apoptotic proteins (i.e. Bcl-2, Bcl-x_L) and are characterized by four short, conserved Bcl-2 homology (BH) domains, known as BH1-BH4. Group II contains pro-apoptotic proteins (i.e. Bax, Bak) and have similar structure to group I, except they do not contain the N-terminal BH4 domain. Group III consists of pro-apoptotic polypeptides (i.e. Bid, Bad) and contain only a single BH3 domain. Group III act by binding the BH3 domain in group I or II. One hypothesis suggests that Bid and Bax, cooperate with lipids to form supramolecular openings on the mitochondrial outer membrane.[13] While others propose that the formation of Bax channels facilitates the release of pro-apoptotic proteins.[14,15] In fact, Bax channels are large enough to release cytochrome c when in oligomerized form.[14,16]

Another theory is based on release via the mitochondrial apoptosis-induced channel (MAC). MAC has been detected in apoptotic cells about the time

cytochrome c is released.[17,18] However a recent study demonstrated that MAC does not appear until a later stage in apoptosis, after the permeabilization of the MOM.[19] The diameter of MAC has been estimated to be ~ 5 nm,[20] which is large enough to release cytochrome c, but not the larger pro-apoptotic proteins. Thus far, the molecular identity of MAC is unknown, however several lines of evidence identifies Bax as a component.[17,18,20]

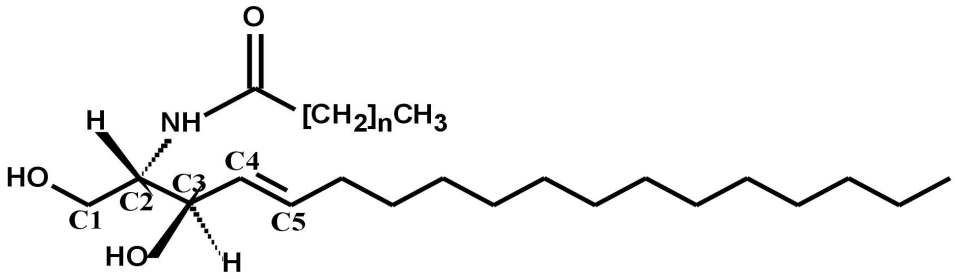


Figure 1: General structure of a ceramide molecule. Ceramide is made up of a backbone sphingoid base and an N-linked fatty acyl chain that can vary from n=1 to more than 23.

Another hypothesis is based on the ability of the sphingolipid ceramide (Figure 1) to form channels.[21,22] Ceramide is known to be involved in the regulation of differentiation, cell cycle arrest, cellular senescence and apoptosis.[23-26] Ceramide can be synthesized in the cell via de novo synthesis from sphinganine and fatty-acyl-CoA or through hydrolysis of sphingomyelin as catalyzed by the enzyme sphingomyelinase. Interestingly, many lines of evidence link ceramide to the process of apoptosis. For example, total cellular ceramide levels have been shown to increase up to 10 mole percent of the total cellular phospholipids during apoptosis.[24] Many agents which initiate apoptosis also induce ceramide formation, such as TNF,[27] fas

ligands,[28] nitric oxide, ionizing radiation and chemotherapy drugs.[24,29-31] In addition, exogenously added cell permeable ceramide analogues are capable of inducing apoptosis in several cell lines.[22-34] Thus, ceramide appears to play a key role in the apoptotic process.

Ceramide plays a role specifically in the mitochondrial stage of apoptosis. Early generation of de novo derived C_{16}-ceramide in response to B-cell receptor cross-linking was linked to a loss of mitochondrial function and subsequent activation of the apoptotic program.[35] Fumonisin B1 (an inhibitor of ceramide synthesis) completely prevented not only ceramide production, but also disrupted mitochondrial membranes, PARP cleavage, and DNA fragmentation.[35] Short-chain cell permeable ceramide analogues, such as C_2- and C_6-ceramide, have been shown to induce cytochrome c release when added to whole cell cultures[31,36-41] and isolated mitochondrial suspensions.[42-44] Long-chain, naturally occurring C_{16}-ceramide also induced release of cytochrome c[43] and AIF[45] when added to mitochondrial suspensions. Furthermore, cytochrome c release is decreased when cells are treated with inhibitors of ceramide synthesis.[46] Enzymes responsible for ceramide metabolism (ceramide synthase and ceramidase) have been identified in mammalian mitochondria.[47,48] Furthermore, highly purified mitochondria can generate ceramide via ceramide synthase and reverse ceramidase pathways.[49] Thus, the enzymatic machinery exists in mitochondria for the formation and breakdown of ceramide.

Siskind and Colombini reported that C_2- and C_{16}-ceramides form large stable channels in phospholipid membranes[21] (see Figure 2 for a sample trace). The discrete stepwise changes in current under voltage-clamp conditions are characteristic of

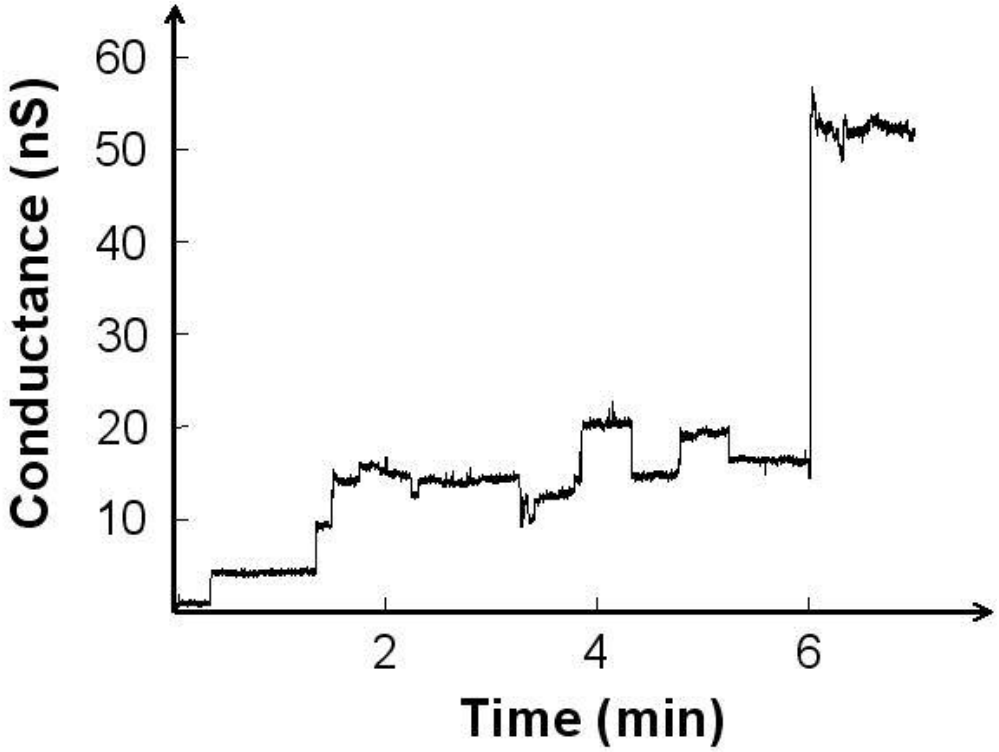


Figure 2: Ceramide conductance trace. 5 μM of C2-ceramide was added to the aqueous phase (1 M KCl, 1 mM $MgCl_2$, 5 mM PIPES, pH = 6.8) in a planar phospholipid membrane experiment. The phospholipid monolayers were composed of the following (w/v): 0.5% diphytanoylphosphatidylcholine, 0.5% asolectin, 0.2% cholesterol. The applied voltage used in this experiment was 10 mV. (Figure from Siskind, 2005[102])

membrane channels. Conductances from 1 to 200 nS have been observed corresponding to pore diameters from 0.8 to 11 nm.[21] The current structural model is based on a fundamental unit of a column of ceramide molecules stabilized by intermolecular hydrogen bonds between the carbonyl oxygens and amide nitrogens on opposite faces of the ceramide molecule.[22] Multiple columns would come together to form an annulus, which is stabilized by a hydroxy-hydrogen-bonded network proposed to line the channel lumen (Figure 3). The channel size would thus depend on the number of ceramide columns making up the annulus. In order to span the

width of the membrane, the columns would have to be made up of 6 to 7 ceramide molecules.

The channel-forming ability of ceramide was tested in isolated rat liver mitochondria.[50] Ceramide-treated mitochondria induced a size-selective protein release from the intermembrane space with a cut-off of 60 kDa. This is consistent with the size of the intermembrane-space proteins that are released from mitochondria

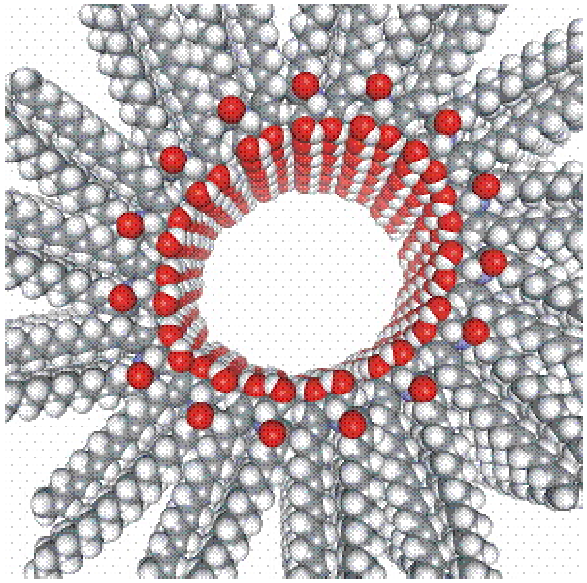


Figure 2: Theoretical structural model of a ceramide channel. A top view of the channel is shown. Each column consists of 6-7 ceramide molecules.

during the induction phase of apoptosis. Importantly, the ceramide precursor dihydroceramide (differs from ceramide by reduction in a double bond) is incapable of inducing apoptosis,[51] does not form channels in phospholipid membranes and does not allow release of intermembrane space proteins from mitochondria.[50] Therefore the apoptotic activity of ceramide correlates directly with its channel-forming ability.

It is possible that ceramides form homogenous channels and/or heterogenous channels with Bax, for example. In planar membrane experiments, ceramides form channels that can pass molecules with a diameter as large as 11 nm, which would correspond to a molecular weight ~300 kDa. However, when ceramide is added to rat liver mitochondria, the molecular weight cut-off is only 60 kDa. The difference may have to do with the fact that planar membrane experiments are performed in a protein-free environment. Therefore, size may be controlled in mitochondria via interactions with other lipids and/or proteins. One possibility may be through an interaction with Bcl-2 proteins, such as Bax.

Pharmacological experiments were performed to see if agents that inhibit MAC and Bax-induced permeabilization also inhibit ceramide-induced permeability. The inhibitors tested were the amphiphilic cations shown in figure 4: propranolol, dibucaine, lidocaine and trifluoroperazine (TFP), as well as the PTP inhibitor cyclosporine A.

Lidocaine and dibucaine are local anesthetics. Local anesthetics function by blocking sodium channels, thereby preventing depolarizations of nerve cells. The mechanism of blockage is unknown but several theories exist. One theory is that the drug binds to a receptor in or near the ion pore, thereby blocking the channel. [52] Another theory suggests that changes in membrane surface charge due to the anesthetic is important.[52] However this theory does not explain how neutral anesthetics act. Yet another theory proposes that the anesthetics increase membrane fluidity however it is not clear how this would cause a block in sodium conductance.[53]

Propranolol is a non-selective beta-adrenergic receptor blocking agent. It blocks the receptor for epinephrine or norepinephrine, thereby decreasing sympathetic stimulation. Propranolol is used to treat hypertension,[54-55] cardiac arrythmias and angina pectoris;[56] as well as to prevent myocardial infarction.[57-58] TFP is a phenothiazinic drug, which is a type of anti-psychotic drug used to treat conditions such as schizophrenia and bipolar disorder.

Amphiphilic cations have been shown to have protective effects against ischemia-reperfusion injury,[59] as well as anti-apoptotic effects.[60] Both TFP and dibucaine inhibited or delayed PTP;[61] and propranolol inhibited the "mitochondrial megachannel", which is an electrophysiological manifestation of the PTP.[62] Some amphiphilic cations also inhibit channels. For example, propranolol, dibucaine and TFP (TFP being the most effective) inhibited MAC[63] as well as protein import into the mitochondria (possibly through an interaction with the translocase of the inner membrane (TIM)).[64] Propranolol has been shown to inhibit the inner membrane anion channel (IMAC);[65] and both propranolol and dibucaine (propranolol being more effective) inhibited Bax channels.[66] Since these cations have been shown to inhibit different channels, they were tested on ceramide channels. The results in this study show that some amphiphilic cations that inhibit MAC and Bax channels also inhibit ceramide channels; however the inhibition patterns do not conclusively link ceramide to MAC or Bax.

Chapter 2: Materials and Methods

Materials – Wild-type yeast cells (S. cereviciae) were obtained from a local bakery. Asolectin (polar extract of soybean lipids), C_2-ceramide and C_{16}-ceramide were supplied by Avanti Polar Lipids (Alabaster, AL). 4-Carboxyfluorescein (CF) and *p*-xylene-bis-pyridinium bromide (DPX) were from Molecular Probes (Eugene, OR). Cytochrome c, antimycin A, 2,4-dinitrophenol (DNP), sodium ascorbate, bovine serum albumin (BSA, fatty acid depleted), dibucaine hydrochloride, lidocaine hydrochloride and cholesterol were from Sigma (St. Louis, MO). DL-Propranolol was from Acros Organics (Geel, Belgium). Trifluoperazine dihydrochloride (TFP) was from MP Biomedicals (Solon, OH). Cyclosporine A (CsA) was a generous gift from Sandoz Pharmaceuticals (Holzkirchen, Germany).

Mitochondria Isolation - Male Sprague-Dawley rat liver mitochondria were isolated by standard differential centrifugation as previously described[67] with minor changes. Cell debris was sedimented at 600 g and mitochondria at 9700 g. The isolation buffer used was 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA, 0.5% (w/v) BSA (fatty acid depleted bovine serum albumin) and 5 mM HEPES, pH 7.4. The buffer used for the last spin and final resuspension did not contain BSA. Yeast mitochondria were isolated by standard differential centrifugation as previously described[68] with minor changes. The Percoll gradient was found to be unnecessary and was omitted from later experiments. Also the buffer used for the last high speed spin and final resuspension was 0.65 M Sucrose, 0.1mM EGTA, 10 mM HEPES, pH 7.2. Protein concentration was determined spectroscopically.[69]

Preparation of Reduced Cytochrome c - 11 mg of cytochrome *c* and 4 mg sodium ascorbate was mixed in 500 μL of 200 mM HEPES, 10 mM EGTA, pH 7.5. The reduced cytochrome *c* was separated from the ascorbate on a Sephadex G-10 gel filtration column.

Detecting Mitochondrial Outer Membrane Permeabilization - The method of Siskind et al., 2002;[50] was used to assess the permeability of MOM to cytochrome c. Either rat liver mitochondria (10 μg of protein) or yeast mitochondria (40 μg of protein) were suspended in 750 μL of isolation medium containing 1.5 μM antimycin A and 0.5 mM 2,4-dinitrophenol. After different treatments, permeabilization of MOM was found from the initial oxidation rates, obtained from the decrease in absorbance at 550 nm after the addition of reduced-form of cytochrome c. Intactness was calculated from the rates obtained from intact and hypotonically-shocked mitochondria, and only mitochondria with an intactness greater than 80% was used. Ceramide was added either as 9 – 36 nmoles of C_2-ceramide (stock solution: 0.25 - 1 mg/mL DMSO) or 36 – 147 nmoles of C_{16}-ceramide (stock solution: 2 mg/ml isopropanol). Within in one experiment, the ceramide concentration was constant. However, different mitochondria isolations had different sensitivities toward ceramide, therefore the amount of ceramide used varied in order to obtain a comparable rate of cytochrome c oxidation. Also, the C_{16}-ceramide concentration used was higher than the C_2-ceramide concentration because there is much less insertion of the long-chain ceramide in the mitochondrial membrane (L. Siskind, unpublished results). Besides permeabilization increase, C_{16}-ceramide slightly increased light scattering.

Therefore, the rate change at 600 nm was subtracted from the rate change at 550 nm for all experiments performed with C_{16}-ceramide to ensure that only cytochrome c oxidation was being measured. Propranolol, dibucaine, lidocaine and TFP were added from an aqueous solution, and CsA was added from an ethanol vehicle (stock solution: 133μM). All experiments that yielded inhibitory results on ceramide were tested on hypotonically-shocked mitochondria to determine if effects were due to inhibition of cytochrome oxidase or a rereduction of cytochrome c. None of the compounds changed the rate when added to the shocked mitochondria alone. However, in the presence of ceramide, propranolol and dibucaine had small inhibitory effects at higher concentrations. In order to correct for this, an inhibition factor, *f* was calculated: $f = \frac{\upsilon_0}{\upsilon_{\max}}$, where υ_0= rate of shocked mitochondria in the presence of ceramide and the drug and $\upsilon_{\max}$ = the rate of shocked mitochondria in the presence of the vehicle. The corrected rate *r* was calculated: $r = \frac{\upsilon_1}{f}$, where υ_1 = the rate obtained from intact mitochondria treated with ceramide and the drug. Vehicle controls were performed as appropriate.

Preparation of Liposomes – 93% asolectin and 7% cholesterol (by weight) was dissolved in chloroform, dried with a N_2 stream and was put under vacuum for 2 hours. The liposomes were resuspended in buffer A (1.5 mM CF, 6 mM DPX, 39 mM NaCl, 10 mM HEPES and 1mM EDTA pH 7.0) and were subjected to 4 freeze-thaw-sonication cycles, followed by 11 extrusions through a 200 μm pore size filter.

The nonencapsulated fluorophores were separated from the liposomes using a sephacryl S-200 column and eluted with buffer A.

Determination of Liposome Permeabilization - 50 μL of the liposome suspension (~ 0.1 mg of lipid) were diluted into 2 mL of Buffer A. Fluorescence was measured as a function of time using a Deltascan spectrofluorometer (Photon Technology Instruments). The λ_{ex} was 496 nm and the λ_{em} was 516 nm. Propranolol was added at 15 seconds and ceramide was added at 75 seconds. Increase in fluorescence was due to release of CF from the liposomes and its dilution from the quenching agent, DPX. The fractional fluorescence *fl* was calculated from the following equation: $fl = \frac{F - F_0}{F_{\max} - F_0}$, where F = each measured fluorescence intensity, F_0 = the initial fluorescence of intact liposomes and $F_{\max}$ = the maximal fluorescence after the membranes have been solubilized with 30 μL of 5% Triton-X 100. At lower propranolol concentrations there was an initial increase in fluorescence that remained level with time. However, this effect disappeared at higher concentrations. Therefore this initial rise was subtracted out of the data, and the results shown are only the effect of propranolol on ceramide.

Chapter 3: Results

Some amphiphilic cations inhibit ceramide-induced permeabilization in rat liver mitochondria

C_{16}- and C_2-ceramide have been shown to form channels in the outer membrane of rat liver mitochondria.[50] Channel formation is inferred from an increase in the permeability of this membrane to cytochrome c. This permeability increase was assessed by measuring the rate of oxidation of cytochrome c by cytochrome oxidase, as a decrease in absorbance at 550 nm. Addition of ceramide to a mitochondrial suspension results in the development of permeability to cytochrome c after a 5 to 10 minute incubation period.

To assess the effect of amphiphilic cations on ceramide-induced permeabilization of mitochondria, rat liver mitochondria were pretreated with propranolol, dibucaine, TFP or lidocaine prior to ceramide addition. Propranolol and dibucaine inhibited the rate of cytochrome c oxidation caused by ceramide (Figures 4a and 4a) and the inhibition was found to be dose-dependent (Figures 4b and 4b). Note that both inhibitors were equally effective on both long- and short-chain ceramide. TFP was found to be ineffective on C_{16}- ceramide-induced permeabilization up to a concentration of 12 μM. Lidocaine had little effect on C_2-ceramide (23% inhibition with 500 μM and 38% inhibition with 900 μM) and no effect on C_{16}-ceramide-induced permeabilization (results not shown). CsA inhibited C_{16}-ceramide-induced permeabilization in a dose-dependent manner (Figure 6). However, the addition of C_{16}-ceramide to isolated mitochondria did not cause

swelling; and actually slightly increased absorbance at 600 nm (results not shown). Therefore the inhibition of C_{16}-ceramide-induced permeabilization by CsA is not through an effect on PTP. The IC_{50} for dibucaine, propranolol and CsA (and Hill coefficients) found for inhibition of C_{16}-ceramide-induced permeabilization are 235 μM (0.81), 415 μM (1.01) and 3.4 μM (0.54), respectively.

Since the Hill coefficients for dibucaine and propranolol were essentially 1, this indicates a 1:1 complex between these agents and the ceramide-induced permeability pathway. The hundreds of ceramides required to form a ceramide channel are not condusive to a simple 1:1 stoichiometry. This may indicate the presence of another factor that confers sensitivity to these agents. The fractional Hill coefficient observed with CsA could indicate an indirect, distal effect.

Propranolol inhibits C_2- but not C_{16}-ceramide-induced liposome permeabilization

It is possible that the inhibitory effect of propranolol on ceramide involves other mitochondrial components such as proteins. Therefore its effect was tested on protein-free liposomes. Liposomes loaded with CF and its quencher DPX were pre-incubated with propranolol for 1 minute, followed by ceramide addition. As seen in Figure 7a and 7b, propranolol inhibits C_2-ceramide-induced permeabilization in a dose-dependent manner. The fluorescence increase has 2 components. The fast initial component is not inhibitable. Nevertheless, in calculating the % inhibition we considered the entire response. Thus the maximal inhibition appears at 55%. Note that the inhibition occurred at lower concentrations in liposomes than in

mitochondria. For example, 50 μM of propranolol yields ~ 19 % inhibition in liposomes, but no significant inhibition in rat liver mitochondria. This higher sensitivity to the inhibitors may indicate a fundamentally different mechanism. This is confirmed by the failure of propranolol to inhibit channel formation by C_{16}-ceramide. The results are expressed as "relative ratios". These are defined as the fractional fluorescence of propranolol + ceramide divided by the fractional fluorescence of ceramide alone (Figure 7c). A ratio greater than 1 is indicative of potentiation and less than 1 is indicative of inhibition. As seen from the relative ratios in Figure 7c, propranolol potentiates the effect of C_{16}-ceramide in a concentration-dependent manner.

Propranolol and dibucaine inhibit C_2- but not C_{16}-ceramide-induced permeabilization in yeast mitochondria

The different results obtained from experiments on rat liver mitochondria and those on liposomes indicate the presence of a factor in mitochondria that might confer a particular sensitivity to the amphiphilic cationic inhibitors. This factor might not be present in yeast mitochondria because yeast cells do not contain the same apoptotic factors found in mammalian mitochondria. If this hypothetical factor were required to sensitize mitochondria to ceramide channel formation, yeast mitochondria may be refractory to ceramide addition.

Yeast mitochondria were found to be as responsive to ceramide permeabilization of their outer membrane as mammalian mitochondria (Figure 8a). However, their response to propranolol and dibucaine resembled that observed in the

liposome experiments. The effects of propranolol or dibucaine on C_{16}- and C_2-ceramide-induced permeabilization of MOM is expressed using a relative ratio as defined above. As seen in Figures 8b and 8c, propranolol and dibucaine inhibit C_2- but not C_{16}-ceramide-induced permeabilization. Similar to the results found in liposomes, the effect of C_{16}-ceramide-induced permeabilization is in fact potentiated. Thus, the action of these inhibitors in mammalian mitochondria may be through a factor found there and not in yeast mitochondria or liposomes.

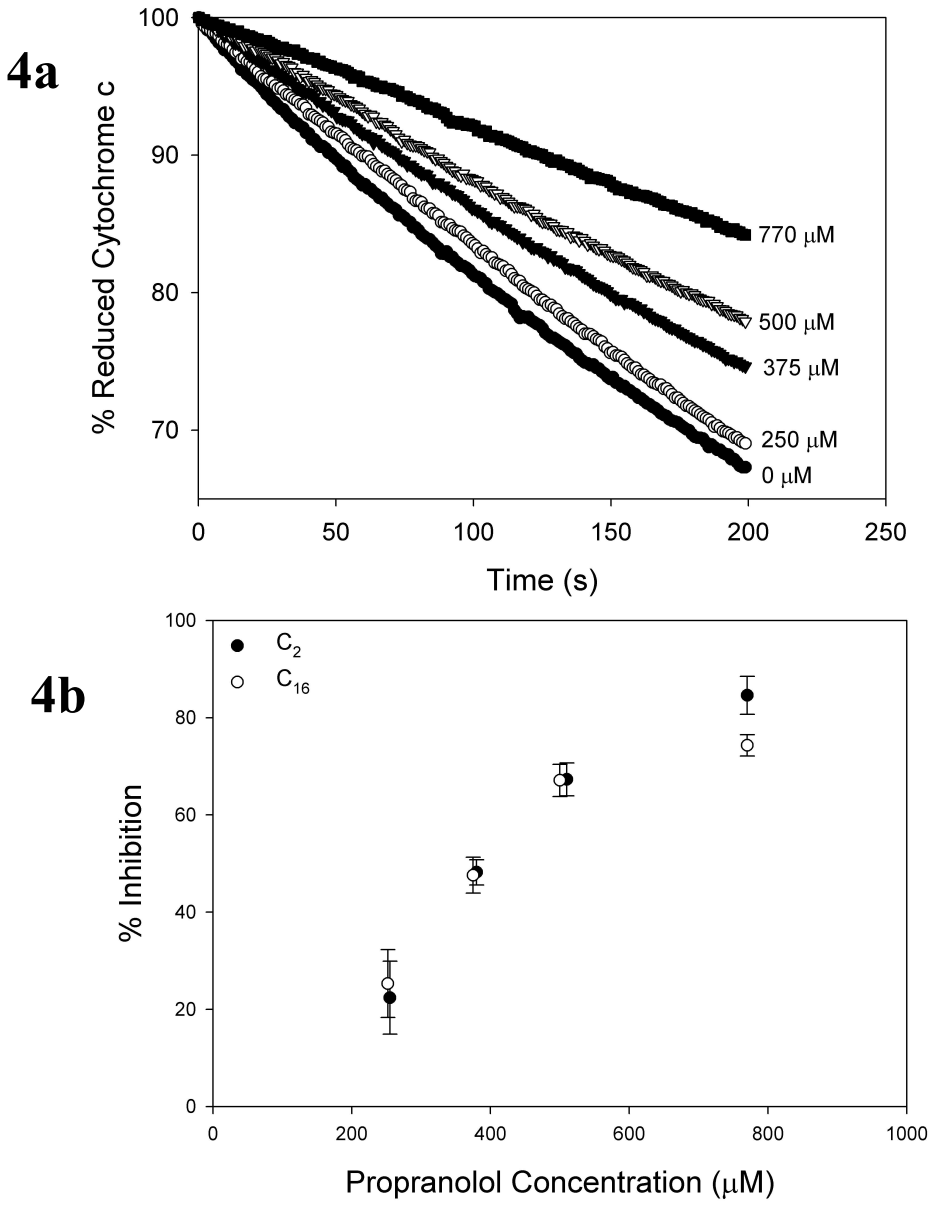


Figure 4. Propranolol inhibits ceramide-induced permeabilization of MOM in rat liver mitochondria. 10 μg of mitochondrial protein in 750 μL of isolation buffer were incubated with propranolol for 1 min prior to a 5 or 10 min incubation with C_2- or C_{16}-ceramide, respectively. **(a)** Different concentrations of propranolol were added 1 min prior to a 10 min incubation with 108 nmoles of C_{16}-ceramide. The reduced form of cytochrome c was added and absorbance measured at 550 nm as a function of time. **(b)** The percentage inhibition of ceramide-induced permeabilization as a function of propranolol concentration is shown for C_2-ceramide (C_2) and C_{16}-ceramide (C_{16}). Mean and SEM were calculated from several repeats within 2 to 3 independent experiments. The concentration range of C_{16}- and C_2-ceramide used was 108 – 147 nmoles and 11 – 18 nmoles, respectively. The concentration of ceramide used within one set of experiments was kept constant.

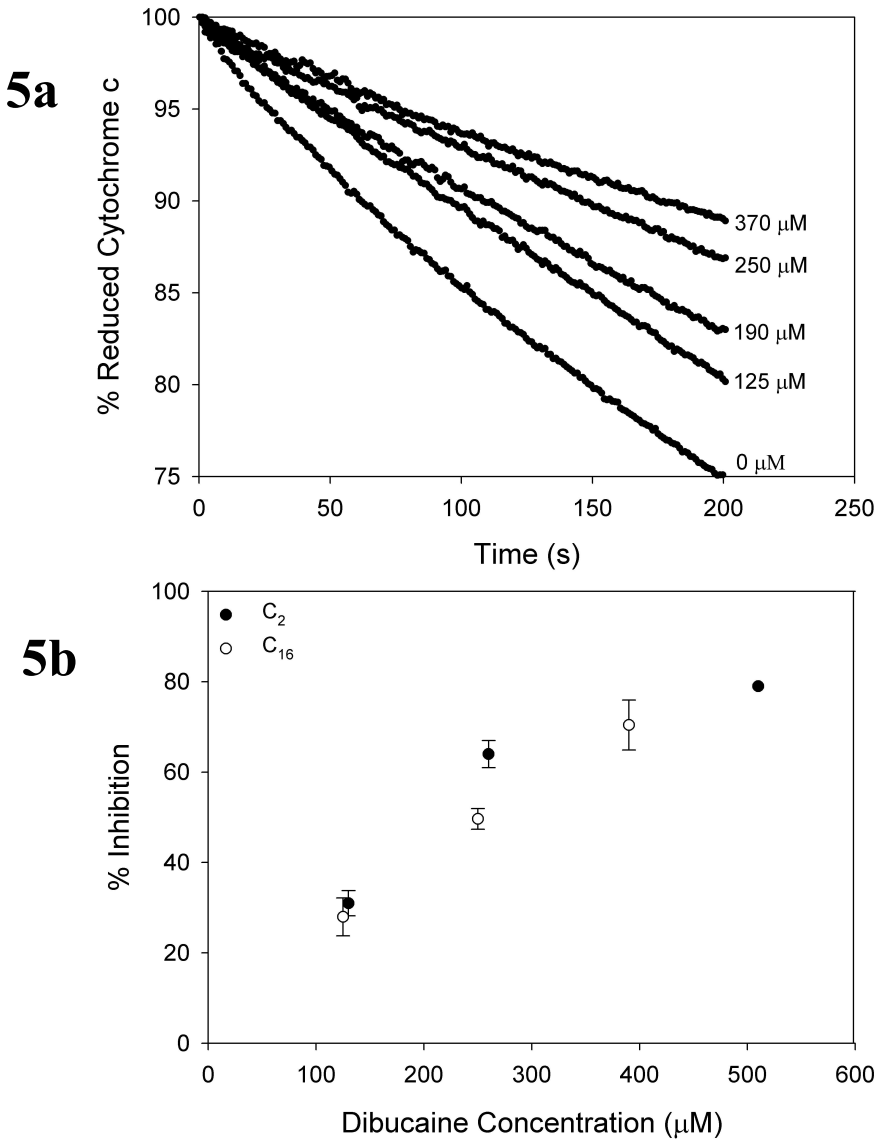


Figure 5. Dibucaine inhibits ceramide-induced permeabilization of MOM in rat liver mitochondria. Experiments were performed as described in Figure 4. **(a)** Different concentrations of dibucaine were added 1 min prior to a 10 min incubation with 108 nmoles of C_{16}-ceramide. **(b)** The percentage inhibition of ceramide-induced permeabilization as a function of dibucaine concentration is shown for C_2-ceramide (C_2) and C_{16}-ceramide (C_{16}). Mean and SEM were calculated from several repeats within 2 to 3 independent experiments. The concentration range of C_{16}- and C_2-ceramide used was 108 – 147 nmoles and 9 – 18 nmoles, respectively.

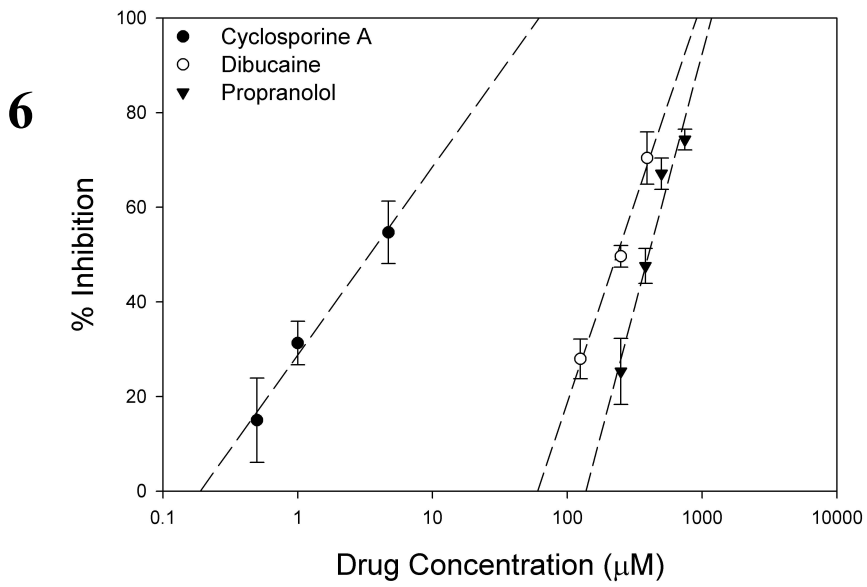
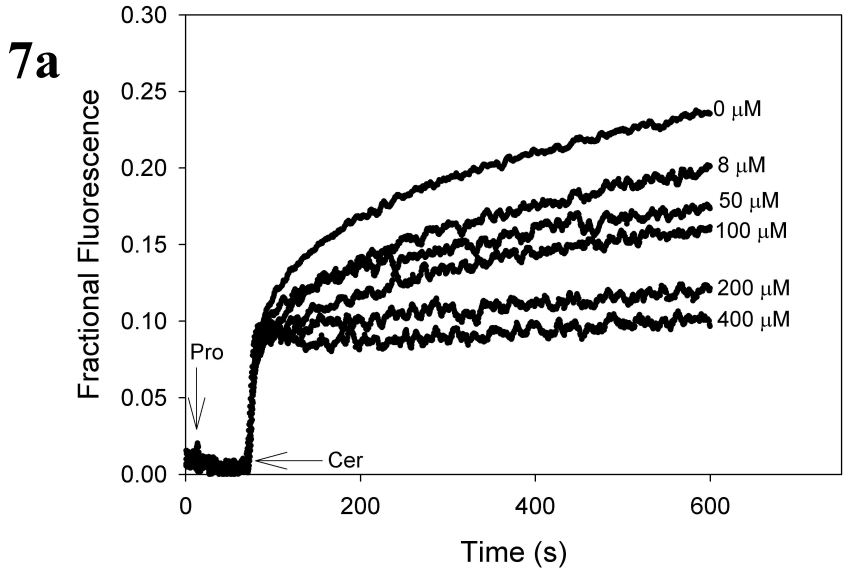


Figure 6. Inhibitory effects of CsA, dibucaine and propranolol on C_{16}-ceramide-induced permeabilization of MOM in rat liver mitochondria. Experiments were performed as described in Figure 4. The inhibitor was added 1 min prior to a 10 min incubation with 57 - 147 nmoles of C_{16}-ceramide. Mean and SEM were calculated from several repeats within 2 to 3 independent experiments. The data were best fit for CsA, dibucaine and propranolol with IC_{50} values of 3.42, 233 and 417 μM; Hill coefficients of 0.54, 0.81 and 1.01; and correlation coefficients (R^2) of 0.92, 0.99 and 0.90, respectively.



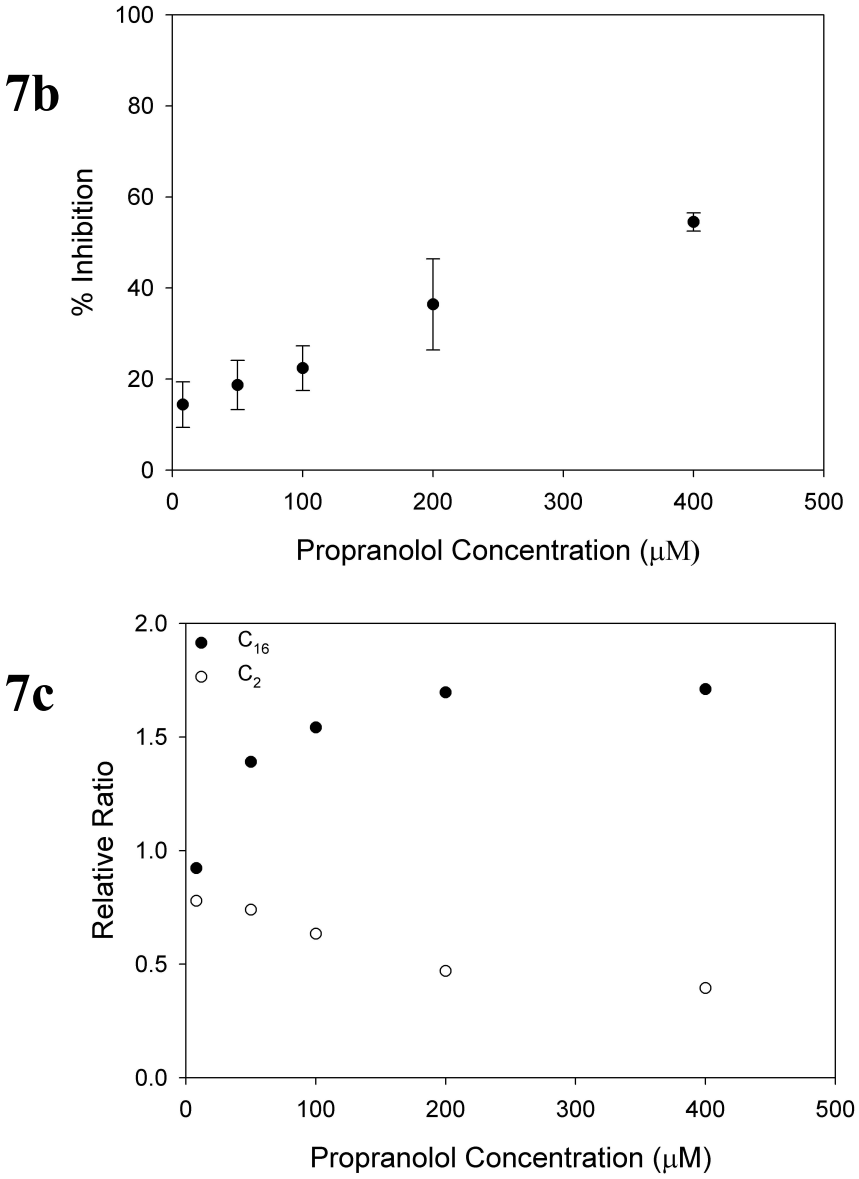
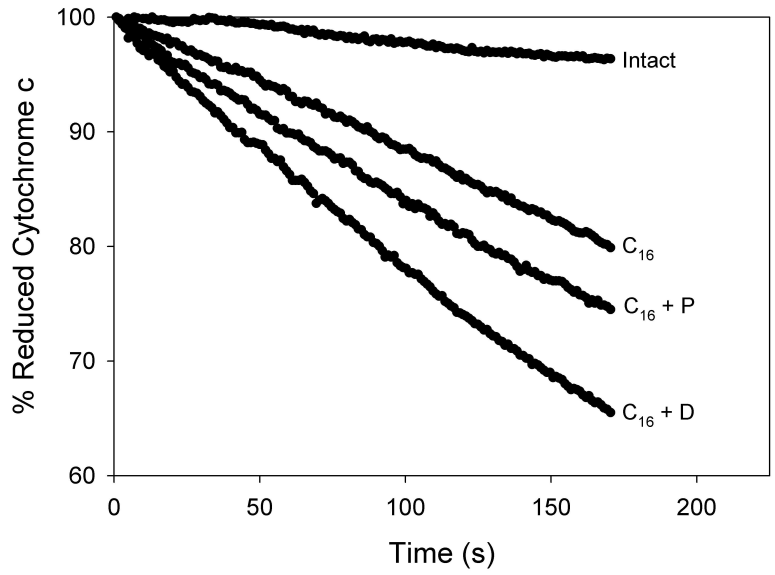


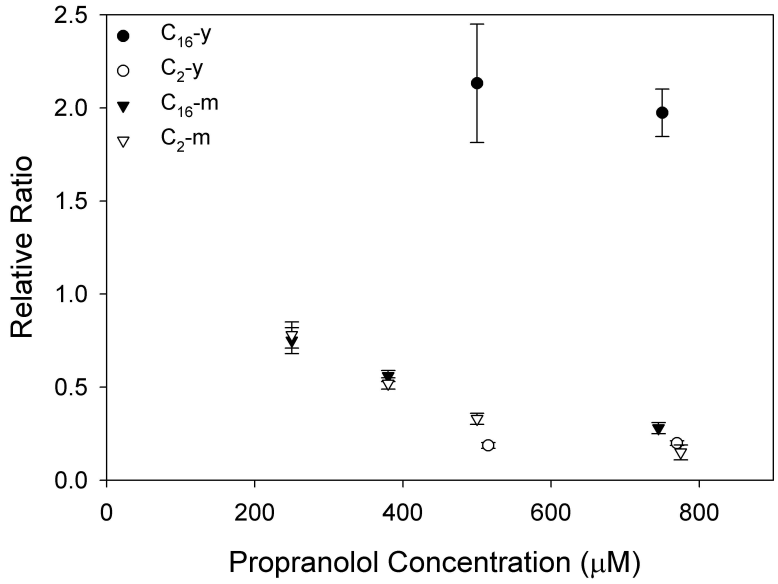
Figure 7. Propranolol inhibits C_2- but not C_{16}-ceramide-induced liposome permeabilization. Propranolol was incubated with liposomes (~0.1mg lipids) composed of 93% asolectin and 7% cholesterol, followed by the addition of C_2-ceramide (C_2) or C_{16}-ceramide (C_{16}). **(a)** Representative experiment of propranolol concentration-dependence inhibition of permeabilization induced by 29 nmoles of C_2-ceramide. Permeabilization results in a fluorescence increase due to the dilution of CF from its quencher DPX. Data was normalized to 0 at baseline fluorescence and 1 at maximal fluorescence, which was achieved with 0.07% Triton-X 100. **(b)** The percentage inhibition of C_2-ceramide-induced permeabilization as a function of propranolol concentration is shown. Mean and SEM were calculated from several repeats within 2 independent experiments. The concentration of C_2-ceramide used was 14 and 29 nmoles. **(c)** Effects of propranolol on C_{16}- and C_2-ceramide-induced

permeabilization is expressed using a relative ratio. Relative ratio is the fractional fluorescence of propranolol + ceramide divided by the fractional fluorescence of ceramide alone. The concentration of C_2-ceramide used was 14 and 29 nmoles and the concentration of C_{16}-ceramide was 190 nmoles. Data shown is the average of 2 – 4 data points from 2 independent experiments.

8a



8b



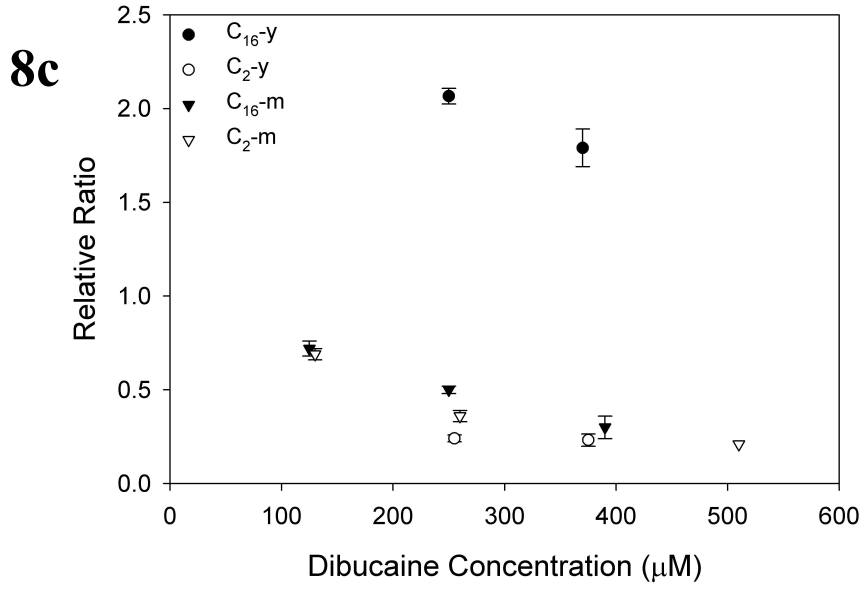


Figure 8. Propranolol and dibucaine inhibit C_2- but not C_{16}-ceramide-induced permeabilization of MOM in yeast mitochondria. 40 μg of mitochondrial protein in 750 μL of isolation buffer were incubated with propranolol or dibucaine for 1 min prior to a 5 or 10 min incubation with C_2- or C_{16}-ceramide, respectively. (a) 112 nmoles of C_{16}-ceramide (C_{16}) causes permeabilization to the MOM of yeast. Intact mitochondria were incubated with isopropanol. 250 μM dibucaine (D) and 500 μM propranolol (P) potentiate the effect of ceramide. Effects of propranolol (b) or dibucaine (c) on C_{16}-ceramide and C_2-ceramide-induced permeabilization is expressed using a relative ratio. Relative ratio is the cytochrome c oxidation rate of the drug + ceramide divided by the oxidation rate of ceramide alone. The relative ratios shown for rat liver mitochondria (C_{16}-m, C_2-m) were calculated based on the experiments in Figures 4 and 5. The mean and SEM for results obtained with yeast mitochondria were calculated from several repeats within 3 independent experiments. The concentration of C_{16}- and C_2-ceramide (C_{16}-y, C_2-y) used in yeast mitochondria were 36 – 147 nmoles and 29 nmoles, respectively.

Chapter 4: Discussion

During the induction phase of apoptosis, pro-apoptotic proteins are released from the intermembrane space of mitochondria. The mechanism by which these proteins are released from mitochondria is not well understood. Several theories explain this as an increase in the permeability of MOM by the formation of pores capable of translocating proteins. For example, some Bcl-2 proteins, which are known regulators of apoptosis, have been proposed to form such channels (for review see 12,70,71). The pro-apoptotic protein Bax has been shown to form channels in artificial membranes with a peak conductance of up to ~ 2 nS.[17,72,73] In a later study, Bax was shown to produce a conductance of up to 10 nS, with a mean conductance of ~ 5 nS.[20] Furthermore, Bax channels are large enough to release cytochrome c when in an oligomerized form.[14,16] Bax in the presence of another pro-apoptotic Bcl-2 protein tBid has been proposed to reorganize membrane lipids to form large lipidic pores, capable of releasing even larger pro-apoptotic proteins.[74] In fact, it has been shown that Bax in the presence of tBid strongly promotes the rate of transbilayer phospholipid diffusion, thereby reorganizing lipids.[74,75]

Another theory of how pro-apoptotic proteins are released from the mitochondrial intermembrane space is via MAC. MAC has been detected in apoptotic cells about the time cytochrome c is released.[17,18] However a recent study demonstrates that MAC did not appear until a later stage in apoptosis, after the permeabilization of MOM.[19] MAC's peak conductance was found to be 2.5 nS,[17] but later studies reported a peak conductance as high as 10 nS, with a mean conductance of 3.3 to 4.5 nS depending on cell type.[18,20] The diameter of MAC has been estimated

to be ~ 5nm, which is large enough to release cytochrome c, but not the larger pro-apoptotic proteins. The molecular identity of MAC is currently unknown, however there is evidence that Bax is a component of MAC. [17,18,20]

Still another theory of how pro-apoptotic proteins are released from the mitochondrial intermembrane space is via ceramide channels.[21,50] Short and long chain ceramides form channels in phospholipid membranes and induce protein release from mitochondria up to 60 kDa.[50] The large channel size of up to 11 nm demonstrated in planar membranes is not consistent with the smaller channel size formed in mitochondria. The difference may have to do with the fact that planar membrane experiments are performed in a protein-free environment. Therefore, the size may be controlled in mitochondria via interactions with other lipids and/or proteins. One possibility may be through an interaction with Bcl-2 proteins, such as Bax.

In this study, pharmacological experiments were performed to test whether amphiphilic cations that inhibit MAC and Bax-induced permeabilization also inhibit ceramide-induced permeabilization. Results obtained in mammalian mitochondria showed that some of the amphiphilic cations that inhibited Bax-induced cytochrome c release from rat forebrain mitochondria[66] and MAC conductance from proteoliposomes containing mitochondrial membrane from FL5.12 cells[63] also inhibited ceramide-induced permeabilization in rat liver mitochondria. Table 1 summarizes IC_{50} values, which were determined or extrapolated from %inhibition plots from Martinez-Caballero, et al., 2004;[63] Polster et al., 2003; [66] and from plots

presented in this study. Results were only quantified for BH3 peptide alone (BH3)* in the Bax study, however propranolol and dibucaine (concentrations up to 500 μM) strongly inhibited cytochrome c release induced by Bax in the presence of cBid (B + cBid) and Bax in the presence of BH3 peptide (B + BH3). They were also the strongest inhibitors of BH3-induced permeabilization. This correlates well with their inhibition of ceramide-induced permeability in mitochondria. TFP was not tested in any Bax experiments and lidocaine (up to 500 μM) was tested on BH3-induced permeabilization but was found to be ineffective, consistent with its marginal effect on ceramide-induced permeabilization. The strongest inhibitors of MAC conductance are TFP, dibucaine then propranolol. Lidocaine (up to 300 μM) was tested on MAC and was reported to be ineffective despite a very weak inhibitory trend. All these results are consistent with the observations made with ceramide on rat liver mitochondria, except for TFP. The latter was without detectable effect. Considering the many reported effects of propranolol and dibucaine, the negative result with TFP argues strongly against a relationship between ceramide channels and MAC.

The amphiphilic cation propranolol was tested on C_2- and C_{16}-ceramide-induced permeabilization in liposomes in order to determine if the inhibitory effect can be observed in a protein free system. As shown in Figure 7c, propranolol inhibited C_2- but not C_{16}-ceramide-induced permeabilization. This finding and similar results obtained with yeast mitochondria suggest that the inhibition via amphiphilic cations may be through a factor found only in mammalian mitochondria. In rat liver mitochondria, similar IC_{50} values for the inhibition by propranolol and

* The BH3 peptide is a sequence of amino acids that comprise the BH3 domain of Bax. This peptide caused release of cytochrome c in cell types that had Bax associated with the MOM (Polster et al., 2001).[76]

dibucaine on C_2- and C_{16}-ceramide channels suggested a similar mechanism of inhibition of channels made up of ceramides of different chain lengths. This again argues for a special factor that confers sensitivity to propranolol and dibucaine to both types of ceramide channels. Since the Hill coefficient is close to 1 and ceramide channels are composed of hundreds of monomers, an inhibition-conferring factor could explain the observed stoichiometry. In addition to this putative factor, liposome and yeast mitochondria experiments show that C_2-ceramide channels might also be directly sensitive to these inhibitors.

	IC_{50} (μM)					
	C_2-m	**C_{16}-m**	**C_2-y**	**C_{16}-y**	**MAC[a]**	**BH3[c]**
Dibucaine	**200**	**235**	**<250**	**n.i.**	**39**	**185**
Propranolol	**365**	**415**	**<500**	**n.i.**	**52**	**110**
Lidocaine	**>900**	**n.i.**	**-**	**-**	**>300[b]**	**>500[d]**
CsA	**-**	**3.42**	**-**	**-**	**>10[b]**	**>1[d]**
TFP	**-**	**n.i.**	**-**	**-**	**0.9**	**-**

Table 1. Comparison of IC_{50} values found for ceramide, MAC and Bax channels.
n.i.: no inhibition
[a] Martinez-Caballero et al., 2004
[b] extrapolated from figure 3b in reference a.
[c] extrapolated from figures in Polster et al., 2003
[d] The release of cytochrome c was presented in an immunoblot and not quantified

The pattern of inhibition by amphiphilic cations is consistent with a relationship between Bax-induced permeabilization of mitochondria and that of ceramide. Yet any such link is tenuous because of the promiscuity of these inhibitors[61,64,77-84] and the claim that Bax channels and MAC channels are related. There is substantial evidence linking Bax to MAC. For example, MAC activity was not found in yeast mitochondria unless Bax was expressed in the yeast cells. Also,

immunodepleting Bax in apoptotic HeLa cells resulted in almost no detectable MAC activity.[20] Nevertheless it is possible that Bax may have multiple activities. Indeed, a major distinction between mammalian and yeast cells is the lack of Bcl-2 proteins in yeast. Thus the absence of Bax may account for the insensitivity of ceramide permeabilization of yeast mitochondria to propranolol and dibucaine.

There are several lines of evidence that link ceramide to Bax. An antisense Bax oligonucleotide inhibited ceramide-induced cytochrome c release and ceramide-induced Bax translocation to the mitochondria in HL-60 cells.[85] Furthermore, HL-60 cells which are resistant to ceramide have a reduced expression of Bax.[86] Since Bax in the presence of tBid, was shown to reorganize some lipids,[74,75] further experimentation may show that ceramide reorganization may occur as well. There are contradictory reports of whether Bax is associated with mammalian mitochondria prior to the induction of apoptosis. Polster et al., 2001;[76] reported that Bax is not associated with mitochondria in some cell types (including rat liver mitochondria). While others have shown that monomeric Bax is associated with mitochondria from HeLa cells[15] and rat liver,[19] prior to the induction of apoptosis. If it is the case that Bax is not found in the outer membrane of rat liver mitochondria, interaction of ceramide with Bcl-2 protein(s) is not ruled out. There is another pro-apoptotic Bcl-2 protein, Bak believed to be involved in MOM permeabilization.[87-89] Bak has been shown to be functionally redundant with Bax[20,88,90,91] and is found inserted in the MOM of healthy cells.[92] Pharmacological experiments using C_{16}-ceramide on yeast expressing Bax or Bak may provide further insight into possible interactions between these proteins and ceramide .

CsA, was tested on C_{16}-ceramide induced permeabilization to determine if ceramide might be involved with PTP. CsA is known to bind cyclophilin D, thereby inhibiting the PTP. Experiments with a positively-charged ceramide showed that the effects of this ceramide are sensitive to inhibition by CsA.[93] This led to the hypothesis that ceramide induces cytochrome c release by permeabilizing the mitochondrial inner membrane. This permeabilization would result in the same sequence of events as PTP: i.e. swelling of the matrix compartment, tearing of the outer membrane and thus free flow of proteins between the intermembrane space and the cytosol or medium. In this work CsA was found to inhibit C_{16}-ceramide channels (Figure 6) supporting the notion that ceramide works through PTP. Supporting this conclusion is the fact that dibucaine has been shown to inhibit mitochondrial swelling believed to be due to PTP[94] and propranolol blocked the "mitochondrial megachannel" which is thought to be an electrophysiological manifestation of the PTP.[62] However, our findings are inconsistent with the PTP model because the addition of C_{16}-ceramide to isolated mitochondria did not result in mitochondrial swelling. In fact C_{16}-ceramide caused a small increase in absorbance at 600 nm. Therefore the inhibition of C_{16}-ceramide-induced permeabilization by CsA cannot be through an effect on PTP. Like other inhibitors, CsA has other targets. It binds calcineurin[95] and has been found to increase the production of reactive oxygen species.[96] However these experiments were performed in whole cell environments and it is not clear what might be the other mitochondrial targets of CsA. The significance of the inhibitory effect of CsA on C_{16}-ceramide-induced permeabilization in mammalian mitochondria is not known. However, it is another

difference between ceramide channels and MAC since CsA was reported to only have slight inhibitory effects on MAC.[63]

The practical implications of the inhibition of ceramide-induced permeabilization by amphiphilic cations is the possibility of developing pharmacological agents that could be used to control the initiation of apoptosis. The factor that confers sensitivity to these agents to mammalian mitochondria may be a good drug target. Ceramide is known to be extensively involved in the apoptotic process; and endogenous ceramide levels in cells undergoing apoptosis have been shown to increase prior to the execution phase of apoptosis.[35,97-100] It is preferable to control apoptosis prior to the activation of effector caspases, since undesirable repercussions may result. For example, pharmacological inhibition of executioner caspases has been shown to result in a condition resembling necrosis.[101] Hence, inhibition of apoptosis at the ceramide permeabilization level may be a more effective approach.

Conclusion

This study has shown that amphiphilic cations can be used to inhibit ceramide channels. One surprising result was the finding that there is a factor in mammalian, but not in yeast mitochondria, that allows C_{16}-ceramide to respond to the inhibitors propranolol and dibucaine. The pattern of inhibition argues that there is a link between ceramide channels and channels induced by Bax. However, the results indicate that ceramide channels are distinct from MAC.

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