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Review

Ceramide channels and their role in mitochondria-mediated apoptosis

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ABSTRACT

A key, decision-making step in apoptosis is the release of proteins from the mitochondrial intermembrane space. Ceramide can self-assemble in the mitochondrial outer membrane to form large stable channels capable of releasing said proteins. Ceramide levels measured in mitochondria early in apoptosis are sufficient to form ceramide channels in the outer membrane. The channels are in dynamic equilibrium with non-conducting forms of ceramide in the membrane. This equilibrium can be strongly influenced by other sphingolipids and Bcl-2 family proteins. The properties of ceramide channels formed in a defined system, planar phospholipid membranes, demonstrate that proteins are not required for channel formation. In addition, experiments in the defined system reveal structural information. The results indicated that the channels are barrel-like structures whose staves are ceramide columns that span the membrane. Ceramide channels are good candidates for the protein release pathway that initiates the execution phase of apoptosis.

1. Introduction

The ability of mitochondria to initiate apoptosis by releasing proteins into the cytosol is an establish fact. Many have marveled that mitochondria are so vitally important to both the life and death of cells. Yet to those interested in molecular mechanisms, even more fundamental questions come to mind: How are proteins released from mitochondria? Is this release mechanism used only once in the life of a cell? If so, how does a cell insure that this death-initiating process does not become activated accidentally in view of the fact that in the absence of appropriate extracellular signals the default pathway for mammalian cells is death by apoptosis? The importance of regulating apoptosis is highlighted by the existence of a family of proteins whose primary task is to regulate this process. The Bcl-2 family includes both pro- and anti-apoptotic proteins [1], just what one would expect for a process that is highly controlled. Yet nature seems to love to defy rational expectations and flirt with the edge of disaster.

Unlike proteins whose complexity easily allows for all manner of regulation, lipids are far simpler structures (at least they seem to be) and thus ill-suited to serve as the effectors of the mitochondrial protein release process initiating the essentially irreversible execution phase of apoptosis. After all, without adequate control this most critical life or death decision could be made incorrectly with disastrous consequences. Apoptosis of normal cells can lead to loss of function such as in neurodegenerative diseases. Failure of defective cells or cells infected by viruses to undergo apoptosis can lead to cancer or the spread of the virus. Yet, remarkably, one lipid, ceramide (Fig. 1), is capable of performing this function. This review is focused on this remarkable discovery.

2. Ceramide and apoptosis

The role of ceramide in the induction of apoptosis has been clear for some time [2–5], well before ceramide's ability to form channels came to light [6]. The elevation of ceramide levels in cells [7] and more precisely in mitochondria [8,9] early in apoptosis, is an important correlation. The ability of added ceramide to induce cells to undergo apoptosis (e.g. Refs. [3,10]) and isolated mitochondria to release intermembrane space proteins [11–13], indicates that ceramide is pro-apoptotic. The combination of both types of findings is rather persuasive. The question of how ceramide is acting is complex because there are many effects [5,14] but one of these is relatively simple. Ceramide monomers can self-assemble to form large, stable channels capable of allowing proteins to cross membranes [13,15].

3. Formation of ceramide channels

Protein translocation through membranes can occur in a variety of ways: protein translocation via a carrier-type mechanism, endo/exocytosis, membrane damage, and channel formation. A definitive demonstration of channel formation following ceramide addition to a membrane is the measurement of discontinuous changes in membrane permeability of a magnitude that exceeds what one carrier could transport. The fastest carriers transport 10^4 to 10^5 ions per second [16]. Since channels produce a continuous water-filled pathway through the membrane, the flow of solutes can proceed at a much faster rate. The addition of ceramide to phospholipid membranes results in discontinuous

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Abbreviation: MOM, mitochondrial outer membrane

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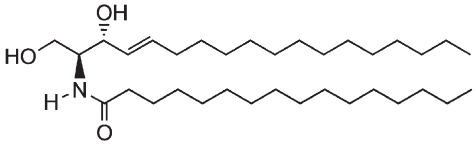

Fig. 1. Structure of C_{16}-ceramide. Ceramide is a sphingolipid composed of sphingosine amide linked to a fatty acid. Illustrated is the structure of a typical ceramide found in mammalian cells where the fatty acyl chain is 16 carbons (palmitate).

increases in ion flow typically between 10^7 and 10^{10} ions per second (Fig. 2). These are much higher rates than those of known carriers and thus are the result of channel formation.

Since ceramide is very poorly soluble in water, insertion into phospholipid membranes has been achieved in three ways: 1) dispersal of the ceramide and phospholipids (the common glycerolipids) in an organic solvent followed by removal of the solvent and formation of liposomes or planar membranes [6,17]; 2) dissolution of ceramide alone in an organic solvent that is miscible with water and then dispersing a small amount of this solution nearby preformed phospholipid membranes in the hope that some of the ceramide will insert into the membrane before it self-associates into micellar-like structures (e.g. Refs. [6,13]); 3) generating the ceramide in membranes containing sphingomyelin by treating these with sphingomyelinase (e.g. Ref. [18]). Method number 2 has been used extensively to study the formation of ceramide channels in phospholipid membranes. The results shown in Fig. 2 were obtained with method 2. In the main figure, the time of ceramide addition is indicated. The "noisy" record is due to the vigorous stirring used to disperse the ceramide. Note that there is typically a long lag time before channel formation (sometimes hours). The increase in membrane conductance is a complex mix of discontinuous, step increases, typical of single-channel insertion, and very small, almost continuous increases in conductance. The first insert shows a particularly "clean" record showing mainly rather uniform channel-like insertions. This is not typical.

The simplest interpretation of the stepwise increments in membrane conductance illustrated in Fig. 2 is the formation of a heterogeneous population of channels, as originally reported [6]. However, more detailed studies [15] showed that rather than a population, the results are consistent with the formation of a single enormous channel.

First of all, occasional observations (Fig. 2 innermost inset) indicated that if each stepwise increment were a single channel then these can act in a cooperative manner and close or disassemble in unison (note the drop in conductance to baseline in one step). A more appealing interpretation is that each increment after the very first represents the growth of one channel, a channel that can collapse all at once. Secondly, measurements of channel selectivity revealed that the initial conductances favored cations over anions but as the overall conductance increased, the selectivity declined so that the overall conductance showed no preference for cations over anions [15]. This is inconsistent with a population of small channels because the overall selectivity of such would be a weighted average of the individual selectivities. However, if one channel was growing in size then the selectivity should decline as more ions move through the channel at greater and greater distances from the channel wall. Thirdly, and perhaps most compelling, is the observation of stochastic behavior [15]. The addition of La^{+3}, an agent that results in channel disassembly, does not result in an exponential decrease in membrane permeability, as would be expected for a population of channels. Rather, what is observed (Fig. 3a) is a delay followed by a structured disassembly. The delay is not constant but stochastic. Single structures respond in a probabilistic manner. For example, carbon 14 decays to nitrogen 14 with a half life of 5730 years but an individual atom could decay in 1 min or wait for 10,000 years. This variable delay reflects a probability of decay. Similarly, following La^{+3} addition, a ceramide channel shows a variable delay prior to initiating its disassembly. When the results of many experiments were combined, the delay itself shows an exponential dependence on time, consistent with a first-order process. The exponential fit is quite good (Fig. 3b) if one corrects for an initial delay arising from the time needed for the added La^{+3} to reach the channel. Although the solution next to the membrane is stirred, there is an unstirred layer of approximately 50 μm [19] next to the membrane and the diffusion time is a few seconds.

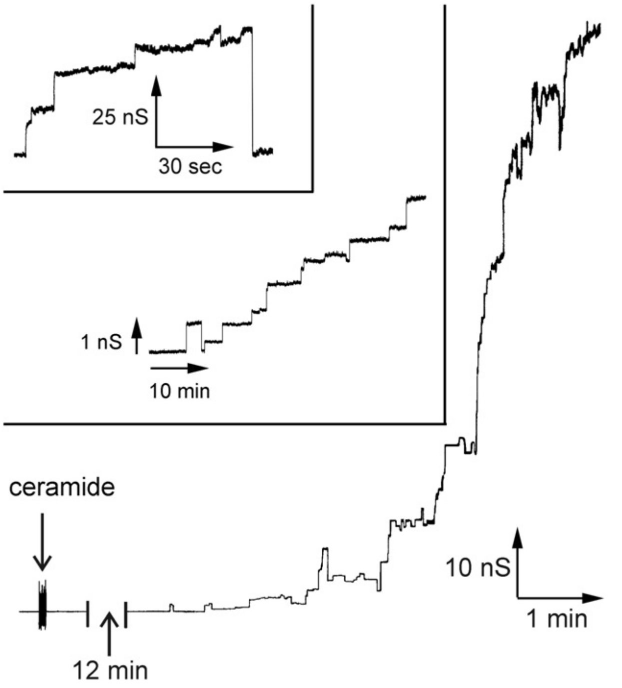

Fig. 2. The formation and dynamics of ceramide channels formed in planar phospholipid membranes. The main figure shows the increase in conductance following the dispersal of ceramide into the aqueous phase next to the membrane. Typically a delay precedes the conductance increments. The outer inset shows a particularly orderly increase in conductance observed some time after ceramide addition (not shown). The inner inset shows conductance increments from baseline followed by a sudden return to baseline. The records represent 3 separate experiments. This figure is a compilation or results published previously [15,26].

4. Structure of the ceramide channel

In principle, the structure of the ceramide channel could be ill-defined, more like a "leak", or it could be a well-organized structure. The properties of the highly saturated acyl chains of the ceramide molecule, exacerbated by the trans double bond, result is a melting point much higher than that of typical cell membranes resulting in a strong propensity for lateral phase separation. An island of solid lipid surrounded by a sea of liquid crystal sometimes results in mismatched interfaces and thus membrane defects. The leakiness of vesicles containing high mole fractions of ceramide has been attributed to such defects [20]. However, channel formation in the low mole-fraction regime (less than 1 mol%) is the result of a highly-organized structure.

Compelling evidence for a highly-organized structure arose from the observation that ceramide channels disassemble with a pattern that gives insight into the fundamental structure of the channel [15]. The reduction in channel conductance, whether spontaneous or induced by La^{+3}, consists of a variety of decrement sizes. Most of these are small and the smaller decrements are more frequent than the larger ones. This is as expected. Unexpectedly, among the large decrements there is a pronounced preference for decrements that are multiples of a fundamental unit: 4.0 nS (Fig. 4). This result was a surprise because a channel would be expected to disassemble by losing segments from the structure forming its wall and the circumference varies linearly with pore radius while the conductance should depend of the cross-sectional area and this is a square function of the radius. However, especially for large channels, the access resistance (the resistance ions face in reaching the mouth of the

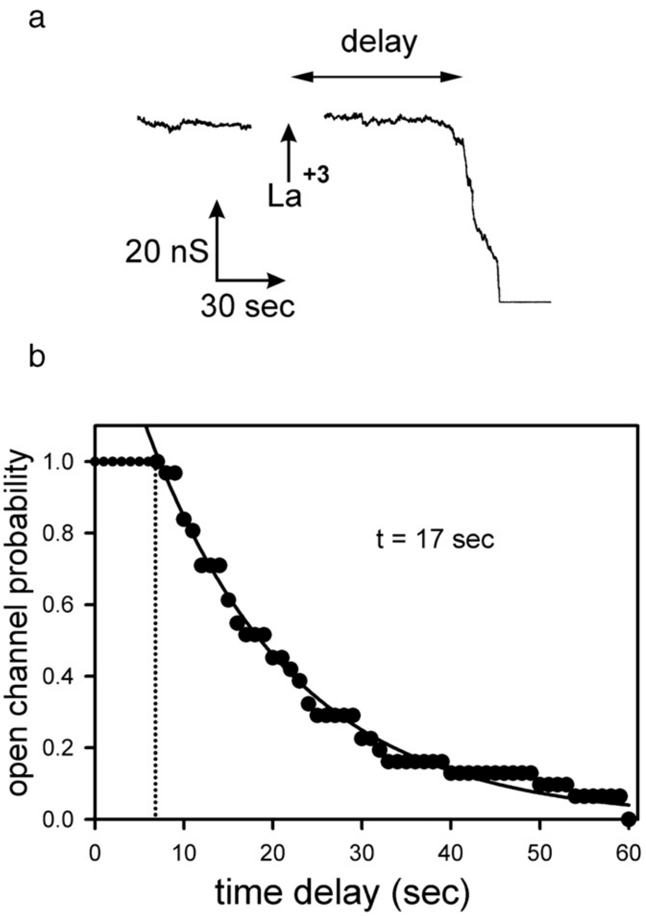

Fig. 3. Lanthanum-ion-induced disassembly of a ceramide channel (a) and an exponential fit to delay times prior to channel disassembly (b). Panel (a): A channel was formed in a planar membrane as in Fig. 1 (not shown). After the conductance stabilized, $LaCl_3$ was added to a final concentration of 10 µM. After a delay, the conductance decayed to baseline. Panel (b): The results of 31 independent experiments as in panel (a) were used to determine the probability of finding the ceramide channel unaffected for the indicated time after $LaCl_3$ addition. The time indicated (17 s) is the time constant of the single exponential fit to the data. The dotted line is proposed to be the time needed for La^{+3} to diffuse through the unstirred layer so as to reach the channel. This figure is a compilation of results published in reference [15].

channel) must be included in the determination of the conductance. In addition, if the channel is very large then the length of the channel can be neglected yielding a relationship that depends on radius (or circumference). Thus, for a very large barrel-stave channel disassembling by losing staves of the barrel wall, the conductance drop should be quantized and vary linearly with the number of staves lost at one time. From a working model (Fig. 5), the losing of one stave should reduce the conductance by 2 nS. The finding that there is a preference for multiples of 4 nS would indicate that the channel prefers to disassemble by losing an even number of columns (Fig. 4, top numbers corresponding to most frequent large conductance decrements). Regardless of the details, these findings argue strongly for a channel with a high degree of order. Indeed, a theoretical analysis shows that the pattern evident in the sizes of the large decrements could not easily be obtained by simulation and certainly not without a highly ordered channel [15].

In any scenario, hundreds of ceramide molecules must come together to a channel capable of allowing proteins to cross the membrane. In the working model (Fig. 5), 6 ceramides form a column capable of spanning the hydrophobic part of the membrane (about 3 nm). These are held together by the hydrogen-bonding of the amide bond formed when sphingosine is acylated. Note that the ability of amide linkages to organize matter is well known in that these are responsible for the secondary structure of proteins. The columns have the right biophysical properties to self-assemble to form a barrel-stave channel. This structure can increase or decrease in size by adding or removing columns. Since the acyl chains are aligned in the plane of the membrane, some bending of the channel slightly toward an hourglass shape is likely for it to interface properly with the phospholipid bilayer. The phospholipids would also need to be distorted. Molecular dynamic simulations show how this can take place and also indicate that the structure is inherently stable because it easily survives 10 ns of simulation [21].

The quantized decrements indicating a preference for disassembly of an even number of columns have led to a revision of the working model from one composed of columns in parallel to one composed of anti-parallel columns. It was realized that a column of ceramide monomers held together by hydrogen-bonding through the amide linkage results in aligning the dipoles of the amide linkages forming a large effective dipole spanning the membrane. If adjacent columns were oriented in opposite directions, these dipoles would result in a favorable, attractive interaction, explaining why columns might prefer to leave the channel in pairs.

The ceramide channel functions in an environment and thus is influenced by its environment. It is, in fact, at the interface between two phases: the aqueous environment and the membrane's liquid crystal phase. The dynamic equilibrium between ceramide in the channel and in other membrane forms should be influenced by the characteristics of both of these environments but an understanding of these influences is still in its infancy. Potent effects of specific lipids have been described. Both sphingosine [22] and dihydroceramide [17] favor ceramide channel disassembly or interfere with ceramide channel formation. The influence of sphingosine is particularly well documented [22]. A ceramide channel was first formed in a phospholipid membrane. When its size had stabilized, sphingosine was added and the conductance of the membrane decreased progressively over a period of about 20 min and often to baseline conductance. This demonstrates a drastic disassembly of the channel. Often the conductance would subsequently rise but with the characteristics of sphingosine channels (small size and anion preference). A simple interpretation is that sphingosine intercalates into the ceramide channel inducing instabilities. The inhibitory effect of dihydroceramide can be interpreted in a similar way. Here the inhibitory effect is more remarkable because dihydroceramide differs

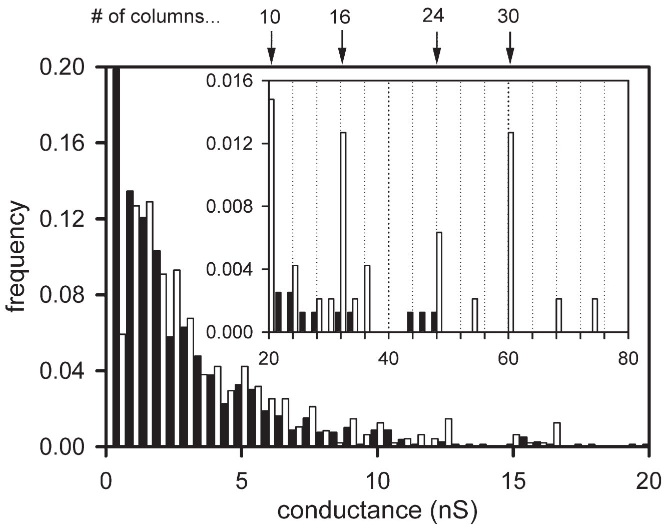

Fig. 4. Frequency of occurrence of increments (black bars) or decrements (white bars) in conductance of ceramide channels. The main figure shows the frequency of conductance changes that were smaller than 20 nS. The inset shows the frequency of large conductance changes. The dotted lines indicate the position of multiples of 4 nS. The number of columns that the ceramide channel would need to lose to account for the most frequent large conductance decrements is shown on top. These data were collected from 18 independent experiments. The figure is a revision of Fig. 3 from reference [15]. A similar preference for multiples of 4 nS was observed in lanthanum ion induced decrements [15].

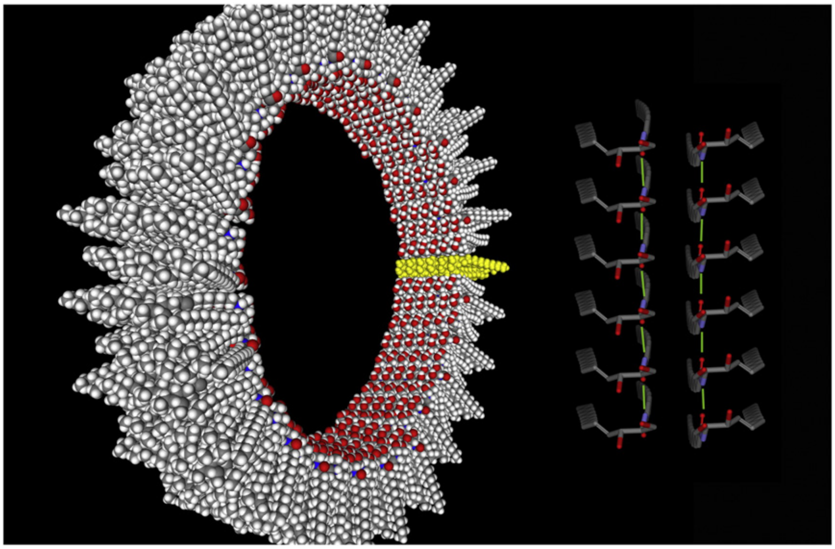

Fig. 5. Illustration of a model of a 48 column ceramide channel. One column is shown in yellow to distinguish it from the entire structure. On the right, two columns are illustrated in stick mode to illustrate the hydrogen-bonding between the carbonyl oxygen of one ceramide molecule and amide hydrogen of the adjacent ceramide in the column. The columns are arranged in an anti-parallel mode as are those in the channel.

from ceramide only in the fact that it lacks the trans double bond. Dihydroceramide does not form channels but as little as 10% dihydroceramide added to ceramide will reduce ceramide's ability to form channels [17]. The effects of both of these agents argue more for a highly ordered channel that does not tolerate structural alterations than a strictly environmental effect. However, in vivo the presence of other lipids and their ability to intercalate into the structure of the ceramide channel will influence the ability of ceramide to form channels. The inability of ceramide to form channels in the plasma membrane of erythrocytes [23], a membrane rich in sphingomyelin (ceramide esterified to phosphocholine), could be explained by the same type of interference. Clearly, this explanation is merely educated speculation and needs to be addressed experimentally, especially since it touches on the critical issue of specificity of channel formation to particular membranes.[1]

Ceramide exists with fatty-acid chains of different lengths. The most common forms are a 16-carbon, an 18-carbon and a 24-carbon chain length (referred to as C_{16}-ceramide, C_{18}-ceramide, and C_{24}-ceramide). Chemically one can generate ceramides where the sphingosine is acylated to fatty acids of virtually any chain length. Cells clearly distinguish between ceramides of different chain lengths and thus the length of the acyl chain may be importance in ceramide channel formation. In fact, with regard to ceramide's ability to permeabilize the MOM, C_2-ceramide and C_{16}-ceramide were found to be equally potent once one compensated for the difference in ability to be delivered to the MOM. Differences in effects of these in cells are at least partially due to the high specificity of metabolic enzymes.

5. Ceramide channel formation in the mitochondrial outer membrane (MOM)

The addition of ceramide to mitochondria results in the release of proteins into the medium but does it result in the permeabilization of the MOM? In order to answer this question, one needs to measure the rate of protein translocation through the MOM. The very small mitochondrial intermembrane space precludes a direct flux measurement. Thus, following the pioneering work of Lech Wojtczak et al. [24], Siskind et al. [13] used the rate of oxidation of exogenously-added reduced cytochrome *c* to assess the permeabilization of the MOM. Addition of ceramide resulted in a dose- and time-dependent increase in the rate of cytochrome *c* oxidation and thus demonstrated that ceramide increased the permeability of the MOM to cytochrome *c*. This increase in permeability could be reversed by removing the ceramide. When C_2-ceramide (short-chain ceramide where the acyl group is just 2-carbons long) was used, it could be removed by adding fatty-acid depleted albumin. This resulted in a reduction of the permeability of the MOM [13]. The permeability could also be reduced by inhibiting ceramide channel formation by adding sphingosine [22] or the anti-apoptotic protein Bcl-xL [25]. This reversal of the permeabilization induced by ceramide indicates that the permeabilization is due to a controllable permeability pathway, probably a ceramide channel.[2]

The ceramide-induced protein release from mitochondria indicates the formation of single ceramide channels in individual mitochondria. This mirrors the single channel seen in the phospholipid membrane

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[1] The specificity of ceramide channel formation to certain membranes has two aspects: 1) location of ceramide formation and 2) ability to form channels in that membrane environment. Like phospholipids, ceramide is highly water insoluble and faces a large energy barrier to transfer between membranes. For example, transfer from the outer to the inner mitochondrial membrane should be a very slow process unless catalyzed in some way. Thus, the presence of ceramide synthesizing machinery, hydrolyzing enzymes, and ceramide transport processes in a particular membrane will strongly influence the steady-state level of ceramide in that membrane and the propensity for channel formation. In addition, although we can only measure the total ceramide content one should really know the ceramide activity in the membrane, i.e. the level of free ceramide in the 2-dimensional solution. The presence of substances that sequester ceramide (lipid–lipid binding, raft formation, binding to proteins, etc.) will affect the activity. In addition some lipids or proteins may specifically destabilize ceramide channels (e.g. sphingosine and Bcl-xL…see below). The problem is quite complex.

[2] In the mitochondrial experiments, the sphingosine was added to the mitochondria before the ceramide and one might worry that sphingosine is merely interfering with ceramide insertion into the mitochondria. This sequence of addition was necessary because sphingosine inhibits cytochrome oxidase activity and thus the adenylate kinase release assay had to be used. In this assay, adenylate kinase is only released once and a reduction in permeability after the fact cannot be measured. Although the results obtained with experiments in phospholipid membranes (Section 4, last paragraph) cannot be explained by an interference of sphingosine with ceramide insertion because sphingosine was added later, other results with isolated mitochondria also provide a compelling support for a direct destabilization of ceramide channels by sphingosine. When sphingosine was added to isolated mitochondria, half the sphingosine was converted to ceramide in 10 min [26] yet the outer membrane permeability remained very low indicating that the ceramide that was formed was being inhibited to form channels or perhaps was located in another membrane (the inner membrane or the MAM). However, addition of fatty-acid depleted albumin to mitochondria pretreated with sphingosine resulted in a large increase in outer membrane permeability as the albumin bound up and removed the sphingosine from the membrane leaving the ceramide to form channels.

experiments. The evidence for this conclusion comes from the measurement of release of adenylate kinase from isolated rat liver mitochondria. Unlike cytochrome *c*, adenylate kinase does not have any particular affinity for membranes. Thus, when the MOM is permeabilized, the release should essentially be "all or none". That is because of the small volume of the intermembrane space. Thus when ceramide added to isolated mitochondria results in partial release of adenylate kinase (e.g. Ref. [13]), then it indicates that some mitochondria have a ceramide channel and other do not. Statistically, such a situation means that mitochondria having 2 channels would be a relatively rare event.

The dose of ceramide needed to form channels in isolated mitochondria is comparable to the level of ceramide reported in mitochondria isolated from cells early in the apoptotic process [23]. The mole fraction of ceramide to phospholipids was measured after addition of radiolabeled ceramide to isolated mitochondria. The permeabilization of the MOM was also measured under the same conditions. A relationship between the mole fraction of ceramide in mitochondria and permeabilization of the MOM was constructed (Fig. 6) and it shows that at the mole fraction found in mitochondria early in apoptosis, there should be substantial permeabilization of the MOM. Thus, neglecting other influences, the ceramide level present in the MOM of cells undergoing apoptosis is large enough to contribute substantially to protein release.

The complexity of mammalian mitochondria and the presence of a variety of proteins and other factors raise the question of whether ceramide is acting directly or indirectly. The fact that ceramide forms channels in phospholipid membranes provides confidence that it would also form channels in mitochondria. However, since ceramide does not permeabilize the plasma membrane of erythrocytes [23], the situation might be more complex. Mammalian mitochondria contain Bcl-2 family proteins so these might form the protein release pathways upon ceramide insertion. However, experiments with mitochondria isolated from yeast (lacks Bcl-2 family proteins) show similar permeabilization following ceramide addition, indicating that it is ceramide that forms channels in the mitochondrial outer membrane [25]. That is not to imply that the Bcl-2 family proteins are not involved, merely that they are not necessary for ceramide channel formation.

Bcl-2 family proteins influence the ability of ceramide to form channels as would be expected from their function in cells. Anti-apoptotic proteins, Bcl-xL, Bcl-2, and CED-9 all interfere with ceramide channel formation [25]. The results are consistent with the formation of a 1:1 complex between Bcl-xL or CED-9 and the ceramide channel. The result seems to be a shift in the equilibrium constant between ceramides in the channel and those in other forms in the membrane. An attractive hypothesis is that the binding of Bcl-xL distorts the highly hydrogen-bonded structure resulting in the transmission of the tension throughout the channel in an allosteric-like manner. This would change the energetics of the dynamic equilibrium and thus explain the propensity for disassembly. Bax, a pro-apoptotic protein, acts synergistically with ceramide to enhance membrane permeabilization both in the MOM and in phospholipid membranes [27]. The Bax/ceramide channel may be quite different from that formed by ceramide alone and Bax alone. The added stability would favor channel growth. Thus while ceramide alone has the biophysical characteristics necessary to form large stable channels without the aid of proteins, a system has evolved to regulate this spontaneous process.[3]

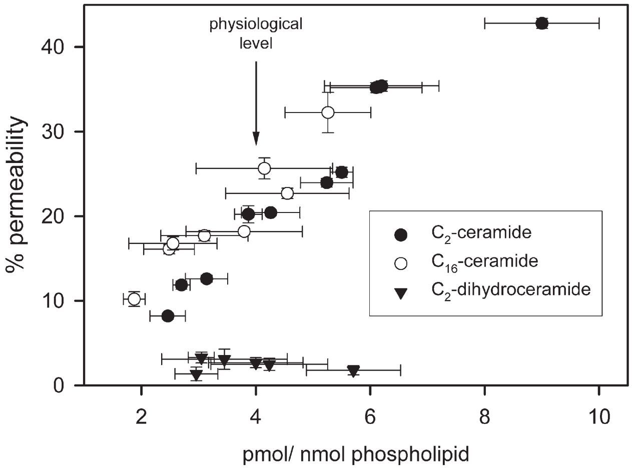

Fig. 6. Relationship between ceramide delivery to mitochondrial membranes and permeabilization of the mitochondrial outer membrane to cytochrome *c*. The permeability was assessed as the rate of cytochrome *c* oxidation and expressed as a percent of the rate observed when the outer membrane was damaged by hypotonic shock. The amount of added ceramide that was incorporated into the mitochondrial membranes was measured using radiolabeled ceramide. The error bars are standard deviations. The figure is revised from reference [23].

6. Conclusion

Ceramide channels represent a novel mechanism by which cells form permeability pathways through membranes. The channels are in dynamic equilibrium with ceramide monomers and ceramide aggregates in the membrane. Channel formation and stability are determined both by the amount of ceramide present and by proteins and lipids that influence the stability of these channels. The activity of enzymes that form and break down ceramide and processes that move ceramide from one membrane to another all contribute to determining the steady-state level of ceramide in the mitochondrial outer membrane. These also influence the levels of other sphingolipids, such as sphingosine and dihydroceramide, that influence the stability of ceramide channels. The levels and activity of Bcl-2 family proteins is determined by a variety of cellular factors and conditions within and outside the cell. Thus the probability of forming a ceramide channel is a highly complex function of many variables. That is as it should be since ceramide channel formation is a life or death decision. Thus, despite the apparent simplicity of the ceramide structure, the formation of channels by ceramide does not degrade the ability of cells to make an informed decision on whether or not to undergo apoptosis.

Acknowledgements

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[3] The standard view is that Bax and Bak form the mitochondrial protein release channel. Strong support for this comes from the fact that cells lacking both Bax and Bak are known to be highly resistant to apoptosis. This fact appears to demonstrate the lack of importance of ceramide in forming the protein release pathway. However, in the parental cells, inducers of apoptosis elevate cellular levels of ceramide. This elevation does not occur in the Bax/Bak knock-outs [28]. It turns out that Bak is needed to elevate the ceramide synthase activity and elevate ceramide levels [28]. Furthermore, sphingomyelinase knock-out cells produce lower levels of ceramide and these cells are resistant to UV light-induced apoptosis even though they have both Bax and Bak [29,30]. Thus, the importance of ceramide cannot be dismissed.

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