



Review

Regulation of ceramide channels by Bcl-2 family proteins

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ABSTRACT

Mitochondrial outer membrane permeabilization to proteins, an irreversible step in apoptosis by which critical proteins are released, is tightly regulated by Bcl-2 family proteins. The exact nature of the release pathway is still undefined. Ceramide is an important sphingolipid, involved in various cellular processes including apoptosis. Here we describe the structural properties of ceramide channels and their regulation by the anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family. The evolutionarily conserved regulation of ceramide channels by Bcl-2 family proteins, consistent with their role in apoptosis, lends credibility to the notion that ceramide channels constitute the protein release pathway.

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1. Introduction

Apoptosis, a form of programmed cell death, is a complex and exceedingly important cellular process. A key step in this process is the release of pro-apoptotic proteins from mitochondria into the cytosol thus initiating the execution phase of apoptosis. This is a highly regulated step because it commits the cell, in an essentially irreversible way, to death. This review is focused on a controversial aspect of this process, the identity of the pathway by which these proteins are released. We present results favoring an unconventional pathway for this release: channels formed by a unique sphingolipid, ceramide. The supporting experimental results are compelling and require a great deal of published research to be reexamined in a new light. We ask that the reader keep an open mind and not try to examine the results through the lenses of existing dogmas.

2. An expanded role for Bcl-2 family proteins

Bcl-2 family proteins regulate the release of proteins from the mitochondrial intermembrane space and thus regulate a key, decision-making step in the apoptotic process. There is a general con-

sensus that some of these are pro-apoptotic and others anti-apoptotic. It is also clear that these proteins act in different ways to favor or inhibit apoptosis, helping to orchestrate the conditions needed to achieve apoptosis or to disrupt these conditions. With time the entire picture will emerge but, until then, it is important to examine each of these actions with an open mind. Recently, it was discovered that pro and anti-apoptotic proteins are capable of regulating the ability of ceramide to form channels both in defined systems and in the mitochondrial outer membrane. This review will focus on this novel aspect of the actions of Bcl-2 family proteins with the confidence that the evolutionary selection pressure that generated and maintains this emergent property is based on regulating the onset of apoptosis, ensuring that it occurs only when the appropriate conditions are present in the cell.

3. Channel formation by the sphingolipid, ceramide

Ceramide is a member of a family of lipids called sphingolipids. Unlike the more commonly studied membrane lipids referred to as phospholipids, sphingolipids are not based on esterification of glycerol but on sphingosine and its amide linkage to fatty acids. The amide linkage, identical to the peptide bond of proteins, has great potential for hydrogen bonding, having one hydrogen bond donor and one acceptor. This is perfect for generating assemblies and ceramide channels are proposed to be formed by a ring of columns each consisting of six ceramides interconnected by the hydrogen bonds of the amide linkage (Fig. 1). The two hydroxyls

Abbreviations: MOM, mitochondrial outer membrane; ER, endoplasmic reticulum; BH, Bcl-2 homology domain

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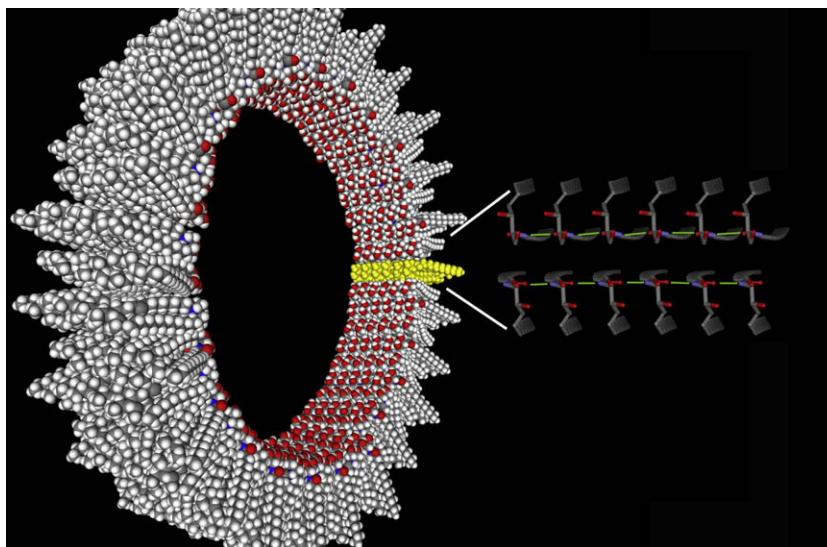


Fig. 1. A model of the structure of a ceramide channel. The ceramide channel is a water-filled cylinder that spans the membrane. It is composed of a variable number of columns each consisting of six ceramide molecules. One of the columns is colored in gold to illustrate the fundamental unit. Ceramides within a column are held together by hydrogen bonds (green) between the amide linkages. Adjacent columns have an anti-parallel orientation as shown in the enlarged section. Note that in the enlarged region, the hydrogens were not included for clarity. Channel growth is achieved by the incorporation of additional columns. Color coding: red – oxygen, blue – nitrogen, grey – carbon and white – hydrogen.

and perhaps bridging water molecules are likely involved in connecting adjacent columns. Indeed, from model building one can conclude that the inner surface of the channel is lined with a hydroxyl–hydrogen-bonded network, interfacing nicely with the aqueous phase. As is the case for the alpha helix of proteins, aligning the amide linkages results in a long dipole. Imbedded in the low dielectric constant of the membrane, the influence of the dipole will be stronger and extend over longer distances. Experiments [1] strongly support the notion that this dipole forces the columns to be arranged in an anti-parallel fashion.

Addition of ceramide to phospholipid membranes results in the formation of a conductance pathway, a permeability that grows with time finally reaching a steady value (Fig. 2). This indicates the formation of a channel that starts out small and becomes larger as more ceramides are recruited. The conductance increases large range from small and non-descript to large discrete events indicating insertions ranging from small aggregates or individual monomers to assemblies of pre-formed columns. The final steady value likely

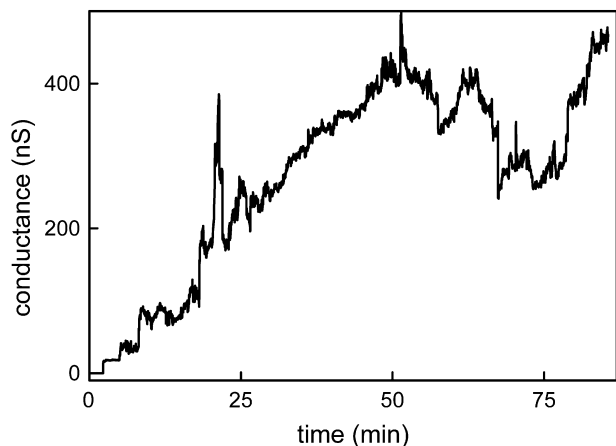


Fig. 2. The formation of a ceramide channel following the addition of C₁₆-ceramide to the aqueous phase bathing a phospholipid membrane. The medium consisted of 1.0 M KCl, 1 mM MgCl₂, 5 mM PIPES pH 6.9. The membrane was composed of diphytanoylphosphatidyl choline:asolectin:cholesterol, 1:1:0.1.

reflects a dynamic equilibrium between ceramide molecules in the structure of the channel and ceramide monomers or non-channel forming ceramide aggregates in the membrane. This is supported by observing increases and decreases in conductance even after a steady value is achieved. In addition, channel disassembly can be induced by removing ceramide monomers from the medium when short-chain ceramide was used [2] to form the channel. (The short-chain ceramide is sufficiently water-soluble that the addition of fatty-acid depleted albumin can bind it and cause channel disassembly by mass action.) Experiments are consistent with a highly-organized and rigid structure, deriving its stability from hydrogen bonding. Presumably, on average the intrastructural hydrogen bonds are somewhat more stable than those with water allowing an enthalpic advantage to overcome what is likely an entropic disadvantage. A very small free energy change arising from bonding associated with a single ceramide unit would be greatly magnified by the hundreds of ceramides forming one channel. Channel growth results in the formation of more intrastructural hydrogen bonds but also results in a change in channel curvature and thus changes in the length and angle of these bonds (i.e. bond energy). It may be that an optimal curvature exists, below and above which the bonding is less stable. The overall structure is destabilized by addition of lanthanum ions. By mechanisms yet unknown, lanthanum chloride induces channel disassembly in discrete steps without affecting the ceramide concentration in the membrane [1]. The high charge density of this trivalent cation would be expected to strongly influence the structure and stability of a ceramide channel if it bound to the its polar inner surface: from electrostatic repulsion between nearby bound La⁺³ ions to disrupting/distorting the hydroxyl–hydrogen-bonded network forming the polar lining of the channel following ligation of the ion by the ceramide hydroxyls.

The loss of ceramide conductance following the addition of LaCl₃ occurs in a way that directly demonstrates that only one large ceramide channel is formed in the phospholipid membrane. When low levels of LaCl₃ were added (2 μM final), the conductance declined but only after a delay that was generally far longer than the diffusion time for lanthanum to reach the membrane. Furthermore, the delay was variable in duration among the various experiments [1]. Thus the delay was stochastic or probabilistic, a

property characteristic of a single structure. The presence of a population of channels would result in an exponential decay of conductance as each individual channel lost its conductance. Such an exponential decay was generated by pooling together results from more than 30 experiments so as to artificially generate a population of channels. Thus, the stochastic behavior of individual experiments demonstrates that the ceramide conductance represents the growth of a single large channel in the membrane.

This conclusion was supported by other observations. For example, selectivity measurements of membranes with low ceramide conductance showed substantial cation preference. When more ceramide was added to increase the conductance the selectivity declined and finally became non-selective. If the increase in conductance had been due to the formation of more ceramide channels then the selectivity should not have changed. If the additional ceramide was incorporated into the existing channel resulting in channel enlargement, the selectivity would decline because ions traversing the channel would interact less with the channel wall [1].

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

Channel formation in the MOM can be *detected* by measuring the release of proteins [2–5] but permeabilization of the outer membrane can be measured [2] by recording the rate of oxidation of exogenously added cytochrome *c* by cytochrome oxidase (active site on the outer surface of the inner membrane). This cytochrome *c* accessibility assay measures the permeabilization of the outer membrane to proteins. This can be measured at any time during an experimental protocol and the initial rate of oxidation will measure the permeability at that time. One can follow both an increasing permeability as channels form and a decreasing one as they are disassembled [6].

The dispersal of ceramide (dissolved in isopropanol) into a mitochondrial suspension results in a gradual permeabilization of the outer membrane to cytochrome *c* with a half time of about 5 min [2]. The permeabilization occurs at the elevated ceramide levels that are found in mitochondria early in apoptosis [7,8]. From adenylate kinase release experiments [2], it is clear that at the levels of ceramide used in these experiments, only a portion of the mitochondria become permeabilized because only a portion of the adenylate kinase was released. Thus the permeabilized mitochondria probably contain only one or at most two channels and this realization is important in interpreting the results of ceramide channel modifiers.

The probability of ceramide channel formation and the stability of existing channels are at the mercy of the steady-state concentration of ceramide in the membrane. The latter is, of course, determined by the rates of ceramide synthesis, degradation, and transfer between membranes. In the mitochondrial outer membrane, where these channels form, there are enzymes capable of both ceramide synthesis and hydrolysis [9–11]. The tight interaction between the MOM and endoplasmic reticulum (ER) membranes provides the opportunity for ceramide exchange, as already demonstrated [12], between the major location of ceramide *de novo* synthesis (the ER) and the MOM. Thus, there are ample opportunities to regulate the steady-state level of ceramide in the MOM and indeed this level becomes elevated early in apoptosis in a number of systems. However, other sphingolipids can also influence channel formation and stability. Both dihydroceramide and sphingosine interfere with ceramide channel formation [13,14]. Finally, and most importantly, Bcl-2 family proteins can also influence the stability of ceramide channels, probably through influencing the dynamic equilibrium between ceramides in a channel structure and ceramide in other forms [4,15].

5. The anti-apoptotic protein, Bcl-xL, favors channel disassembly

Bcl-xL (26 kDa) is an anti-apoptotic Bcl-2 family protein structurally classified as having four Bcl-2 homology (BH) domains. It acts directly on ceramide channels formed in phospholipid membranes causing them to disassemble [4]. The pure, full-length Bcl-xL protein was expressed in *Escherichia coli* and only the protein that remained soluble in the cytosol was used. Denatured protein in inclusion bodies was not used. No detergent was used in the purification process as this was found to cause spurious effects [4]. Addition of the Bcl-xL protein to a preformed ceramide channel resulted in rapid channel disassembly until all the membrane permeability was lost. The conductance did not immediately drop to baseline as would be expected for a blocking of the channel (Fig. 3). Rather there was a structured reduction in conductance consistent with a time-dependent channel disassembly. It is important to note that published results [1] provide compelling evidence that a single ceramide channel is formed in the membrane. The disassembly following Bcl-xL addition is consistent with there being a single channel because the record is consistent with a single process. In addition, the calculated size of the ceramide channel based on the conductance as compared to the size of Bcl-xL means that Bcl-xL binding to the inside of the channel would only reduce the conductance by a small value (ineffective block). More insight into the mechanism of disassembly comes from experiments performed on isolated mitochondria.

6. Anti-apoptotic proteins inhibit ceramide permeabilization of the mitochondrial outer membrane

The permeabilization of the MOM to cytochrome *c* by ceramide is inhibited by the addition of Bcl-xL [4]. This inhibition is independent of the sequence of addition (Fig. 4). Pre-incubation of rat liver mitochondria with Bcl-xL inhibits the ability of C₁₆-ceramide to permeabilize the outer membrane to cytochrome *c*. The degree of inhibition depends on the amount of added Bcl-xL. Importantly, the same levels of permeabilization were observed (Fig. 4) even when the MOM was first permeabilized by adding the ceramide. Thus, not only did the Bcl-xL reverse the ceramide-induced permeabilization but the same end point was achieved, indicating that an equilibrium was reached. A fit of the inhibition curve to the Hill

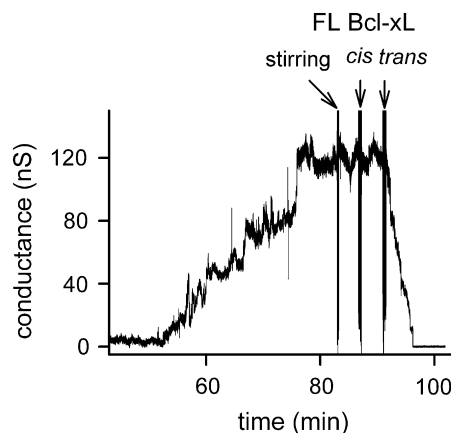


Fig. 3. Disassembly of a ceramide channel formed in a phospholipid membrane by the addition of the anti-apoptotic Bcl-2 family protein, Bcl-xL. FL refers to Full Length, implying that the Bcl-xL used is a wild-type protein without any modifications. This is used to distinguish it from other mutants that have deleted domains. The conductance of the membrane induced by addition of C₁₆-ceramide was measured as a function of time. The medium consisted of 1.0 M KCl buffered to pH 7.0. See Ref. [6] for further details.

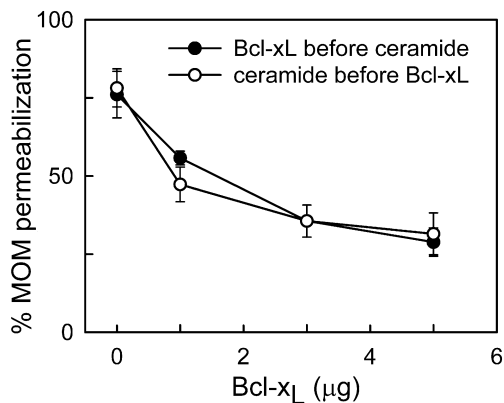


Fig. 4. Bcl-xL reduces the ceramide-mediated permeabilization of the outer membrane of mammalian mitochondria. The same amount of C₁₆-ceramide was added to all experiments. In one set of experiments, ceramide was added first and allowed to achieve the maximal permeabilization. This was followed by the addition of Bcl-xL. In the other set, Bcl-xL was added first. Regardless of the sequence of addition, the same final permeability was observed. The permeabilization of the outer membrane to cytochrome c is expressed as a percent of the maximal permeabilization achieved by damaging the outer membrane with a mild hypotonic shock. Reprinted from Ref. [6].

equation yields a power dependence very close to 1, indicating a 1:1 complex between Bcl-xL and the channel. The possibility exists that Bcl-xL is acting as a channel blocker but because of the large excess of Bcl-xL over the estimated number of channels in the mitochondria, any blocking action must be transient, resulting from the binding and unbinding of Bcl-xL. Considering the results of the phospholipid membrane experiments, a blocking mechanism seems unlikely. The possibility that Bcl-xL forms hetero-oligomers with non-channel-forming ceramide complexes thus favoring these and causing ceramide disassembly by mass action, also seems unlikely because the molar amount of ceramide present was 3 orders of magnitude larger than Bcl-xL. In addition, such a scenario could not explain the Hill coefficient. A more likely mechanism would be an effect of Bcl-xL binding on the channel stability. If the binding of Bcl-xL were to distort the structure of the channel, then the distortion would spread throughout the hydrogen-bonded structure. This would result in a shift in the dynamic equilibrium between ceramides forming the channel and those in other forms in the membrane.

That Bcl-xL is acting specifically was shown by testing a variety of Bcl-xL truncation mutants that had previously been tested for their ability to influence the apoptotic process [16,17]. The mutants known to be ineffective on apoptosis were also ineffective on the ceramide-induced permeabilization of the MOM. One mutant that converted Bcl-xL to a pro-apoptotic protein, increased the ability of ceramide to permeabilize the MOM. Thus altered versions of Bcl-xL behave as expected, attesting to the specificity of the effect of Bcl-xL on ceramide channels.

Apoptosis in *Caenorhabditis elegans* is also associated with mitochondrial outer membrane permeabilization and regulated by Bcl-2 family proteins that are homologous to mammalian equivalents. CED-9, the nematode homolog of mammalian Bcl-xL, localizes to mitochondria to inhibit apoptosis. In isolated rat liver mitochondria, CED-9, also inhibits the ability of ceramide to permeabilize the mitochondrial outer membrane and does so in a dose-dependent manner [4]. This shows that the inhibition is not confined to Bcl-xL but extends to other anti-apoptotic proteins.

Experiments with yeast (*Saccharomyces cerevisiae*) mitochondria provide evidence for a specific interaction between anti-apoptotic proteins and ceramide channels. Since yeast do not produce proteins equivalent to the Bcl-2 family, mitochondria from yeast

cells have been used as a “clean” system in which to study the interactions between ceramide channels and anti-apoptotic proteins. Interference from other Bcl-2 family proteins cannot occur. Human Bcl-2 expressed in yeast travels to the MOM. Isolated mitochondria from yeast expressing Bcl-2 have Bcl-2 incorporated into their membranes as shown by western blot. These mitochondria are resistant to permeabilization by ceramide as compared to mitochondria isolated from control cells [4]. Bcl-xL added to control yeast mitochondria inhibits ceramide permeabilization in the same way as it does in experiments with mammalian mitochondria. Again, the dose dependence shows a Hill coefficient close to 1 and Bcl-xL truncation mutants act as expected [4]. These results, as well as the results of experiments performed on phospholipid membranes, provide strong evidence that anti-apoptotic proteins are acting directly on ceramide channels to destabilize them.

7. The pro-apoptotic protein, Bax, and its role in mitochondrial outer membrane permeabilization

Bax is a 21 kDa monomeric cytosolic pro-apoptotic Bcl-2 family protein that has only three of the four BH domains, hence classified as a BH1-3 type Bcl-2 protein, as compared to BH3 only pro-apoptotic proteins, that have only the BH3 domain. That structure of Bax is characteristic of BH1-3 pro-apoptotic proteins [18]. It is typically located in the cytosol but translocates to mitochondria early in the apoptotic process. This translocation is followed by conformational changes often referred to as Bax activation that eventually lead to mitochondrial outer membrane permeabilization to proteins or MOMP. Typically the conclusion is that Bax itself forms the channel that allows protein permeabilization or that Bax acts with another pro-apoptotic protein called Bak to form the pathway. However, there is evidence for the role of ceramide associated with the Bax activity. Several studies have found that increasing mitochondrial ceramide results in Bax translocation to mitochondria and subsequent activation [7,19,20]. This translocation and/or activation of Bax by ceramide does not require other cytosolic factors as this also happens in isolated mitochondria [19]. In fact, Bax has been found to localize to ceramide enriched microdomains of mitochondria [21]. But these studies have not resolved the exact role of ceramide in activating Bax or inducing MOMP. Is ceramide a structural component of the MOMP channel along with Bax? Or does it act as a second messenger to activate Bax? Both ceramide and Bax are independent channel formers in membranes. So, under circumstances where both ceramide and Bax are present, which molecule forms the protein permeable MOMP channels? Results are consistent with the conclusion that activated Bax and ceramide can synergistically induce permeabilization of MOM. This synergy was also observed in yeast mitochondria and planar phospholipid membranes [15].

8. Bax and ceramide act synergistically

When isolated mitochondria were pretreated with Bax activated either with the detergent octyl glucoside, or with an activated form of the BH3 only protein, Bid, (a protein that activates Bax in vivo and in vitro) these mitochondria are much more sensitive to C₁₆-ceramide permeabilization. The degree of permeabilization is greater than the combined effect of each treatment alone (Fig. 5). This synergy indicates a possible direct interaction between Bax and ceramide, perhaps cooperativity in channel formation. When tested on phospholipid membranes, activated Bax alone did not form channels easily and similarly for C₁₆-ceramide. When both were present, channel formation seemed to occur more readily, indicating a lowering of the energy barrier for permeabilization. More importantly, the addition of activated Bax to a cera-

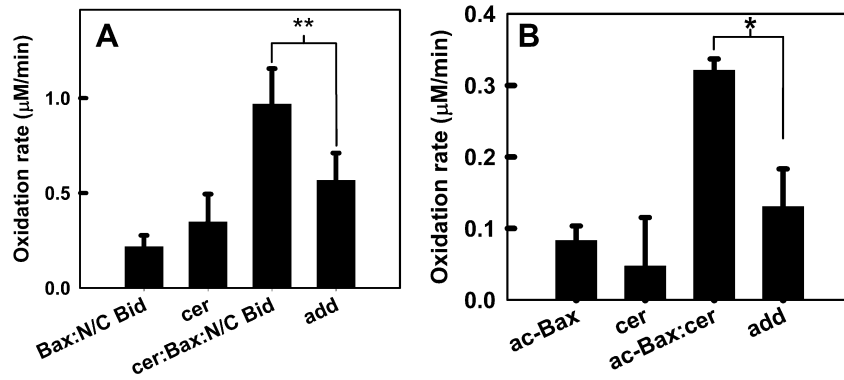


Fig. 5. Bax activated by either processed Bid (N/C Bid) (A) or detergent (0.7% octyl glucoside) (B) acts synergistically with C₁₆-ceramide to permeabilize the mitochondrial outer membrane to cytochrome c. The permeabilization resulting from the presence of both ceramide and activated Bax simultaneously is higher than the sum of the permeabilizations induced by either activated Bax or ceramide alone. The statistical significance is at the 99% level for (A) and at the 95% level for (B). Reprinted from Ref. [15].

mid channel resulted in a large increase in conductance (Fig. 6). This increase was the result of the growth of the preformed channel because disassembly of the combined channel with lanthanum chloride resulted in channel disassembly with stochastic behavior. There was a delay of variable length followed by a structured disassembly [15]. As described for the ceramide channel, such stochastic behavior is characteristic of a single structure. This behavior is not consistent with the presence of multiple pathways or separate pathways for ceramide and Bax but rather with one pathway, one channel.

Insight into the nature of this synergistic interaction was obtained with dose–response experiments on isolated mitochondria. The ceramide-induced permeabilization increases monotonically with the level of activated Bax used to preincubate the mitochondria. However, this is only the case at low levels of added ceramide, i.e. levels that produced a very small amount of permeabilization. Regardless, the monotonic increase reaches a saturating level of permeabilization suggesting a stoichiometric interaction between activated Bax and ceramide. Interestingly, the concentration of activated Bax at which this saturating permeabilization is achieved depends on the amount of added ceramide. As the amount of added ceramide increases, the activated Bax concentration at which the saturation of permeabilization is achieved decreases. In other words, when more ceramide is present, a lower concentration of Bax is required to achieve maximal MOMP, indicating that

the affinity of activated Bax for ceramide might increase as the amount of ceramide increases. A Hill analysis was used to determine the apparent affinity between the ceramide channel and activated Bax. A plot of the Bax concentration that resulted in half-maximal effect versus the amount of ceramide added showed that the apparent affinity does increase with increasing amounts of ceramide (Fig. 7). It must be remembered that as the amount of added ceramide increases, the permeabilization induced by ceramide also increases. So, it is likely that activated Bax is affecting the permeabilizing structure formed by ceramide. This is consistent with the observation that at high ceramide levels, activated Bax does not induce any further stimulation of ceramide induced MOMP. These observations are not readily explained by the hypothesis that ceramide acts by enhancing Bax activation. On the contrary, these results lend themselves to the conclusion that activated Bax acts on a ceramide channel and increases its size. When an activated Bax molecule binds to a ceramide channel, the channel could come under stress, a stress that is relieved by the expansion of the channel. This expansion goes on until the channel is of an optimum size with a radius of curvature that matches something in the structure of activated Bax. This conjecture explains why permeabilization by

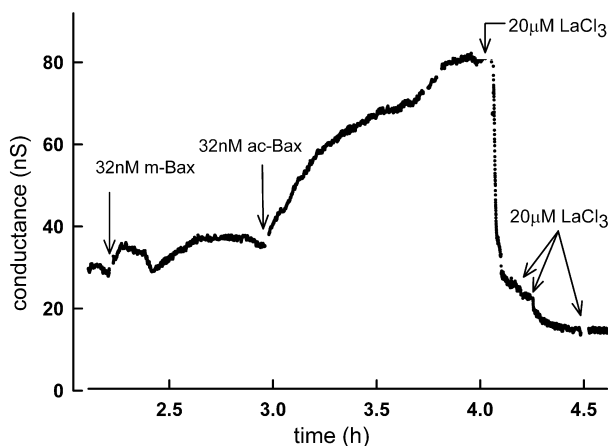


Fig. 6. Activated Bax (ac-Bax) induces a large increase in the size of a ceramide channel formed in a phospholipid membrane. Addition of monomeric Bax (m-Bax) had no effect. The addition of LaCl₃ induced channel disassembly. Reprinted from Ref. [15].

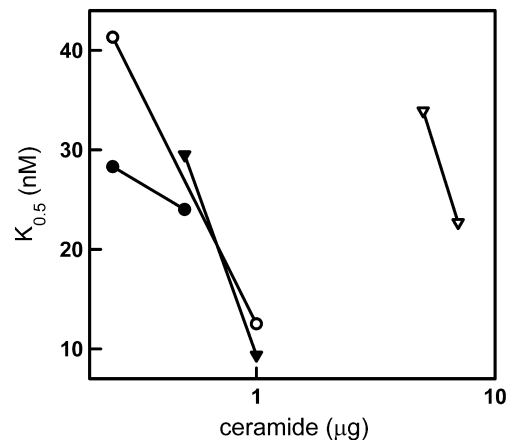


Fig. 7. K_{0.5}, the concentration of activated Bax that produces a half-maximal enhancement of the ceramide permeabilization of the mitochondrial outer membrane to cytochrome c, declines with increasing dose of ceramide. K_{0.5} was obtained from a Hill analysis of the data. Each set of data points represents an experiment performed on a different day with a different mitochondrial preparation from rat liver (total of 4 independent experiments). As the amount of added C₁₆-ceramide was increased, less activated Bax was required to achieve the same level of permeation enhancement. This result is consistent with the model of activated Bax enlarging a ceramide channel by binding more tightly with a channel of larger size. Reprinted from Ref. [15].

high levels of ceramide is not influenced by the presence of activated Bax. If the channel formed by ceramide itself is large enough, the binding of activated Bax might be accommodated by the channel completely and thus there would be no stress to drive the channel to a larger size.

According to this model, activated Bax and ceramide are structural components of the same MOMP channel, but the combined channel retains some of the properties of the ceramide channel. Consistent with this, we found that trehalose, a disaccharide that specifically inhibits ceramide induced permeabilization also inhibits the combined permeabilization of ceramide and activated Bax. This model predicts that the interaction between activated Bax and ceramide is direct. This is certainly true because the interaction exists when tested in phospholipid membranes. Another requirement of this model is that activated Bax and ceramide form a single permeabilizing structure. This condition was also verified in the phospholipid membrane system where the disassembly of the permeabilizing structure formed by activated Bax and ceramide by lanthanum chloride was shown to be stochastic event [15], indicating a single structure. The molecular nature of this combined channel is difficult to imagine because the components are so different in size and structure.

9. Physiological relevance of ceramide channels

Ceramide is a vital sphingolipid that participates in a slew of cellular events including cell senescence, apoptosis, differentiation, cell cycle arrest and raft formation during cell signaling [22]. Focusing on apoptosis, exogenously-added ceramide has been found to be able to induce apoptosis in a variety of cell types. Moreover, cellular ceramide levels rise in response to various apoptotic stimuli [23–27] so endogenous levels often rise when apoptosis is induced. Since ceramide is virtually insoluble in water, it can be isolated in different membrane compartments. The mitochondrion is a particularly important site where an increase in the ceramide level is associated with apoptosis [7,19,27,28]. While mitochondria are equipped with their own ceramide synthesizing machinery [9,28], migration of acid sphingomyelinase to mitochondria has been observed in response to UV irradiation [29] resulting in elevation of mitochondrial ceramide. The localization of ceramide to the MOM during apoptosis is also correlated with MOMP [7,19]. Several studies have found that isolated mitochondria can be permeabilized by adding ceramide [2,6,12,30]. Targeted elevation of mitochondrial ceramide can also lead to mitochondrial permeabilization and the subsequent steps of apoptosis [31]. Anti-apoptotic Bcl-2 proteins, namely Bcl-2 and Bcl-xL have been found to negatively regulate ceramide synthesis during apoptosis [32–34]. Thus the case for ceramide's importance in causing apoptosis in cells is very strong. The aspect where there is lack of consensus is that ceramide *channels* are involved in MOMP.

The association of ceramide with Bcl-2 family proteins in the context of apoptosis is also very strong. The mechanistic basis for this association will turn out to be complex. One aspect, mentioned above, is that mitochondrial ceramide acts as a platform for Bax translocation and activation [7,19,21]. Other observations can be understood within the context of Bcl-2 family proteins acting on ceramide channels. Traditionally, the mechanism of Bcl-xL mediated inhibition of MOMP has been assumed to be entirely mediated by direct and indirect inhibition of Bax activation by BH3 only proteins, some of which directly activate Bax while some others sequester anti-apoptotic proteins. However, studies with mutants of Bcl-xL that do not bind to any Bcl-2 family proteins [35–37], which would be expected to not affect permeabilization, still inhibit MOMP, suggesting that additional levels of regulation exist by which Bcl-xL can inhibit MOMP. The effect of Bcl-xL on the cera-

mid channel qualifies as an additional regulatory step. Regarding Bax, it is known that activated Bax can induce protein release from isolated mitochondria without requiring ceramide [37–39] but activated Bax induced protein release in isolated mitochondria occurs over unphysiological timescales of several hours. No real-time permeabilization is detected with Bax [38]. There is a considerable time gap between Bax translocation to the MOM and the induction of permeabilization [40,41]. Additional signals apart from those that cause Bax translocation are necessary to induce the conformational changes in Bax associated with MOMP [41,42]. Studies have also found that most of the Bax translocates to MOM downstream of MOMP [43–45]. Valentijn et al. [42] show that Bax inserts into the MOM through a C-terminal targeting signal but that Bax can insert into the MOM even in the absence of this targeting signal once the mitochondria have been permeabilized, in a caspase independent manner. These authors and others [46] have concluded that activated Bax is a receptor for cytosolic Bax. However, it is also likely that cytosolic Bax binds to ceramide channels downstream or concomitant with MOMP in a target-signal-independent fashion, especially because a constitutively membrane localized Bax mutant that cannot induce permeabilization does not recruit cytosolic Bax to membranes. The results can now be interpreted considering the possibility of interaction with ceramide channels and our results hence spawn new a new line of thinking. In a system that normally lacks Bcl-2 family proteins, cell death can be induced in yeast by the over-expression of Bax but this cell death was inhibited when ceramide levels were reduced by co-expression with sphingomyelin synthase [47]. The interplay between ceramide and Bax in this highly simplified system is compelling. There is still further evidence for interplay between ceramide and pro-apoptotic proteins. While either Bax or Bak has been found to be mandatory for the induction of apoptosis in response to various stimuli, acid sphingomyelinase knockout cells (reduced ability to generate ceramide) are resistant to UV induced apoptosis even in the presence of both Bax and Bak [19,48]. Finally, although cells deficient in both Bax and Bak are very refractory to pro-apoptotic signals, these signals also fail to elevate cellular levels of ceramide. The failure to raise the cellular ceramide level is due to the fact the Bax is necessary to elevate the activity of ceramide synthase [49].

10. Conclusion

The hypothesis that ceramide channels are involved in protein release from mitochondria early in apoptosis is strongly supported by the regulation of these channels by the Bcl-2 family proteins. The regulation observed is in the direction concurrent with their role in apoptosis: anti-apoptotic proteins inhibit ceramide channels while the pro-apoptotic proteins enhance these channels. It is indeed remarkable that inhibiting ceramide channels is an evolutionarily conserved property of Bcl-2 type anti-apoptotic proteins across species (Bcl-xL of mammals and CED-9 of *C. elegans*). In addition Bax affects the ceramide channel at concentrations where by itself it has minimal effect on the permeabilization of mitochondria. This indicates that the interaction with the ceramide channel is physiologically more important than acting on its own. Thus, in the process of developing a global understanding of the apoptotic process, one must include ceramide channels as an intrinsic and probably a key component.

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