Bax and Bcl-xL exert their regulation on different sites of the ceramide channel

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The present study demonstrates the important structural features of ceramide required for proper regulation, binding and identification by both pro-apoptotic and anti-apoptotic Bcl-2 family proteins. The C-4 = C-5 *trans*-double bond has little influence on the ability of Bax and Bcl-xL to identify and bind to these channels. The stereochemistry of the headgroup and access to the amide group of ceramide is indispensible for Bax binding, indicating that Bax may interact with the polar portion of the ceramide channel facing the bulk phase. In contrast, Bcl-xL binding to ceramide channels is tolerant of stereochemical changes in the headgroup. The present study also revealed that Bcl-xL has an optimal interaction with long-chain ceramides that are elevated early in apoptosis, whereas short-chain ceramides are not well regulated. Inhibitors specific for the hydrophobic groove of Bcl-xL, including 2-methoxyantimycin A_3, ABT-737 and ABT-263 provide insights into the region of Bcl-xL involved in binding to ceramide channels. Molecular docking simulations of the lowest-energy binding poses of ceramides and Bcl-xL inhibitors to Bcl-xL were consistent with the results of our functional studies and propose potential binding modes.

Key words: ABT-263, ABT-737, apoptosis, Bcl-2 family protein, ceramide analogue, ceramide channel, 2-methoxyantimicin A_3, mitochondrion.

INTRODUCTION

The intrinsic pathway of apoptosis, a type of programmed cell death, is regulated at the mitochondrial level by the Bcl-2 family of proteins. Permeabilization of the MOM (mitochondrial outer membrane) leads to the release of key IMS (intermembrane space) proteins, such as cytochrome *c*, which irreversibly commit the cell to the execution phase of apoptosis. The Bcl-2 proteins have pro-survival members, such as Bcl-xL, Bcl-2 and Mcl-1, that maintain the integrity of the MOM in normal cells and pro-apoptotic members, such as Bax, Bak and Bid, that initiate the apoptotic process and facilitate IMS protein release when the cell is damaged or an apoptogenic signal is delivered. Whether the anti-apoptotic or pro-apoptotic Bcl-2 proteins prevail will determine whether IMS proteins are released by a pore through the MOM and thus the fate of the cell. There are multiple pathways proposed for the release of IMS proteins from mitochondria [1–4], and even Bax itself is able to form channels [5,6]. Whatever the nature of the pathway, it must be tightly regulated to prevent unwanted cell death. One such proposed pathway for IMS protein release is formed by the bioactive sphingolipid ceramide.

The involvement of ceramide in apoptosis is widely recognized, and intracellular ceramide levels increase in response to many different apoptotic stimuli; specifically, it is the elevation of ceramide in mitochondria that is pro-apoptotic [7–10]. Elevation of ceramide in mitochondria can occur in a number of ways [11,12], including transfer of ceramides from mitochondria-associated membranes [13]. In fact, whereas Bax/Bak-knockout cells are known to be highly resistant to apoptosis [14,15], it was shown recently that this resistance is accompanied by a deficit in ceramide generation, as Bak regulates ceramide metabolism by activating a critical ceramide synthase to elevate mitochondrial ceramide levels [16]. Moreover, whereas metabolism of ceramide to other forms, such as glucosylceramide, can render cells less susceptible to cytotoxic agents [17–19], blocking ceramide metabolism can sensitize multidrug-resistant cells to death [20–22]. Thus the steady-state level of ceramide is an important step in the apoptotic programme in a variety of cell types [18,23], and cytotoxic stimuli [8,9,20] and ceramide channel formation can be demonstrated at these physiological levels of ceramide in mammalian mitochondria and yeast mitochondria [1,24,25]. Ceramide channels have also been visualized in liposomes using transmission electron microscopy [26]. These structures are stable and have been shown to be large enough for the release of all known IMS proteins released from mitochondria during apoptosis [25].

The current model of the ceramide channel highlights the structural features that are important for channel formation [27] (see Figure 1 for structures of ceramide and analogues). The amide nitrogen and carbonyl group allow for the formation of ceramide columns. Adjacent ceramide columns are held together by the C-1 and C-3 hydroxy groups forming the lumen of the channel. The length of the hydrocarbon chains of ceramide, although physiologically important [28,29], is relatively unimportant for channel formation [30]. The sphingoid base is typically 18 carbons in length with a C-4 = C-5 *trans*-double bond. The ceramide species in mammalian cells have fatty-acyl chains that range from 12 to 24 carbons in length. It is important to note that ceramide channels are highly stable rigid structures that are held together by

Abbreviations used: C_2-Cer, *N*-acetyl-D-*erythro*-sphingosine; C_{16}-Cer, *N*-palmitoyl-D-*erythro*-sphingosine; $C_{18:1}$-Cer, *N*-oleoyl-D-*erythro*-sphingosine; C_{20}-Cer, *N*-arachidoyl-D-*erythro*-sphingosine; C_{24}-Cer, *N*-lignoceroyl-D-*erythro*-sphingosine; DNP, 2,4-dinitrophenol; IMS, intermembrane space; 2-MeAA_3, 2-methoxyantimycin A_3; MOE, Molecular Operating Environment; MOM, mitochondrial outer membrane; MOMP, MOM permeabilization..

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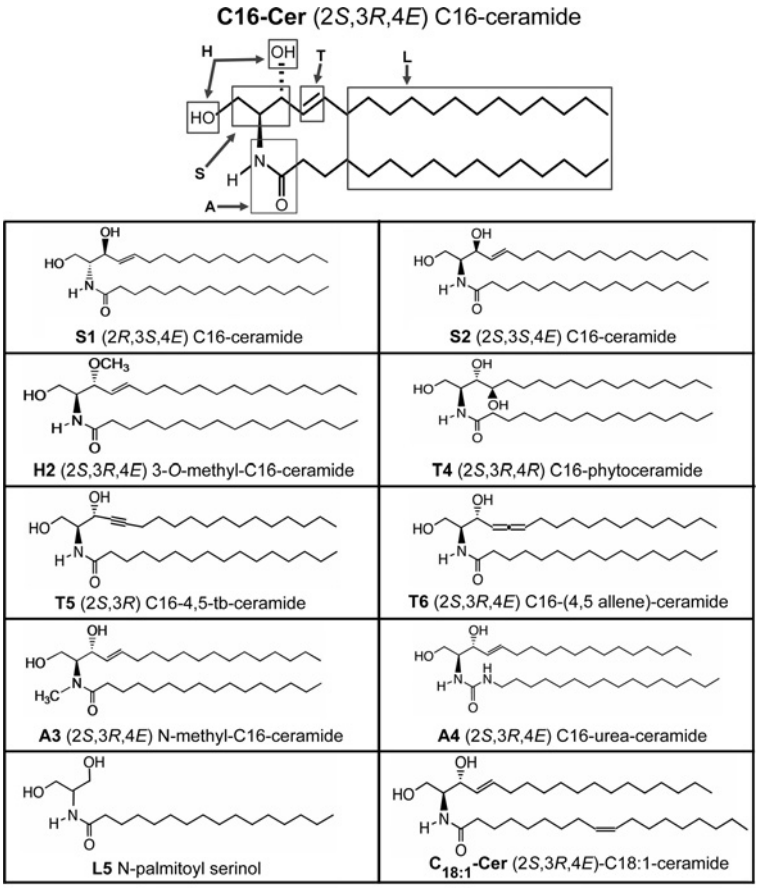

Figure 1 Structures of ceramides and analogues used

Coded boxes highlight the key areas of the ceramide molecule that are important for ceramide channel formation (top). The codes indicate the location on the ceramide molecule where changes were introduced (**S** for stereochemistry, **H** for hydroxy group, **T** for *trans*-double bond, **A** for amide linkage, **L** for hydrophobic chain length) and the analogues are named throughout the paper using these short codes followed by a number as indicated.

a vast network of hydrogen bonds [27,31] and not simply lipidic pores of a transient non-specific nature.

It was found that ceramide channels are regulated by the Bcl-2 family of proteins. The pro-survival Bcl-2 members, Bcl-xL and Ced-9 (the *Caenorhabditis elegans* homologue of Bcl-2) were shown to antagonize channel formation and disassemble ceramide channels [24]. Conversely, a pro-apoptotic Bcl-2 member, Bax, can enhance ceramide channels directly [31]. The regulation of ceramide channels was demonstrated by the addition of purified Bcl-2 family proteins to isolated mammalian mitochondria (which have other Bcl-2 family proteins naturally present in those membranes), yeast mitochondria (which do not have potentially interfering Bcl-2 family proteins), and planar phospholipid bilayers (which are devoid of any proteins or specialized lipids). These results highlight the ability of Bcl-2 family proteins to identify and regulate ceramide channels. These interactions are highly specific and do not require other proteins or specialized lipids, demonstrating for the first time that a channel formed of lipids can be regulated by a protein. Remarkably, this regulation was in accordance with the known physiological function of these Bcl-2 proteins.

In the present study, we set out to understand further the interaction of ceramide channels and Bcl-2 family proteins. We provide insights into the specific sites on the ceramide channel where Bax and Bcl-xL exert their regulatory effects. Moreover, to investigate the binding site on Bcl-xL for ceramide, we tested specific inhibitors of Bcl-xL that bind to a known location on the protein. Molecular docking studies were conducted with ceramides and Bcl-xL inhibitors to probe for the ceramide-binding site and reveal the lowest-energy binding modes of these molecules to Bcl-xL. We offer mechanistic insights as to how the specific interactions that we discovered could lead to the regulation of ceramide channels and discuss the implications of these results.

EXPERIMENTAL

Reagents

C_2-Cer (*N*-acetyl-D-*erythro*-sphingosine), C_4-Cer (*N*-butyryl-D-*erythro*-sphingosine), C_8-Cer (*N*-octanoyl-D-*erythro*-sphingosine), C_{16}-Cer (*N*-palmitoyl-D-*erythro*-sphingosine), $C_{18:1}$-Cer (*N*-oleoyl-D-*erythro*-sphingosine), C_{20}-Cer (*N*-arachidoyl-D-*erythro*-sphingosine) and C_{24}-Cer (*N*-lignoceroyl-D-*erythro*-sphingosine) were obtained from Avanti Polar Lipids. Antimycin A, DNP (2,4-dinitrophenol), and fatty-acid-depleted BSA were purchased from Sigma. The analogues of D-*erythro*-C_{16}-ceramide and the analogues **T4** and **L5** (Figure 1) were synthesized as described previously [32–36]. Horse heart cytochrome *c* was purchased from Acros, 2-MeAA$_3$

(2-methoxyantimycin A_3) was purchased from Enzo Life Sciences, and ABT-737 and ABT-263 were purchased from Chemie Tek.

Preparation of rat liver mitochondria

Rat liver mitochondria were isolated from male Sprague–Dawley rats by differential centrifugation of tissue homogenates as described previously [37] with modifications described in [25]. BSA medium was used to wash the liver during the excision and initial centrifugation steps and was removed by centrifugation at 8700 ***g*** (twice) in BSA-free medium. The final mitochondrial pellet was resuspended in ~3–5 ml of ice-cold sucrose-free isotonic FH buffer (280 mM mannitol, 0.1 mM EGTA and 2 mM Hepes, pH 7.4). The mitochondrial intactness was determined from the rate of cytochrome *c* oxidation compared with the rate measured after mild hypotonic shock [38] as described below. The animal use protocols were approved by the Institutional Animal Care and Use Committee. The animals were killed by a procedure consistent with the Panel on Euthanasia of the AVMA (American Veterinary Medical Association). The animal facility used to house the animals is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care).

Purification of recombinant proteins

Recombinant human Bax was purified as described previously [39] with modifications described in [31]. One change made was to reduce the Tris concentration from 20 mM to 10 mM in both dialysis steps; this change had no effect of the activity of the protein. The protein was shell-frozen in small aliquots in thin-walled glass tubes using ethanol/solid CO_2 and stored at $-84\,^{\circ}C$. A fresh monomeric Bax sample was thawed before each experiment. Standardizing the concentration of Bax to 5–10 μg/ml with FH buffer (10 mM Tris/HCl, pH 8.0, solution could also be used) before activation improved reproducibility between different Bax preparations. This monomeric Bax was activated using 10 % β-octyl glucoside to a final concentration of 1 % and incubated for 30 min on ice; this activated Bax was used in the experiments throughout the study. Full-length recombinant Bcl-xL was isolated as described previously [40] and the following changes are recommend. Only 5 ml of overnight culture was inoculated into 1 litre of LB (Luria–Bertani) medium and this was allowed to incubate with shaking until the D_{600} reached 0.600. The culture was then induced with 0.01 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 2 h at 37 °C. After the cells were harvested by centrifugation, the pellet was resuspended in 40 ml of PBS and incubated on ice for 20 min with 140 units of lysozyme and 35 μM PMSF (final concentration). The cells were then subjected to two passes through a French press and centrifuged to remove cell fragments. The lysate was then incubated with glutathione–agarose beads for 2 h at 4 °C and packed into a column. The beads were washed with 10 column volumes of ice-cold PBS containing 35 μM PMSF and another 10 column volumes of 20 mM Tris/HCl (pH 8.0). Then, 10 ml of the same buffer supplemented with 5 units of biotinylated thrombin were added to the column and allowed to incubate overnight at 4 °C. The protein was eluted, and 80 μl of streptavidin beads were added to remove the remaining thrombin. After a 30 min incubation with rotation, the beads were removed by centrifugation and the sample was filter-sterilized. Glycerol (10 %) was added to the protein and 100 μl aliquots were rapidly shell-frozen in ethanol/solid CO_2. The published method included the addition of Triton X-100 to the cells along with the PMSF and lysozyme before French pressing of the cells. This greatly increased the yield (1.4 mg/ml with Triton X-100 compared with 165 μg/ml without Triton X-100). Both methods resulted in proteins with identical function in our assays.

Cytochrome *c* oxidation assay

The ability of cytochrome *c* to translocate through the MOM and become oxidized by cytochrome *c* oxidase (found on the mitochondrial inner membrane) is the rate-limiting step, thus this assay is a measurement of the permeability of the MOM. Whereas measurements from mitochondrial release assays indicate that mitochondria were permeabilized at some time point, perhaps transiently, one of the advantages of using this dynamic cytochrome *c* oxidation assay is that real-time permeability of the MOM can be measured. Thus this assay allows us to garner more information about the nature and stability of the channels in question. This assay was performed as described previously [41] with the following modifications to optimize reproducibility as mitochondria were found to be more stable at higher concentrations. In a typical experiment, unless noted otherwise in the Figure legends, mitochondria were diluted in FH buffer at 4 °C to a concentration of 0.5 mg/ml, in small batches just before the assay. Then, 50 μl aliquots were dispersed in 650 μl of room temperature (??°C) reaction buffer [160 mM mannitol, Q1 pH 7.25, 60 mM KCl, 5 mM DNP and 1.3 μM antimycin A (antimycin A was tested in our system with Bcl-xL and ceramide and no effect was seen at the highest concentration tested which was 2.7 μM)]. The final mitochondrial protein concentration was 36 μg/ml. The mitochondria were allowed to acclimate at room temperature for 5 min in a microfuge tube. Then ceramide or a ceramide analogue (dissolved in propan-2-ol at 1 mg/ml) was delivered to the mitochondria, while the suspension was vortex-mixed for 30 s to achieve effective dispersal of the sphingolipid. After dispersal, the mixture was incubated for 10 min at room temperature followed by addition of cytochrome *c* (12 μl; final concentration 27 μM) and immediate measurement of the absorbance at 550 nm for a period of 2 min. The initial rate of decline of absorbance of reduced cytochrome *c* was used as a measure of the permeability of the MOM to cytochrome *c*; ε_{550} (red-ox) = 18.5 $mM^{-1}\cdot cm^{-1}$. The amount of vehicle added was less than 4 % of the sample volume, and all vehicle controls were treated in an identical way. Rates were corrected for the rate of oxidation observed with vehicle alone and this was very close to the untreated rate arising from a small number of damaged mitochondria. The maximal rate of cytochrome *c* oxidation was assessed by subjecting the mitochondria to mild hypotonic shock as described previously [38] with the following modifications. Briefly, mitochondria were diluted in double-distilled water to 1 mg/ml and incubated on ice for 10 min before adding 2×FH buffer (FH buffer with double the amount of additives) to restore the isotonic conditions. Then, 50 μl of this 0.5 mg/ml mitochondrial stock was added to the reaction buffer and tested for cytochrome *c* oxidation as described below. In these experiments, the intactness of the mitochondrial preparations was greater than 85 %. The rate of cytochrome *c* oxidation was converted to the percentage permeabilization of mitochondria by taking this rate as a percentage of the maximal rate of oxidation obtained by subjecting the mitochondria to mild hypotonic shock. The permeabilization induced by ceramide alone is given for each experiment in the Figure legends. It is important to note that even a relatively low percentage of permeabilization of mitochondria, as seen with our Bax experiments, is physiologically relevant

[1,42,43], and even low levels of cytochrome *c* release are sufficient to induce apoptosis in whole cells [44,45].

It should be pointed out that, unlike liposomes, mitochondria are complex organelles whose function decays with time. Thus we are limited in what experiments can be performed within the functional time window. Also, it is necessary to monitor the state of the mitochondria. Before experiments are performed, the mitochondria are tested for the ability of C_{16}-Cer to permeabilize the MOM and for the ability of the Bcl-2 family proteins to alter this permeabilization. These tests are repeated as time passes to ensure that any results obtained in the experimental trials are meaningful. The disadvantages of using a 'living' organelle are more than compensated for by performing experiments in the natural membrane environment. Clearly, the greatest level of understanding is reached by performing experiments at various levels of complexity, from the intact cell to the pure components in a defined system.

Testing Bcl-2 proteins on channels formed by ceramide and its analogues

The sensitivity of isolated mitochondria to permeabilization by added ceramide or analogues varied from one preparation to another. Therefore experiments with analogues were always performed in parallel with experiments with C_{16}-Cer. Furthermore, the amounts of C_{16}-Cer and analogue added were adjusted (dilutions were made to keep the volume added to the sample constant at 10 μl) so as to induce approximately the same extent of MOMP (MOM permeabilization). This was done because some analogues of ceramide may insert more or less readily into mitochondria or have a different propensity for channel formation. By finding conditions that result in the same degree of MOMP, we achieve the same level of channel formation. The permeabilization was measured as the rate of cytochrome *c* oxidation. Bax, Bcl-xL, 2-MeAA_3, ABT-737 and ABT-263 were added along with the mitochondria for 5 min of incubation at room temperature before sphingolipid dispersal. Stock solutions of 2-MeAA_3 (1.9 mM in propan-2-ol), ABT-737 (0.7 mM in DMSO) and ABT-263 (0.7 mM in DMSO) were diluted to the appropriate concentration with the same solvent so that the volume added to reach the concentration indicated in the Figures remained constant (10, 5 and 5 μl respectively). Results are shown as a percentage change comparing the effect of adding a Bcl-2 protein in combination with ceramide (or an analogue) compared with the addition of ceramide (or the analogue) alone.

Molecular docking simulations of potential Bcl-xL inhibitors

Modelling, simulations and visualizations were performed using MOE (Molecular Operating Environment) version 2011.10 (Chemical Computing Group). Simulations were performed on a Dell E8500 with an Intel Core 2 Duo @ 3.16 GHz using Windows XP operating system. All other computational procedures were performed using a Dell XPS M1530 with an Intel Core2 Duo processor T8300 @2.40 GHz with 2 GB RAM using Windows Vista operating system. The structural file used as input for analysis and docking simulations was PDB code 1PQ1. Before analysis and simulations, the main-chain protein was protonated at pH 7.5 with a salt concentration of 0.2 M and the structures were energy-minimized using the Amber99 forcefield and Born solvation model. Simulations focused on the entire protein surface and Bcl-xL was held rigid while the ligand was flexed. Initial placement calculated 500 poses per molecule using triangle matching placement with London dG scoring, the top 250 poses were then refined using forcefield placement and Affinity dG scoring. The resulting interaction after refinement is called E score 2. This is a measure of the strength of the interaction, with stronger interactions yielding a more negative number. Ligands were prepared in MOE, protonated at pH 7.5, and chirality formalized within the ligand database. The top poses generated from each ligand were then minimized in place as a system with Bcl-xL to allow the protein to flex as well as the ligand. System minimization also used the Amber99 forcefield and Born solvation model.

Statistical analysis

All results shown are means $\pm$ S.E.M. for three or four trials, and each experiment was repeated on three separate mitochondrial preparations. Student's *t* test was performed to obtain *P* values and identify a statistically significant change from the additive value (for Bax experiments) or individual C_{16}-Cer or analogue addition (for Bcl-xL experiments). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

RESULTS AND DISCUSSION

Previous results [24,31] have indicated that the recognition between the ceramide channel and Bcl-2 proteins is highly specific. These findings prompted the present study in which we used a dynamic cytochrome *c* accessibility assay to assess the influence of Bax and Bcl-xL on the ability of ceramide and its analogues to permeabilize the MOM. To determine the specific site(s) of interaction of these Bcl-2 proteins on ceramide channels, we prepared ceramide analogues with changes in different functional groups thought to be important for channel formation. The analogues that retained channel-forming ability are illustrated in Figure 1. Experiments with ceramide and an analogue were performed in parallel. Before each experiment, the amounts of ceramide or ceramide analogue were adjusted so as to induce approximately the same extent of MOMP. In this way, we compensated for any differences in the propensity for channel formation and tested the Bcl-2 family proteins on the same degree of channel presence. As a result, the ability of the Bcl-2 family proteins to alter the MOMP induced by ceramide could be compared with that for the analogue.

When different doses of ceramide or ceramide analogues were applied to achieve a comparable degree of permeabilization, the amount of Bcl-2 family protein added was kept the same. This is because the amount of protein added greatly exceeded the number of channels present (for additional details, see the Supplemental Online Data at http://www.BiochemJ.org/bj/444/bj4440000add.htm). Thus the binding is not of very high affinity or stoichiometry. Previous studies showed that the channels are responsive to the effective concentration of added protein in a manner consistent with a dynamic equilibrium. Thus despite adding different amounts of the sphingolipid to achieve the same degree of permeabilization, it makes sense to use the same effective dose of Bcl-2 family protein to detect differences in effect on channel-mediated permeability.

The results described were obtained at room temperature, whereas apoptosis, in mammalian cells, typically takes place at 37 °C. Thus differences in rates of reaction, fluidity, etc. resulting for a difference in temperature could easily affect the reported values, but are unlikely to change the fundamental conclusions about the specificity of the sites of interaction with ceramide associated with Bax and Bcl-xL. Indeed, key experiments reproduced at 37 °C yield similar results as seen at 23 °C (see Figures 4A and 5C, and Supplementary Figures S1A

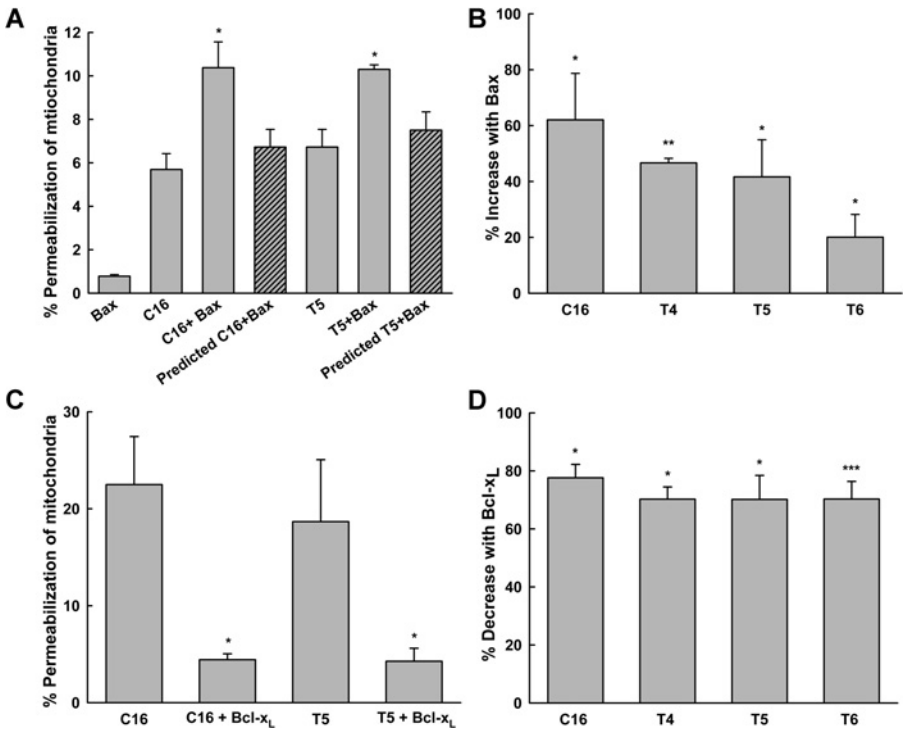

Figure 2 Testing the ability of Bax and Bcl-xL to regulate channels formed in isolated rat liver mitochondria by ceramide analogues with changes in the C-4 = C-5 *trans*-double bond or extent of unsaturation

The cytochrome *c* oxidation assay was used to determine the degree of MOMP induced by the addition of ceramide or an analogue and the enhancement or inhibition of this permeability in the presence of Bax or Bcl-xL respectively. (**A**) A typical Bax/C_{16}-Cer/analogue experiment: the amount of C_{16}-Cer ('C16', 10 μg) and **T5** (5 μg) added was adjusted so that they induced approximately the same extent of MOMP when added alone. The addition of Bax (3.5 nM) along with C_{16}-Cer ('C16 + Bax' column) and **T5** ('T5 + Bax' column) resulted in enhancement of the MOM permeability induced by ceramide and the analogue. These combinations tested are compared with the permeability predicted by adding the effect of the respective individual treatments ('Predicted' columns, i.e. 'Bax' + 'C16' = 'Predicted C16 + Bax'). (**B**) Bax (3.5nM) can enhance channels formed by **T4** (5 μg), **T5** (5 μg) and **T6** (5 μg) and is shown as a percentage increase in MOMP. (**C**) A typical Bcl-xL/C_{16}-Cer/analogue experiment is shown here and is conducted similarly to that in (**A**). Bcl-xL (0.4 μM) inhibits C_{16}-Cer (10 μg) channels and **T5** (6 μg) channels to a similar extent. As Bcl-xL does not induce a detectible MOMP (see Figure 7A), the combination treatments of 'C16 + Bcl-xL' and 'T5 + Bcl-xL' were compared with the individual treatments of C_{16}-Cer and **T5** respectively and subject to the same statistical analysis as stated below. (**D**) Bcl-xL (0.4 μM) inhibited C_{16}-Cer (10 μg), **T4** (7.5 μg), **T5** (6 μg) and **T6** (5 μg) channels to the same extent and is shown as a percentage decrease in MOMP.

and S1B at http://www.BiochemJ.org/bj/444/bj4440000add.htm), indicating that the physical properties of this interaction remain at physiological temperatures.

The importance of the *trans*-double bond

The following changes were introduced to the C-4 = C-5 *trans*-double bond of the sphingoid backbone of ceramide: C_{16}-phytoceramide (**T4**), which has a hydroxy group at the C-4 position instead of the double bond; C_{16}-4,5-tb-ceramide (**T5**), which has a triple bond in place of the double bond; and C_{16}-(4,5-allene)-ceramide (**T6**), which is a 4,5-cumulated diene. Previous results showed that the addition of either detergent-activated Bax or tBid-activated Bax along with C_{16}-Cer to isolated rat liver mitochondria resulted in a much greater permeabilization of the MOM than either entity alone, indicating that Bax enhances the formation of ceramide channels [31]. We looked for a similar result on ceramide analogues. An example of the result of a typical paired experiment with C_{16}-Cer and an analogue (**T5**) is shown in Figure 2(A). Detergent-activated Bax ('Bax') induces a small permeabilization, whereas C_{16}-Cer ('C16') produces a more significant permeabilization. However, when both are added together ('C16 + Bax'), the resulting permeabilization is much greater than the value obtained by adding the values of the 'Bax' and 'C16' bars, 'Predicted C16 + Bax'. The permeabilization induced by **T5** was enhanced by the addition of Bax to a similar extent, indicating that the modification had not influenced the ability of Bax to act on **T5** channels. Similar paired experiments were performed with the other analogues. Figure 2(B) shows that Bax enhanced the permeabilization induced by **T4** to the same extent as **T5**, but was 40 % less effective on **T6**.

In Bcl-xL experiments (Figure 2C), as in the Bax experiments, the doses of ceramide and analogue used were adjusted so that the extent of MOMP induced was approximately the same. When Bcl-xL is present ('C16 + Bcl-xL' and '**T5** + Bcl-xL'), the permeabilization was greatly reduced in both cases and by the same amount. The percentage decrease in permeabilization is shown in Figure 2(D). Bcl-xL (0.4 μM) inhibited ceramide channel formation in isolated mitochondria by nearly 80 % (Figure 2C). The same dose of Bcl-xL was similarly effective on **T4**, **T5** and **T6**.

Analogues in which changes to the double bond were made by introducing a hydroxy group in its place (**T4**), changing it to a triple bond (**T5**) or adding another double bond next to it (**T6**) were well tolerated by both Bax and Bcl-xL. Thus the location of the C-4 = C-5 double bond and the degree of unsaturation at this position have little influence on the ability of Bax or Bcl-xL to regulate these lipid channels.

Importance of the C-1 and C-3 polar groups

The C-1 and C-3 hydroxy groups of the ceramide are thought to hold adjacent columns of ceramide together to form the lumen of the ceramide channel. 3-*O*-Methyl-C_{16}-ceramide (**H2**) has the C-3 hydroxy group blocked, yet this analogue can still form channels (Figure 3A), suggesting that the C-1 hydroxy group is sufficient to stabilize the channel. Both Bax and Bcl-xL can exert their respective effects on **H2**, indicating that the C-3 hydroxy group is not a site at which either protein interacts (Figures 3A and 3B).

The effect of changing the configuration at C-2 or C-3 of ceramide was explored by using two stereoisomers: the enantiomer (2*R*,3*S*,4*E*)-C_{16}-ceramide (**S1**) and the diastereomer (2*S*,3*S*,4*E*)-C_{16}-ceramide (**S2**). Bax was unable to enhance channels formed by **S1** (Figure 2C). **S1** is the mirror image of the natural ceramide and Bax is unable to recognize channels formed by **S1**. However, Bax was able to enhance channels formed by **S2** to a similar degree as C_{16}-Cer (Figure 2D), where only the orientation of the C-3 hydroxy group is altered. Bcl-xL was able to inhibit channels formed by **S1** and **S2** (Figure 2E), although to a lesser degree than those formed by C_{16}-Cer. These results indicate that the ability of Bax to interact with ceramide channels does not require access to the C-3 hydroxy group (**H2**), but is highly sensitive to the configuration at C-2 of ceramide.

The importance of the amide group of ceramide

In the current model of the ceramide channel, the amide group is thought to form a hydrogen bond with the carbonyl group of another ceramide molecule [27]. These intermolecular interactions allow for the assembly of ceramide columns. Analogue **A3**, *N*-methyl-C_{16}-ceramide, features a methyl group instead of the amide group. MOMP induced by **A3** was not enhanced by the addition of 3.5 nM Bax, whereas parallel experiments with C_{16}-Cer resulted in a 40 % enhancement (Figure 4A and Supplementary Figure S1A). Thus Bax is unable to influence the structure of channels formed by **A3**, possibly due to a requirement for binding to the amide group. Bcl-xL also has a much reduced interaction with **A3**, but the effect is not completely diminished as with Bax (Figure 4B). (2*S*,3*R*,4*E*)-C_{16}-urea-ceramide (**A4**) is a potent channel former as half as much is needed to permeabilize the MOM to the level achieved by a given amount of C_{16}-Cer (Figure 4C). This could be attributed to the enhanced hydrogen-bonding ability afforded by the urea group and thus the potential to further stabilize ceramide columns. This change is opposite to that of A3 in terms of hydrogen-bonding ability of the molecule and Bax addition resulted in a greater enhancement of **A4** channels than C_{16}-Cer channels. Perhaps the increased hydrogen-bonding ability also serves to increase the strength of the interaction with Bax. Bcl-xL inhibits the **A4** channels to the same extent as the C_{16}-Cer channels (Figures 4C and 4D), indicating that Bcl-xL is not sensitive to the modification.

The importance of tail length and apolar bulk

The importance of the apolar region of ceramide for Bcl-2 protein interaction was tested with a minimal analogue, *N*-palmitoylserinol (**L5**). It only has one aliphatic chain. Bax was able to interact with **L5** (Figure 5A) almost as well as C_{16}-Cer. Surprisingly, Bcl-xL was not able to inhibit these channels at all (Figure 5B). Likewise, the permeability induced by a very-short-chain ceramide analogue, C_2-Cer, was also not inhibited by Bcl-xL (Figure 5C and Supplementary Figure S1B). To test whether this unexpected result was due to kinetic delays, the added doses of the ceramides was adjusted to achieve a similar MOMP by C_2-Cer and C_{16}-Cer alone. Incubations of 15 and 30 min with Bcl-xL resulted in the same degrees of inhibition (Figure 5D). No effect of Bcl-xL on C_2-Cer was seen at either time point, whereas the same substantial level of inhibition of C_{16}-Cer permeabilization occurred at each time point. Thus the inability of Bcl-xL to inhibit C_2-Cer channels is not dependent on incubation time.

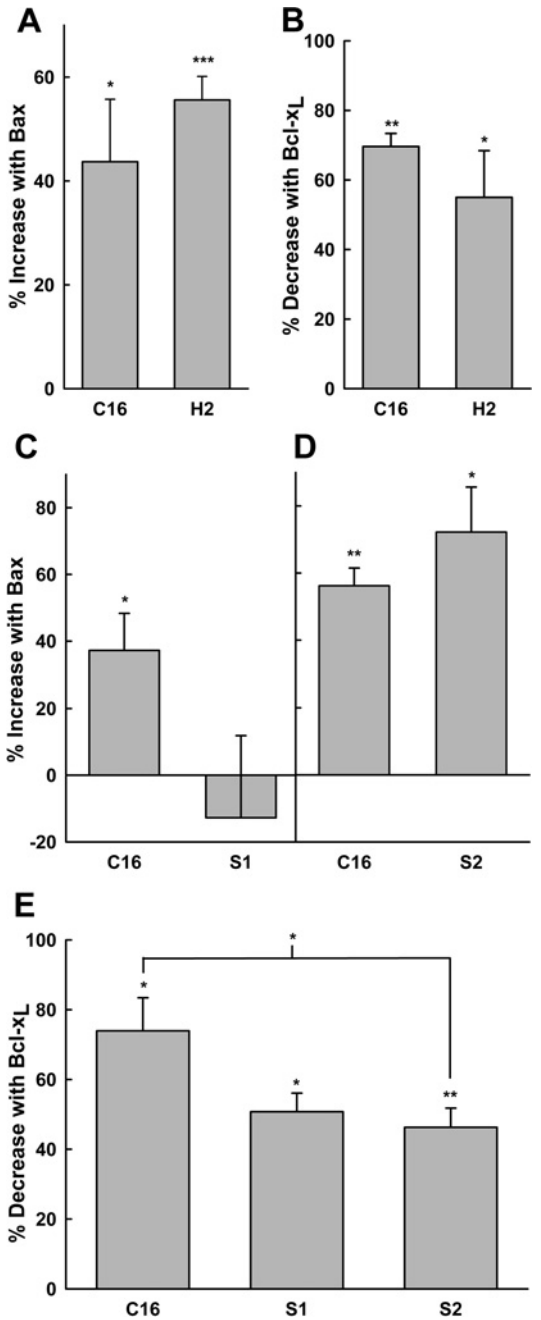

Figure 3 Tests of the ability of Bax and Bcl-xL to regulate ceramide analogues with changes to polar headgroups or stereochemistry

(**A** and **B**) Blocking the C-3 hydroxy group does not reduce the channel's sensitivity to 3.5 nM Bax (5 μg of C_{16}-Cer, 2 μg of **H2**, yielding ~10 % MOMP) or 0.4 μM Bcl-xL (7.5 μg of C_{16}-Cer, 5 of μg **H2**, yielding ~12 % MOMP). (**C**) Bax (2 nM) is sensitive to changes in the stereochemistry found in analogue **S1**. Mitochondria were treated with 10 μg each of **S1** and C_{16}-Cer (~20 % MOMP). (**D**) Channels formed by analogue **S2** (5 μg) interact with Bax (3.5 nM) similarly to those formed by C_{16}-Cer (5 μg) (~8 % MOMP). (**E**) Bcl-xL (0.4 μM) inhibits C_{16}-Cer (3.5 μg) channels more strongly than those formed by **S1** and **S2** (10 and 3.5 μg respectively, yielding ~10 % MOMP).

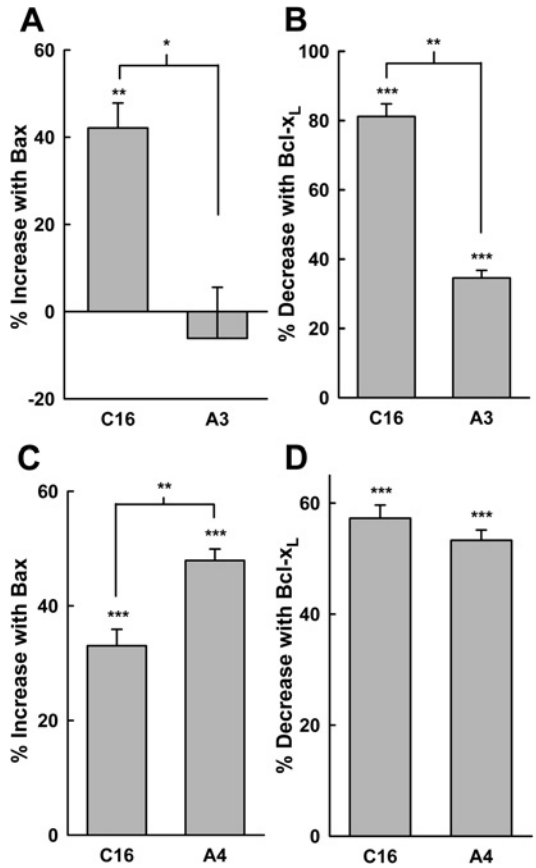

Figure 4 Bax is more sensitive than Bcl-xL to changes in the amide group of ceramide

(**A**) Blocking the amide nitrogen with a methyl group prevents Bax (3.5 nM) from enhancing channels made by **A3** (10 μg of C_{16}-Cer and **A3** were added to induce approximately 10 % MOMP). (**B**) Bcl-xL (0.1 μM) is approximately 50 % less effective on **A3** (20 μg) than is C_{16}-Cer (6 μg); however, Bcl-xL can still cause an approximately 35 % decrease in the permeability induced by **A3** channels (~11 % MOMP). (**C** and **D**) Analogue **A4** contains a urea group, which increases the hydrogen-bonding ability of the molecule. Whereas Bax is more effective on **A4** than C_{16}-Cer, Bcl-xL is similarly effective on both. For these experiments, 3.5 nM Bax, 0.1 μM Bcl-xL and 10 μg of C_{16}-Cer and 5 μg of **A4** were used to induce approximately 22 % MOMP.

Ceramide analogues with tail lengths varying between C_2 and C_{24} were tested and the results show an optimal length for the acyl chain of C_{16}, C_{18} and C_{20}. Bcl-xL is known to have a hydrophobic groove and perhaps the optimal hydrocarbon bulk results in optimal binding. The introduction of an unsaturated bond in the acyl chain at C-9-C-10 of C_{18}-Cer ($C_{18:1}$-Cer) resulted in a large decrease in sensitivity to Bcl-xL inhibition. The kink in the fatty acyl chain may interfere with binding to Bcl-xL, indicating a very specific interaction.

These results have important implications. Various long-chain and very-long-chain ceramides are found in the MOM, and the specific N-acyl chains of the naturally occurring ceramides in membranes are thought to play an important physiological role [28,29,46]. In fact, ceramide synthases show specificity for different acyl-CoA substrates [47–51], and some preferentially generate long-chain ceramides such as C_{16}-Cer or C_{24}-Cer [48,49]. In the early stages of apoptosis, cells are reported to generate primarily C_{16}-Cer along with some C_{18}-Cer and C_{20}-Cer [16,52]. C_{24}-Cer generation occurs at later stages [52]. This fits well with our observation that Bcl-xL interacts optimally with C_{16}-, C_{18}- and C_{20}-Cer, as those species are elevated when the fate of the cell is still being decided. During this initiation stage of apoptosis, before

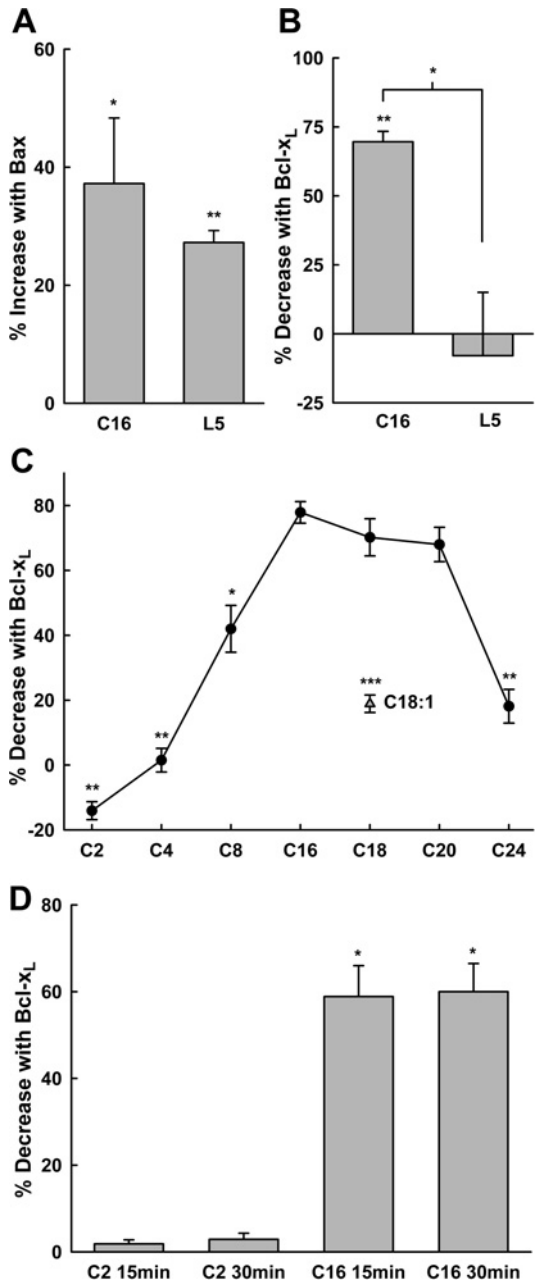

Figure 5 Effects of analogues with altered hydrophobic regions on the regulation by Bcl-xL and Bax

(**A**) Bax (2 nM) enhances MOMP formed by **L5** (10 μg), which has a truncated sphingoid base (10 μg of C_{16}-Cer was used, ~30 % MOMP). (**B**) Bcl-xL (0.4 μM) does not inhibit channels formed by **L5** (3 μg); 7.5 μg of C_{16}-Cer was added (~12 % MOMP). (**C**) Varying the length of the N-acyl chain of ceramide reveals an optimal acyl chain length for Bcl-xL (0.4 μM) inhibition of ceramide channels. of C_2-Cer–C_{18}-Cer and $C_{18:1}$-Cer were each used at 10 μg; C_{20}-Cer and C_{24}-Cer were each used at 7.7 μg added in a volume of 13 μl. Before addition of Bcl-xL, the amount of ceramide or ceramide analogue used induced approximately 19 % MOMP. (**D**) Analysis of the time-dependence of Bcl-xL interaction. The addition of 6 μg of C_2-Cer and 12 μg of C_{16}-Cer in a volume of 12 μl was performed to induce a similar level of mitochondrial permeabilization (~40 %). The addition of 0.1 μM Bcl-xL results in 60 % inhibition of C_{16}-Cer channels, but does not cause any change in the permeability of C_2-Cer channels during the time of a typical experiment, 15 min. Increasing the incubation time by 15 min does not affect the inability of Bcl-xL to recognize C_2-Cer channels.

the mitochondria have become permeabilized, various apoptotic signals result in the elevation of ceramides. Anti-apoptotic Bcl-2 family proteins, such as Bcl-xL and Bcl-2, could prevent

these higher ceramide levels from forming channels resulting in MOMP. Bax activation and heterodimerization with Bcl-xL would relieve this inhibition and, at the same time, any excess Bax could favour ceramide channel formation and/or growth. Clearly, these interactions result in many opportunities for controlling this key decision-making step.

Many studies have been carried out with cell-permeant C_2- and C_6-ceramides to trigger apoptosis in cancer cells. It is also evident that many enzymes that metabolize ceramide do not recognize C_2-ceramides [53,54], thus allowing these molecules to act over a longer time scale. Interestingly, the ability of short- and long-chain ceramides to form channels is about the same, and mixtures of long- and short-chain ceramides are actually more potent [55]. Thus it is possible that these short-chain ceramides may be more effective than long-chain ceramides therapeutically in promoting apoptosis because Bcl-xL is unable to recognize or regulate these species, while Bax retains the ability to promote channel formation by these ceramides.

Inhibitors provide insights into the region of Bcl-xL that binds to the ceramide channel

Cells overexpressing pro-survival proteins Bcl-xL and Bcl-2 are highly resistant to cell death, and many types of cancer indeed display this phenotype. Three known small-molecule inhibitors of Bcl-xL and Bcl-2 are 2-MeAA$_3$, ABT-737 and ABT-263. These inhibitors bind to the hydrophobic groove on Bcl-2 and Bcl-xL [24,56–58], effectively neutralizing the effects of pro-survival protein overexpression and sensitizing cells to death. Since Bcl-xL interacts with the ceramide channel through the hydrophobic region, the inhibitors should interfere with the interaction if binding involves the hydrophobic groove.

Results from the 2-MeAA$_3$ experiments are shown in Figure 6(A). Whereas the highest concentration of 2-MeAA$_3$ tested (2.7 μM) had no effect on ceramide-induced MOMP, Bcl-xL (0.7 μM) inhibited the ceramide-induced MOMP by approximately 50 %. 2-MeAA$_3$ produced a dose-dependent recovery of ceramide-induced MOMP, indicating that 2-MeAA$_3$ might bind to the same site on Bcl-xL as the ceramide channel. Unlike 2-MeAA$_3$, ABT-737 displayed a strong inhibition of ceramide permeabilization at 2.5 and 5 μM, whereas lower concentrations had either no effect or a slightly stimulatory effect on ceramide permeabilization (Figure 6B). In the presence of 0.7 μM Bcl-xL, ABT-737 produced a dose-dependent recovery of ceramide permeabilization with 50 % recovery at approximately 1 μM and complete recovery at 2.5 μM. A strong inhibition of ceramide channels by ABT-737 is evident at 5 μM even in the presence of Bcl-xL. ABT-263, the orally available analogue of ABT-737, was also able to cause a dose-dependent recovery of ceramide permeabilization in the presence of Bcl-xL, but did not inhibit ceramide permeabilization at any of the concentrations tested when Bcl-xL was not present (Figure 6C). Thus the inhibitory effects of Bcl-xL on ceramide channels can be reversed by the addition of Bcl-xL-targeted inhibitors that bind to the hydrophobic groove, suggesting competition and that the hydrophobic groove is likely to be the site at which Bcl-xL binds to the ceramide channel.

Molecular docking of ceramides and inhibitors to Bcl-xL were conducted to determine the lowest-energy site and mode of binding on Bcl-xL. The region of lowest-energy binding was first determined probing the whole surface of Bcl-xL and the hydrophobic groove was the best, or near best, scored region for all ligands tested. The top ten poses for each of the ligands are shown in Figure 7 and the average energy of these were calculated and

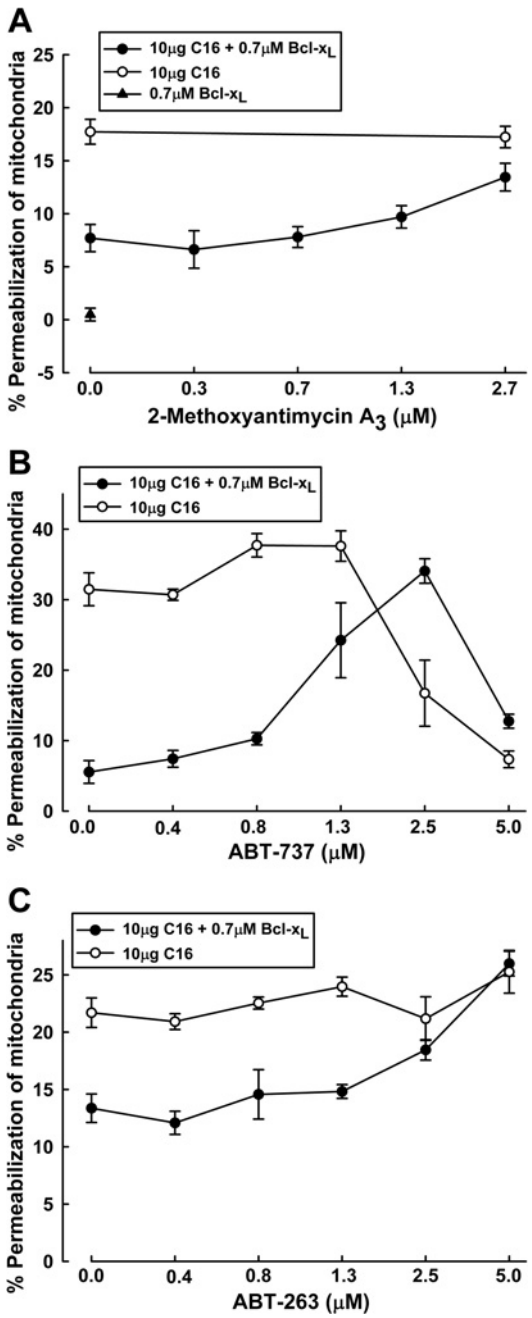

Figure 6 The hydrophobic groove of Bcl-xL is involved in binding to ceramide channels

Inhibitors known to bind to the hydrophobic groove of Bcl-xL were assayed for ability to prevent Bcl-xL binding to ceramide channels in isolated rat liver mitochondria. (**A**–**C**) Increasing concentrations of 2-MeAA$_3$, ABT-737 or ABT-263 were tested with C_{16}-Cer alone (10 μg) and the combination of C_{16}-Cer and Bcl-xL (0.7μM).

are shown as a horizontal bar. The more negative the number, the stronger the interaction. ABT-737 and ABT-263 had the lowest-energy score, and therefore best binding, followed by C_{16}-Cer. Especially noteworthy is the weaker average binding of C_2-Cer compared with C_{16}-Cer, consistent with the experimental results. To explore further the potential interaction between Bcl-xL and C_{16}-Cer, the lowest-energy pose was subjected to global system energy minimization to allow both the protein and the ligand to

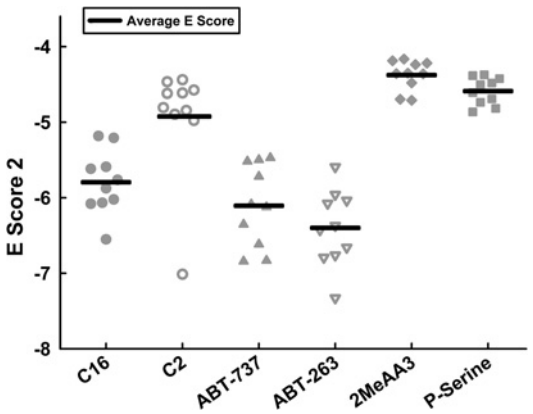

Figure 7 E Score 2 of ten best poses of molecular docking simulations

Docking simulations of Bcl-xL to C_{16}-Cer (C16), C_2-Cer (C2), ABT-737, ABT-263, 2-$MeAA_3$ or palmitoylserine (P-Serine) yielded structures of various degrees of interaction energy. The lower the E Score, the better the interaction. The average of the ten best interacting structures (poses) for each ligand is shown as a horizontal line.

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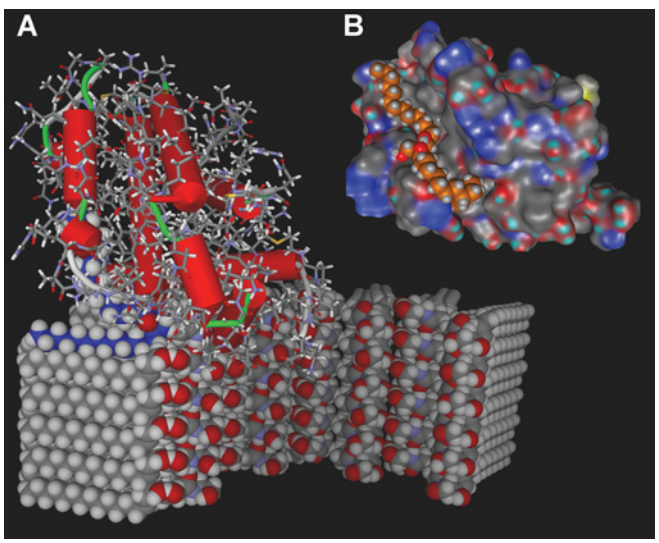

Figure 8 Molecular structures of C_{16}-Cer bound to Bcl-xL from docking simulations and possible interaction with a ceramide channel

(**A**) How Bcl-xL might bind to a ceramide molecule at the end of a ceramide column that is part of a ceramide channel. One of the top poses of C_{16}-Cer bound to Bcl-xL leaves one chain free. This was lined up with a segment of the proposed structure of a ceramide channel, showing how Bcl-xL might bind to the end of one ceramide column, disrupting the column's interaction with the adjacent phospholipid bilayer. (**B**) C_{16}-Cer nestled in the hydrophobic grove of Bcl-xL. This is one of the top ten poses after energy minimization.

flex. These results were considered a plausible prediction of the interaction between ceramide and Bcl-xL (Figure 8B).

One of the top ten poses shows ceramide binding to Bcl-xL with only one chain (Figure 8A). When this is placed on top of a segment of the model of a ceramide channel, this provides mechanistic support for a hypothetical mechanism by which Bcl-xL destabilizes a ceramide channel.

Molecular dynamics simulations [26,27] indicate that the ceramide channel forms an hourglass shape in the membrane, allowing the channel to properly interface with the phospholipid bilayer and thus avoiding exposure of apolar chains to the aqueous environment (Supplementary Figures S2 and S3 at http://www.BiochemJ.org/bj/444/bj4440000add.htm). This results in a curvature in the ceramide columns forming the channel that is positive in the direction normal to the plane of the membrane [27]. This curvature reduces the distortion of the phospholipids in close proximity to the ceramide channel. Thus interfacing the ceramide molecules that would tend to have their long axis parallel with the membrane plane with the phospholipids whose long axis is normal to that plane requires a compromise distortion of both to minimize the overall energy level. This low-energy compromise might be interfered with by the interaction with Bcl-xL. Bcl-xL could bind at this interface, displacing the local phospholipids and allowing the ceramide column to relax by reducing the positive curvature. Evidence from the present study indicates that the hydrophobic groove of Bcl-xL is interacting with the hydrophobic chains of the ceramide molecules in a channel, chains that are buried within the membrane apolar environment. This is consistent with previous results by Siskind et al. [24], who demonstrated that a Bcl-xL deletion mutant lacking the transmembrane domain is unable to disassemble ceramide channels, suggesting that the membrane-active form of Bcl-xL is required for ceramide channel regulation. Thus the binding of Bcl-xL to the end of one column and resulting relaxation of that column (Supplementary Figure S4 at http://www.BiochemJ.org/bj/444/bj4440000add.htm) would result in a mismatch with the curvature of adjacent ceramide columns and thus the generation of mechanical stress. The highly hydrogen-bonded structure of the channel would result in the propagation of the mechanical stress to the rest of the channel (Supplementary Figure S5 at http://www.BiochemJ.org/bj/444/bj4440000add.htm). This high-energy structure would then lead to the destabilization of the channel, shifting the dynamic equilibrium towards channel disassembly. This mechanism is consistent with previous results [24] that indicate the formation of a 1:1 complex between Bcl-xL and the channel, requiring that the effects of this binding propagate through the channel structure in an allosteric manner.

As for Bax, the results indicate that the chirality of the molecule's polar region and specifically the accessibility of the amide group are key for Bax binding. This indicates that Bax may also be acting at the interface of the channel and the phospholipid membrane where there is access to the carboxamide group. However, unlike Bcl-xL, ceramide binding might favour the positive curvature of the ceramide columns, stabilizing the channel at the channel–membrane interface. This stabilization can result in the enlargement of ceramide channels to an optimal size (Supplementary Figure S6 at http://www.BiochemJ.org/bj/444/bj4440000add.htm). Again, these results are consistent with previous findings [31], as the binding of Bax influences the ceramide channel to enlarge to a size that fits optimally with the binding site of Bax. Previous results also indicated that multiple Bax structures bind to a single ceramide channel, presumably all acting to stabilize the ceramide channel in a particular state.

Concluding remarks

Beyond the details, it is worth noting that highly organized lipid assemblies, ceramide channels in this case, take a form dictated by the properties of their monomeric subunits and the environment in which they exist. However, the forms of these superstructures can be modified by interactions with regulating molecules, in this case Bcl-2 family proteins. Thus cells can control these superstructures at various levels: by controlling the supply of specific monomers at the membrane or cell compartment of interest, by changing the lipid environment of the membrane, and by producing proteins (or other regulating factors) that alter the form or stability of the final structure. Perhaps, in thinking about lipids, one must go beyond

thinking about lipid phases, fluidity and phase transitions. Lipids can form superstructures with unique properties and the ability to be regulated much like the structures formed by proteins.

AUTHOR CONTRIBUTION

Meenu Perera designed the study, purified the proteins used, performed all mitochondrial experiments, analysed data and wrote the paper. Shang Lin aided in repeating a portion of mitochondrial experiments. Yuri Peterson designed and ran molecular docking simulations and analysed output data. Alicja Bielawska, Zdzlaw Szulc and Robert Bittman provided ceramide analogues. Marco Colombini provided intellectual guidance and wrote the paper. All authors contributed to editing the paper before submission.

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SUPPLEMENTARY ONLINE DATA

Bax and Bcl-xL exert their regulation on different sites of the ceramide channel

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Comparison of the number of channels per mitochondrion and the amount of Bcl-2 family proteins present: why the effective concentration of Bcl-2 family protein is important for channel regulation

The calculation of the number of channels present is based on the fact that only a fraction of the mitochondrial population is permeabilized as measured by adenylate kinase release. For isolated rat liver mitochondria, there are 7.2×10^9 mitochondria per mg of mitochondrial protein [1]. In an experiment where 160 μg of mitochondrial protein is used and 10 μg of ceramide, 20% MOMP is achieved as measured by cytochrome *c* oxidation, and only 28% of adenylate kinase is released (V. Ganesan and S. Samanta, personal communication). Thus, of the 1.2×10^6 mitochondria, only 0.3×10^6 mitochondria have channels. Statistically speaking, it is unlikely that the mitochondria that were permeabilized would contain multiple channels, whereas the others contain none. Thus the number of permeabilized mitochondria are a good measure of the number of ceramide channels present. However, the amounts of Bax (3.5 nM, 1.5×10^{12} molecules) and Bcl-xL (0.4 μM, 1.5×10^{14} molecules) are well in excess of the amount of mitochondria and channels. Thus a dynamic equilibrium must exist between bound and free protein, and it is the effective concentration of protein that determines the growth or destabilization of the ceramide channels. The amount of added protein is unrelated to the number of channels present. The probability that a channel will have a protein bound to it depends on the free protein concentration, either in aqueous solution or on or in the membrane.

Supplementary Figures

The supplementary Figures provide supporting information and drawings to clarify our interpretation of the results presented. In these drawings, the membrane is shown, for simplicity, as a lipid bilayer. For the sake of clarity, it makes sense to keep the illustrations simple. Clearly, the MOM is more complex, containing proteins and a variety of lipids that may or may not contribute to the structure of the ceramide channel and influence its interaction with the Bcl-2 family proteins.

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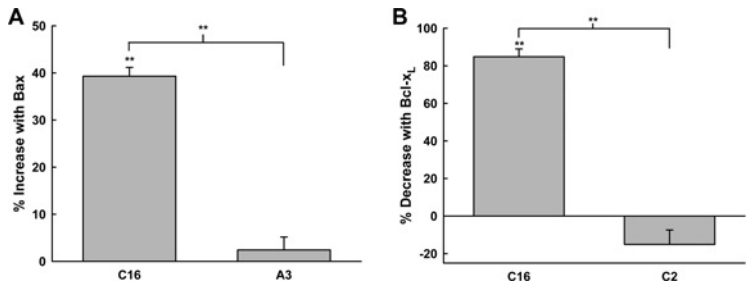

Figure S1 The effect of Bax and Bcl-xL on the ability to regulate channels made of ceramide or ceramide analogues at 37°C

These experiments were conducted in an identical fashion as described in the Experimental section of the main paper, except that the experiments were conducted at a constant temperature of 37°C. (**A**) Bax (3.5 nM) enhances ceramide channels, but not those formed of **A3** (2.5 μg of C_{16}-Cer and 10 μg of **A3** were added in a volume of 10 μl). (**B**) Bcl-xL (0.4 μM) can inhibit channels formed of C_{16}-Cer, but not C_2-Cer (2.5 μg of C_{16}-Cer and 10 μg of C_2-Cer were added in a volume of 10 μl). Results from both (**A**) and (**B**) are consistent with results obtained at room temperature (see Figures 3A and 4B of the main paper respectively)

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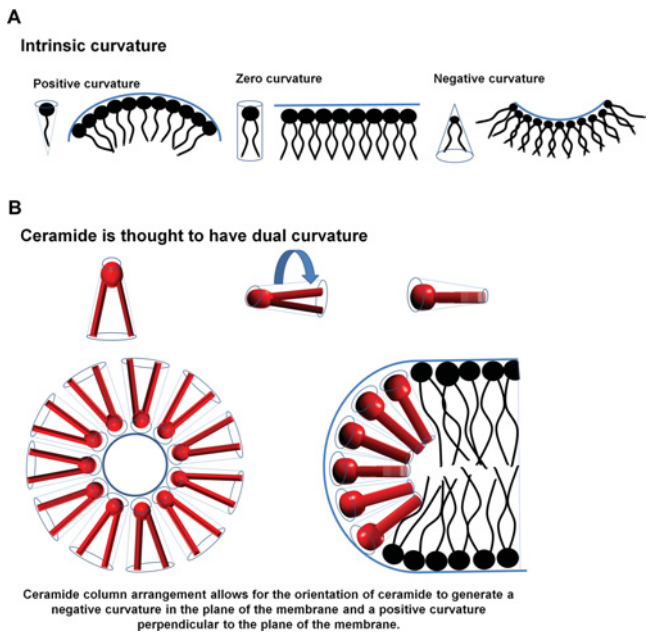

Figure S2 Membrane curvature depends upon the intrinsic curvature of its lipid components

Membrane phospholipids are shown in black and ceramide is shown in red. (**A**) Intrinsic curvature is measure of size of the hydrophilic portion of a lipid relative to the hydrophobic region. Cylindrical lipids where the headgroup and tail region are of similar relative size display zero curvature as shown in the plane of the membrane. Positive intrinsic curvature results when the headgroup of the lipid is larger than the tail region, leading to the formation of positive curvature of the membrane. The opposite is seen for lipids with negative intrinsic curvature. (**B**) Ceramide is thought to have dual curvature; ceramide is known to have negative intrinsic curvature (left, top); however, rotating the molecule reveals a positive curvature (left, bottom). This dual curvature allows for the formation of stable ceramide channels in membranes as revealed by molecular modelling studies conducted by Anishkin et al. [2]. Negative curvature of ceramide allows for the formation of a cylindrical pore in the plane of the membrane (middle), whereas the positive curvature of ceramide allows for the formation of positive curvature perpendicular to the plane of the membrane (right). Together, these features allow for the formation of stable transmembrane ceramide channels in the MOM.

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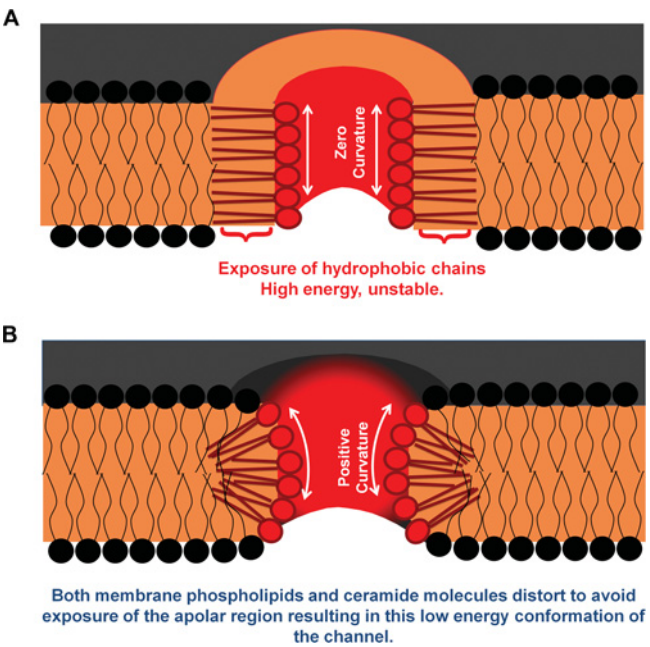

Figure S3 Cross-section of a ceramide channel in the membrane

Membrane phospholipids are shown in black, as is the surface of the membrane, and ceramide is shown in red. The hydrophobic region is shown in tan. (**A**) A ceramide channel is shown here in red as a cylindrical channel formed by columns of ceramide. The columns are held together by a network of hydrogen bonds. If the channel were to have a zero curvature (as shown), or a negative curvature, perpendicular to the plane of the membrane, this would lead to the exposure of the apolar region of ceramide. This is structure is energetically unfavourable and highly unstable in an aqueous environment. (**B**) Molecular modelling studies reveal that ceramide channels could form a positive curvature perpendicular to the plane of the membrane. This stable structure is formed by a distortion of both the membrane phospholipids and the ceramide molecules at the channel–membrane interface. This low-energy compromise effectively protects the hydrophobic region. Any factor that influence the channel between the two states illustrated in (**A**) and (**B**) can affect the stability of the channel and shift the dynamic equilibrium of ceramide to ceramide in the channel form or ceramide in the membrane.

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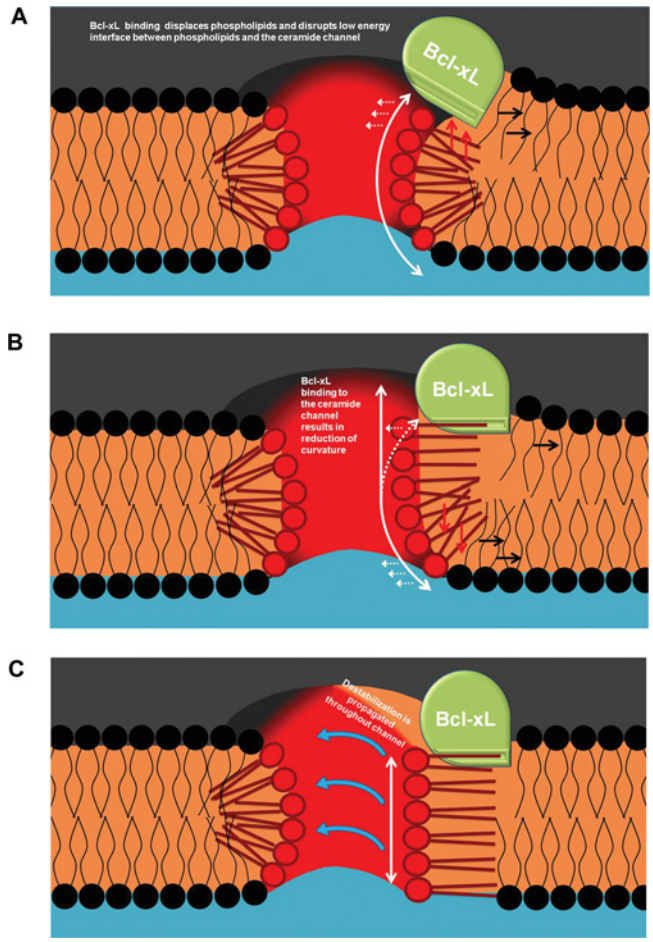

Figure S4 Model of Bcl-xL regulation of a ceramide channel

The membrane phospholipids and the surface of the membrane are shown in black, and ceramide molecules and the ceramide channel are shown in red. The hydrophobic region is shown in tan. (**A**) Bcl-xL binding to the membrane displaces the phospholipids that form a continuous surface with the ceramide channel and contribute to forming the curvature that stabilizes the ceramide channel. Results from the present study indicate that Bcl-xL interacts with the apolar region of the ceramide channel, which is embedded within the membrane, and this interaction involves the hydrophobic groove of Bcl-xL. (**B**) Bcl-xL may bind the apolar region of ceramide and influence positive curvature of the channel, possibly reducing this curvature to a zero or to a negative curvature as seen in (**C**). Reduction of this positive curvature at the site of Bcl-xL binding would result in mechanical stress on adjacent columns, leading to the local exposure of the hydrophobic region (as seen in tan). Ceramide channels are composed of ceramide columns held together by a vast network of hydrogen bonds. As Bcl-xL induces a local destabilizing conformational change, this mechanical stress is propagated throughout the channel via the hydrogen-bonded network (blue arrows), shifting the dynamic equilibrium of ceramide away from channel formation.

COLOUR

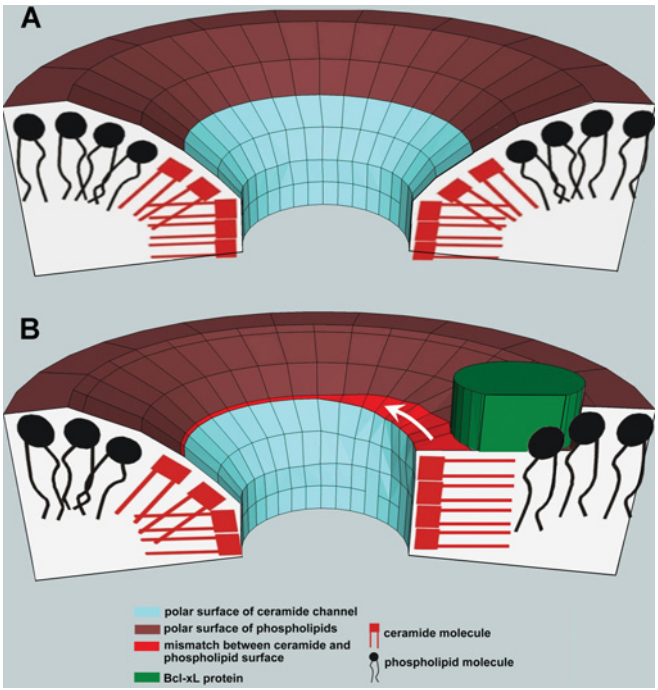

Figure S5 Model of the proposed influence of Bcl-xL binding on the stability of the ceramide channel

The model described in Figure S4 is illustrated differently here using a TrueSpace (Caligari) model. (**A**) The hydrogen-bonded network of ceramide molecules that compose the polar surface of the ceramide channel are shown in blue (also seen in Figure S3B). (**B**) The binding of Bcl-xL (green) to the aliphatic chain(s) of the ceramide at the end of a ceramide column in the channel should displace the phospholipids, allowing the column to relax into a conformation with reduced positive curvature. However, interaction with adjacent columns results in a structural mismatch illustrated as a red fracture area between the ceramide channel and the surrounding phospholipids. This should destabilize the adjacent columns leading to channel disassembly.

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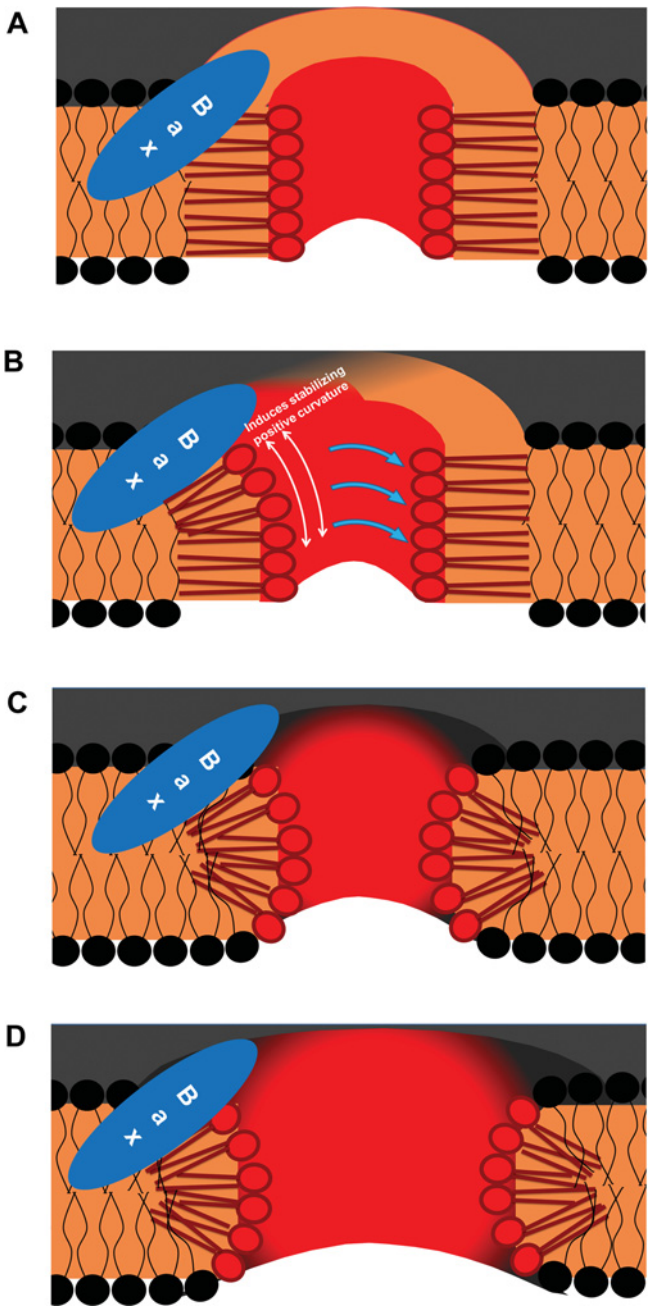

Figure S6 Model of activated Bax regulation of a ceramide channel

The membrane phospholipids and the surface of the membrane is shown in black, and ceramide molecules and the ceramide channel are shown in red. The hydrophobic region is shown in tan. (**A**) Evidence from the present study indicates that Bax appears to be interacting with the top of the ceramide channel, where there is access to the amide group, at the interface of the channel and the membrane. Bax may promote the stable conformation of the channel at this interface, as shown in (**B**), allowing the channel and the membrane to interface correctly. The stabilizing effect of Bax is propagated throughout the channel (blue arrows) via the hydrogen-bonded network of ceramide columns resulting in (**C**). Bax has also been shown to enhance ceramide channel formation and the stabilizing effect of Bax may also allow for the enlargement of ceramide channels to an optimal size as indicated in a study by Ganesan et al. [3].

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