Fatty acids enhance membrane permeabilization by pro-apoptotic Bax

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Fatty acids are known promoters of apoptosis. In the present study, the direct role of fatty acids with regard to their ability to cause membrane permeabilization by Bax was explored. Addition of fatty acids to liposomes in the presence of cations greatly enhanced the permeabilizing activity of Bax, a pro-apoptotic Bcl-2 protein. This provides a putative mechanism for the role of fatty acids in apoptosis. It is not a result of detergent-like properties of fatty acids, since a different micelle-forming amphiphile, dilyso-cardiolipin, was strongly inhibitory. We also demonstrate that there is a synergistic effect on Bax-induced permeabilization between Ca^{2+} and Mg^{2+} , both on the binding of Bax to liposomes as well as on the induction of the leakage of liposomal contents. Micromolar concentrations of Ca^{2+} added externally or submicro-

molar concentrations of free Ca^{2+} present in the medium were sufficient to promote Bax-induced permeabilization synergistically with externally added Mg^{2+} . These results indicate that Bax can induce leakage from liposomes at ion concentrations resembling those found physiologically. The synergistic effects of Ca^{2+} and Mg^{2+} were observed with liposomes with different lipid compositions. Thus the action of Bax is strongly modulated by the presence of bivalent cations that can act synergistically, as well as by micelle-forming lipid components that can be either stimulatory or inhibitory.

Key words: apoptosis, Bax, dilyso-cardiolipin, fatty acid, leakage.

INTRODUCTION

There is a fine balance between the factors that promote apoptosis or programmed cell death, and the factors that oppose this process. There are two sets of proteins, termed Bcl-2 proteins, with some commonality in their structural motif, that modulate this process. One set of proteins has pro-apoptotic functions, such as Bax, while another group is anti-apoptotic, such as Bcl-xL. A major step in the pro-apoptotic action of Bax is the release of proteins from the inter-mitochondrial space [1–3]. There have been many studies of Bax-induced leakage using model liposomal systems [1,4–6]. Although these model systems do not mimic all of the complexities of a biological system, they have been invaluable in elucidating the factors that modulate the interaction of Bax with membranes and the ability of this protein to induce leakage across bilayers. Such studies have contributed significantly to the development of a modified paradigm for the action of Bcl-2 proteins, in which lipids also play a central role [7].

A class of lipid amphiphiles that has been associated with apoptosis is fatty acids [8]. Apoptosis can result both from the delivery of non-esterified ('free') fatty acids derived from ingested lipids and by the action of lipases in the serum. In addition, non-esterified fatty acids are generated endogenously in the cell. Increased generation of fatty acids by the action of phospholipases occurs during apoptosis through the activation of several forms of phospholipase A_2 [9]. It is well established that saturated fatty acids promote apoptosis [10–16]. It had been suggested that one of the mechanisms by which palmitic acid promotes apoptosis is through stimulation of ceramide synthesis [10,13], but it has also been suggested that fatty acids have a role in apoptosis that is independent of ceramide [12,17,18]. Other suggested roles of saturated fatty acids include an increase in the flux of Ca^{2+}

across membranes [19], promotion of the formation of reactive oxygen species [16] and a decrease in the cardiolipin content of the mitochondria [18,20,21]. It has been shown recently that the apoptotic action of fatty acids is dependent on their metabolism, but not as a consequence of the production of ceramide or of reactive oxygen species [18]. One of the actions of palmitate is to lower the concentration of cardiolipin, and as a consequence cytochrome *c* is released into the cytosol to induce apoptosis [18]. The cardiolipin content decreases because of the accumulation of phosphatidylglycerol with saturated acyl chains that are poor substrates for cardiolipin synthesis [20]. A decrease in the cardiolipin content of the mitochondria would facilitate the release of cytochrome *c* that is known to be bound to this lipid. In the case of unsaturated fatty acids, both pro-apoptotic [22–24] and anti-apoptotic [10,11] effects have been observed. Clearly the action of unsaturated fatty acids is likely to involve other components, such as the formation of reactive oxygen species and/or eicosanoid signalling pathways [14]. In addition, oleate is metabolized preferentially to triacylglycerols (triglycerides), and it also promotes the incorporation of palmitic acid into triacylglycerols, decreasing the apoptotic action of saturated fatty acids [18]. In addition to the consequences of fatty acid metabolism, there is also evidence that Bcl-2 proteins are directly involved in the apoptotic action of fatty acids. This has been shown by the finding that overexpression of the anti-apoptotic Bcl-2 proteins Bcl-xL and Bcl-w decreases apoptosis [18]. The possibility of a direct role of fatty acids in the interaction of Bax with membranes has not been explored.

As a consequence of the activation of phospholipase A_2 -catalysed hydrolysis during apoptosis, lysophosphatidylcholine and fatty acids are produced. Both lysophosphatidylcholine [25] and non-esterified fatty acids have been shown to promote

Abbreviations used: Bax Δ C, human Bax- α lacking 20 amino acids at the C-terminus; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; PI, soybean phosphatidylinositol; DOPS, dioleoylphosphatidylserine; LUV, large unilamellar vesicle; ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; DPX, p-xylene-bis-pyridinium bromide; DNS-DHPE, N-dansyl-dihexadecanoylphosphatidylethanolamine.

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apoptosis. It has been suggested that lysophosphatidylcholine can promote the action of Bax by increasing positive membrane intrinsic curvature [4].

The degradation of phospholipids in the mitochondria is promoted by Ca^{2+} , a cation known to facilitate apoptosis [5,26–29]. In addition to the role of Ca^{2+} as a cofactor for phospholipases, this cation may also have other roles in apoptosis. There is evidence from liposomal studies that calcium enhances the perturbing effect of Bax on membranes [3,5,30]. However, the concentration of calcium used in previous *in vitro* assays was much higher than physiological levels. We demonstrate in the present work that Ca^{2+} and Mg^{2+} act synergistically, lowering the Ca^{2+} concentration required to promote membrane permeabilization to physiologically relevant levels. These cations are required for the enhancement of Bax-induced membrane leakage by fatty acids.

EXPERIMENTAL

Materials

Bax Δ C (human Bax- α lacking 20 amino acids at the C-terminus) was produced as described previously using an *Escherichia coli* expression system [31]. The bacteria were lysed with 1 % (v/v) Triton X-100, resulting in oligomerization of the protein. The detergent was removed during purification and the protein finally dialysed against 30 % (v/v) glycerol, 25 mM Hepes and 0.2 mM dithiothreitol. Bax Δ C remains oligomeric even though the detergent has been removed during purification. DNS-DHPE [*N*-dansyl-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt] was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other lipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Cardiolipin was a synthetic tetraoleoyl form. Monolysocardiolipin and dilyocardiolipin were prepared from bovine heart cardiolipin.

Preparation of LUVs (large unilamellar vesicles)

Liposomes of two different lipid compositions were used. One was a simple mixture of equimolar amounts of DOPC (dioleoylphosphatidylcholine), DOPE (dioleoylphosphatidylethanolamine) and cardiolipin. We have used liposomes of this composition previously, so that the present results can be compared directly with the earlier results. In addition, leakage from liposomes with this lipid composition is sensitive to the presence of Bax, so that modulation by other factors can be assessed accurately. We have also used liposomes composed of a lipid mixture resembling that of the mitochondrial membrane [41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI (soybean phosphatidylinositol), 8.9 mol % DOPS (dioleoylphosphatidylserine) and 12 mol % cardiolipin], similar to that reported previously [32]. Lipids were dissolved in chloroform/methanol (2:1, v/v) at the desired molar ratio. The lipid was deposited as a film on the wall of a glass test tube by solvent evaporation with nitrogen. Final traces of solvent were removed for 2–3 h in a vacuum chamber attached to a liquid nitrogen trap. The lipid films were suspended in the appropriate buffer by vortexing at room temperature to form multilamellar vesicles. The lipid suspensions were processed further with five cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA, U.S.A.) in a barrel extruder (Lipex Biomembranes, Vancouver, BC, Canada), at room temperature. LUVs were kept on ice and used within a short time after preparation.

Buffers

Buffer in the absence of bivalent cations was composed of 10 mM Hepes, 0.14 M NaCl and 0.1 mM EDTA, pH 7.4. For some experiments, Ca^{2+} and/or Mg^{2+} was added to this buffer at a concentration of 0.5 mM or greater. In addition, for buffers containing submicromolar concentrations of Ca^{2+} , the EDTA was replaced by mixtures of EGTA and Ca^{2+} , calculated to give a free Ca^{2+} concentration of 100 nM.

Liposomal ANTS (8-aminonaphthalene-1,3,6-trisulphonic acid)/DPX (p-xylene-bis-pyridinium bromide) leakage studies

Aqueous content leakage from liposomes was determined using the ANTS/DPX assay [33]. Lipid films were hydrated with 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl and 10 mM Hepes, pH 7.4. The osmolality of this solution was adjusted to be equal to that of the buffer (10 mM Hepes, 0.14 M NaCl and 0.1 mM EDTA, pH 7.4) as measured with a cryo-osmometer (Advanced Model 3M Oplus Micro-Osmometer; Advanced Instruments Inc., Norwood, MA, U.S.A.). LUVs of 0.1 μm diameter were prepared by extrusion as described above. After passage through a 2.5 cm $\times$ 20 cm column of Sephadex G-75, the void volume fractions were collected and the phospholipid concentration was determined by phosphate analysis. Fluorescence measurements were performed in 2 ml of buffer in a quartz cuvette equilibrated at 37 °C with stirring. Aliquots of LUVs were added to the cuvette to a final lipid concentration of 50 μM , and the fluorescence was recorded as a function of time using an excitation wavelength of 360 nm and an emission wavelength of 530 nm with 8 nm bandwidths. A 500 nm cut-off filter was placed in the emission path. Bax Δ C in buffer was added to the lipid vesicles in the cuvette and the fluorescence was recorded for 1–2 min. When the buffer did not contain Ca^{2+} , potentiation was initiated by external addition of several microlitres of solutions of cations in buffer. When an EGTA/calcium buffer [34] was used, the liposomes were prepared and maintained in this buffer, calculated to contain 100 nM free calcium. The rate of leakage was then measured before and after the addition of 1 mM Mg^{2+} to Bax Δ C. Controls for leakage caused by the bivalent cations in the absence of protein were also performed. At the end of the run, the value for 100 % release was obtained by adding 20 μl of a 10 % (v/v) Triton X-100 solution to the cuvette. Runs were done in duplicate and were in good agreement. Different batches of LUVs prepared on different days retained the same order of potentiation, but varied in absolute values.

Binding of Bax Δ C to membranes

A resonance energy transfer assay between the Trp residues of Bax Δ C and the dansyl group on DNS-DHPE was used to assess the translocation of the protein to a membrane. LUVs were prepared with 5 mol % DNS-DHPE added. The LUVs were diluted to a concentration of 50 μM in a quartz cuvette containing 2 ml of buffer. Vesicles were used immediately after extrusion. The buffer used was either 10 mM Hepes/0.14 M NaCl/0.1 mM EDTA, pH 7.4, or an EGTA/calcium buffer containing 10 mM Hepes and 0.14 M NaCl, pH 7.34, adjusted to contain 100 nM free calcium. The fluorescence was measured at 37 °C using an excitation wavelength of 280 nm. Polarizers were used to eliminate scattering. The emission spectra were recorded three times and averaged, both before and after the addition of 100 nM Bax Δ C, and again after the addition of aliquots of cation solution. The emission maximum was 497 nm. Some batch-to-batch variation in absolute values was observed; however, the

Figure 1 Enhancement of Bax Δ C-induced permeabilization by cation synergism

The rate of leakage of ANTS was measured from 50 μ M LUVs composed of DOPC/DOPE/cardiophilin (1:1:1, by mol) at pH 7.4. **(A)** In Hepes buffer, in the presence of 50 nM Bax Δ C. The protein was added at zero time for curves 5 and 6, and at 40 s for all others, except for curve 1, which had no protein. Cations were added together at approx. 120 s. Curve 1, LUVs with no addition of protein; curve 2, with 500 μ M Ca^{2+} ; curve 3, with 1 mM Mg^{2+} ; curve 4, with 2 mM Mg^{2+} ; curve 5, with 500 μ M Ca^{2+} and 1 mM Mg^{2+} ; curve 6, with 500 μ M Ca^{2+} and 2 mM Mg^{2+} . **(B)** Enhancement of membrane permeabilization by Bax Δ C induced by the synergistic effect of cations, using a Ca^{2+} /EGTA buffer. The lipid concentration was 50 μ M, and that of Bax Δ C was 100 nM. The composition of the LUVs was DOPC/DOPE/cardiophilin (1:1:1, by mol). Curve 1, control LUVs in 10 mM Hepes/0.14 M NaCl/0.1 mM EDTA buffer, pH 7.4, with 1 mM Mg^{2+} added at zero time in the absence of protein; curve 2, control with LUVs and protein in the same buffer; 1 mM Mg^{2+} was added at 100 s; curve 3, enhancement of permeabilization caused by Bax Δ C in a Ca^{2+} /EGTA/Hepes buffer calculated to contain 100 nM free Ca^{2+} ; 1 mM Mg^{2+} was added at 100 s.

relative orders of binding under different conditions were retained in all cases.

RESULTS

Synergistic actions of Ca^{2+} and Mg^{2+} on Bax-induced liposome permeabilization

Mg^{2+} lowered the concentration of Ca^{2+} required to produce significant leakage from liposomes in the presence of Bax Δ C, even at concentrations at which either cation alone had little effect (Figure 1A). This synergism between cations could also

Figure 2 Rate of permeabilization from LUVs approximating the lipid composition of the mitochondrial outer membrane

The lipid composition of the LUVs was 41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS and 12 mol % cardiophilin. **(A)** The rate of leakage of ANTS and DPX was measured at pH 7.4 in the presence of 1 mM Mg^{2+} , 500 μ M Ca^{2+} , 100 nM Bax Δ C and 50 μ M lipid. Mg^{2+} and Ca^{2+} were added at approx. 130 s. Curve 1, Blank with no protein added; curve 2, Bax Δ C alone; curve 3, 1 mM phosphate added. **(B)** The assays for curves 1 and 3 were carried out in an EGTA/ Ca^{2+} buffer (10 mM Hepes, 0.14 M NaCl, pH 7.4, containing 100 nM free Ca^{2+}) after the external addition of 1 mM Mg^{2+} to Bax Δ C. Curve 1, LUVs with no protein or added Mg^{2+} ; curve 2, with protein in a Hepes buffer not containing Ca^{2+} ; 1 mM Mg^{2+} was added after 100 s; curve 3, with protein and 1 mM Mg^{2+} added after 100 s, in buffer containing 100 nM free Ca^{2+} .

be observed in a Ca^{2+} /EGTA buffer calculated to contain 100 nM free Ca^{2+} ; thus submicromolar calcium concentrations still promoted significant permeabilization (Figure 1B). The lipid mixture used here was DOPC/DOPE/cardiophilin (1:1:1, by mol). Using liposomes composed of a lipid mixture resembling that of the mitochondrial membrane (41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS and 12 mol % cardiophilin [32]), we again showed that permeabilization of liposomes occurred with Bax in the presence of micromolar concentrations of Ca^{2+} upon external addition of 1 mM Mg^{2+} (Figure 2A); furthermore, when 100 nM free Ca^{2+} was present in a Ca^{2+} /EGTA buffer (which had no effect on its own), leakage was promoted by the external addition of 1 mM Mg^{2+} (Figure 2B). Although a synergistic effect of Ca^{2+} and Mg^{2+} could still be observed with these liposomes, the extent of ANTS release was much lower than

Figure 3 Cation synergism in the binding of Bax Δ C to liposomes of DOPC/DOPE/cardiophilin

The results indicate the increase in the intensity of emission from DNS-DHPE over that of the liposomes in the absence of protein (arbitrary units). The liposome concentration was 50 μ M; that of Bax Δ C (present in all cases shown) was 100 nM. The increase in intensity is shown as a function of (A) the Ca^{2+} concentration, (B) the Mg^{2+} concentration, and (C) the Mg^{2+} concentration in the presence of 500 μ M Ca^{2+} . Bars labelled 'Bax' do not contain added bivalent cation.

that with liposomes of a simpler lipid composition. We found that phosphate promoted permeabilization further, allowing it to be observed at low concentrations of Ca^{2+} with vesicles composed of lipid mimicking the mitochondrial outer membrane (Figure 2A).

Studies of Bax binding to membranes

The binding of Bax Δ C to liposomes of DOPC/DOPE/cardiophilin (1:1:1, by mol) and/or liposomes with a composition of 41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS and 12 mol % cardiophilin was measured by monitoring the increased fluorescence of DNS-DHPE as a result of resonance energy transfer from the Trp residues in Bax Δ C. There was a synergistic effect between Ca^{2+} and Mg^{2+} when they were introduced externally together (Figure 3C); Mg^{2+} alone had a smaller effect on binding in the absence of Ca^{2+} (Figure 3B), and similarly for Ca^{2+} in the absence of Mg^{2+} (Figure 3A). We also demonstrated this synergism at ion concentrations found physiologically in the cytosol. We achieved this by carrying out binding assays in a Ca^{2+} /EGTA buffer containing 100 nM free Ca^{2+} in 10 mM Hepes and 0.14 M NaCl, pH 7.4, followed by the external addition of 1 mM Mg^{2+} (Figure 4, bars 1 and 3),

Figure 4 Cation synergism in the binding of Bax Δ C in a Ca^{2+} /EGTA buffer containing 100 nM free Ca^{2+}

The results indicate the increase in the intensity of emission from DNS-DHPE in the presence of Bax Δ C over that of the LUVs of DOPC/DOPE/cardiophilin (1:1:1, by mol) in the absence of protein (arbitrary units). The liposome concentration was 50 μ M; that of Bax Δ C (present in all cases shown) was 100 nM. Bars 1 and 3: the assay was carried out in a Ca^{2+} /EGTA buffer in 10 mM Hepes, 0.14 M NaCl, pH 7.4, containing 100 nM free Ca^{2+} , as described in the text, before (bar 1) and after (bar 3) adding 1 mM Mg^{2+} to the protein. Bars 2 and 4: the assay was carried out in 10 mM Hepes buffer, pH 7.4, in the absence of Ca^{2+} , before (bar 2) and after (bar 4) the addition of 1 mM Mg^{2+} to the protein.

compared with assays carried out in Hepes buffer, pH 7.4, in the absence of Ca^{2+} (Figure 4, bars 2 and 4).

Fatty acid promotion of Bax-induced leakage

The Bax-promoted permeabilization in LUVs of DOPC/DOPE/cardiophilin (1:1:1, by mol) in the presence of 500 μ M Ca^{2+} and 1 mM Mg^{2+} was greatly enhanced by the presence of palmitic acid or oleic acid (Figure 5A). We also showed that either palmitic acid or oleic acid promoted permeabilization induced by Bax in a Ca^{2+} /EGTA buffer containing 100 nM free calcium after external addition of 1 mM Mg^{2+} (Figure 5B). Enhancement of Bax-induced leakage by fatty acids does not seem to be sensitive to the lipid composition of the liposomes, also occurring with liposomes mimicking the lipid composition of the mitochondrial outer membrane (Figure 5C).

Lysolipid inhibition of Bax-induced leakage

When fatty acids are generated by the cleavage of phosphatidylcholine by phospholipase A_2 , the other lipid product is lysophosphatidylcholine. We tested the action of this lipid product on Bax-induced leakage as well as on the promotion of ANTIS release by palmitic acid (Figure 5A). Lysophosphatidylcholine alone had little effect or was slightly inhibitory in this system. At higher concentrations, lysophosphatidylcholine enhanced the action of Bax [4]. However, lysophosphatidylcholine was potent in preventing the increased permeabilization caused by the presence of palmitic acid in LUVs (Figure 5A). Controls in the absence of Bax demonstrated that a combination of lysophosphatidylcholine and palmitic acid induced only a low rate of leakage. The lysolipid dilysocardiolipin was very potent in inhibiting Bax-induced leakage, in both the presence and the absence of oleic acid (Figure 5A).

Because of the potent effect of dilysocardiolipin in inhibiting the action of Bax, both monolysocardiolipin and dilysocardiolipin were tested further using different systems. Mono- and di-lysocardiolipin are formed in the mitochondria from cardiophilin. Membrane permeabilization caused directly by mono- and di-lysocardiolipin in liposomes with a lipid composition simulating that of the mitochondrial outer membrane was measured in the presence of Bax Δ C. Two protocols were used for adding

Figure 5 Permeabilization measured at 150 s from liposomes containing 10 mol % fatty acid

The lipid concentration was 50 μ M, and that of Bax Δ C was 100 nM. Error bars correspond to an average of two determinations with the same batch of liposomes. Abbreviations: PA, palmitic acid; OA, oleic acid; LPC, lysophosphatidylcholine; DLCL, dilyscardiophilin. (A) Liposomes of DOPC/DOPE/cardiophilin containing fatty acid; 1 mM Mg²⁺ and 500 μ M Ca²⁺ were added together to Bax Δ C in HEPES buffer, pH 7.4, at approx. 120 s, and the rate of leakage of ANTS was followed for 150 s. (B) Liposomes of DOPC/DOPE/cardiophilin containing fatty acid, as indicated. A Ca²⁺/EGTA buffer containing 100 nM free Ca²⁺ was placed in the cuvette; 1 mM Mg²⁺ was added to Bax Δ C after 110 s only for the last three bars. (C) Liposomes composed of 41.8 mol % DOPC, 28.4 mol % DOPE, 8.9 mol % PI, 8.9 mol % DOPS and 12 mol % cardiophilin, containing 10 mol % fatty acid. A mixture of 1 mM Mg²⁺ and 500 μ M Ca²⁺ was added to Bax Δ C after 150 s, and the rate of leakage of ANTS was followed for 150 s.

the lysolipids; either they were incorporated as part of the lipid film or they were added in buffer solution, just prior to the addition of Bax Δ C. Qualitatively similar results were obtained whether the lysocardiophilin was added into the lipid film or in solution. Dilyscardiophilin was found to be more potent in inhibiting the action of Bax (Figure 6). Subsequently, the same experiment was carried out with these amphiphiles and t-Bid. This Bcl-2 protein was much less efficient than Bax in inducing leakage, and a low rate of ANTS release was measurable. We were able to demonstrate that monolysocardiophilin, when added externally, stimulated leakage (Figure 6D), in agreement with the findings of Esposti et al. [21]. However, dilyscardiophilin, when incorporated into the lipid film, inhibited the action of t-Bid, as it did with Bax Δ C (Figure 6D). It should be noted that even though the measurements with t-Bid are presented with a scale

Figure 6 Lysocardiophilins and the induction of ANTS release by Bax

Leakage was measured using a Ca²⁺/EGTA buffer containing 100 nM free Ca²⁺, with addition of 1 mM Mg²⁺ where indicated. Concentrations were 50 μ M LUVs and 100 nM Bax Δ C or 80 nM t-Bid. (A) Curve 1, LUVs of DOPC/DOPE/cardiophilin (1:1:1, by mol); Bax Δ C added at zero time; Mg²⁺ added at 115 s; curve 2, same as for curve 1, with 10 mol % monolysocardiophilin incorporated into the lipid film; curve 3, same as curve 1, with 10 mol % dilyscardiophilin incorporated into the lipid film. (B) Curves 1, 2 and 3: same as (A), except the lysocardiophilins in curves 2 and 3 were added externally to the LUVs from a buffer solution at a concentration of 5 μ M (10 mol % total lipid), together with Bax Δ C at zero time. The final bulk concentrations of lysocardiophilins in the cuvette are the same for all three panels. (C) Curves 1, 2 and 3: same as (A), except the lipid composition of the LUVs corresponded to that of the outer membrane of the mitochondria. (D) LUVs (50 μ M) of a lipid composition corresponding to that of the mitochondrial outer membrane, with 80 nM t-Bid added at zero time. Note a 10-fold decrease in scale. Curve 1, monolysocardiophilin added externally from a buffer solution at a concentration of 5 μ M, together with t-Bid at zero time; curve 2, dilyscardiophilin added externally from a buffer solution at a concentration of 5 μ M, together with t-Bid at zero time; curve 3, no lysolipid addition; curve 4, 10 mol % monolysocardiophilin incorporated into the lipid film; curve 5, 10 mol % dilyscardiophilin incorporated into the lipid film.

10 times smaller, the extent of permeabilization found was reproducible.

Fatty acids increase the binding of Bax to membranes

Fatty acids increased the binding of Bax Δ C to liposomes of DOPC/DOPE/cardiophilin (1:1:1, by mol) in the presence of bivalent cations, in comparison with that found for similar liposomes in the absence of added amphiphiles (Figure 7). There was a small increase in the binding of Bax Δ C in the presence of palmitic acid and Mg²⁺ alone, in the absence of Ca²⁺ (results not shown).

Lysocardiophilins modulate the binding of Bax Δ C to membranes

Bax Δ C binding to membranes was also evaluated both by incorporating the lysocardiophilins into the lipid film and by adding them from buffer solutions. The strongest inhibition of binding of Bax Δ C occurred with dilyscardiophilin added in solution

Figure 7 Binding of Bax Δ C to liposomes of DOPC/DOPE/cardiophilin (bars 1 and 3) and with 10 mol% palmitic acid (bars 2 and 5) or oleic acid (bars 3 and 6)

The liposome concentration was 50 μ M; 100 nM Bax Δ C was present in all cases shown. Results indicate the increase in the intensity of emission from DNS-DHPE over that of the liposomes in the absence of protein (arbitrary units). Experiments were carried out in a Ca^{2+} /EGTA buffer in 10 mM HEPES/0.14 M NaCl, pH 7.4, containing 100 nM free Ca^{2+} . Bars 1–3, buffer only; bars 4–6, in the presence of externally added 1 mM Mg^{2+} .

Figure 8 Binding of Bax Δ C (A) or t-Bid (B) to LUVs with a composition simulating the mitochondrial outer membrane

Experiments were carried out in a Ca^{2+} /EGTA buffer in 10 mM HEPES/0.14 M NaCl, pH 7.4, containing 100 nM free Ca^{2+} . (A) Binding to liposomes with the addition of 10 mol% monolysocardiophilin or dilysoardiophilin; 100 nM Bax Δ C was present in all cases. Bars 1 and 6, LUVs with Bax Δ C; bars 2 and 7, LUVs with 10 mol% monolysocardiophilin incorporated into the lipid film; bars 3 and 8, LUVs with 10 mol% dilysoardiophilin incorporated into the lipid film; bars 4 and 9, 10 mol% monolysocardiophilin added from a buffer solution at the same time as Bax Δ C; bars 5 and 10, 10 mol% dilysoardiophilin added from a buffer solution together with Bax Δ C, at zero time; bars 1–5, buffer only; bars 6–10, in the presence of externally added 1 mM Mg^{2+} . (B) Binding of externally added monolysocardiophilin and dilysoardiophilin; 80 nM t-Bid was present in all cases. Bars 1 and 4, LUVs with t-Bid; bars 2 and 5, 10 mol% monolysocardiophilin added from a buffer solution together with t-Bid at zero time; bars 3 and 6, 10 mol% dilysoardiophilin added from a buffer solution together with t-Bid at zero time; bars 1–3, buffer only; bars 4–6, in the presence of externally added 1 mM Mg^{2+} .

(Figure 8A). Surprisingly, in the case of t-Bid, both mono- and di-lysocardiophilin increased its binding to liposomes (Figure 8B). Enhancement of the binding of t-Bid to liposomes and to mitochondria was also observed by Esposti et al. [21].

DISCUSSION

Although Bax is normally present in the cytosol of cells, it does not translocate efficiently to mitochondria and initiate apoptosis unless there is a triggering stimulus. There are a variety of mechanisms and modulators by which Bax is activated. Ca^{2+} is one of the many factors involved in promoting the apoptotic action of Bax [26–29,35,36]. We have also shown that Ca^{2+} sensitizes the action of Bax in model liposomal systems [5]. However, in this earlier work, potentiation of the action of Bax was observed only at Ca^{2+} concentrations above 3 mM. We have considered experimental factors that may sensitize a system to Ca^{2+} . In the present paper, we show that submicromolar levels of calcium facilitated Bax-promoted membrane leakage when millimolar concentrations of Mg^{2+} were also present (Figures 1B and 2B). We suggest that the basis of the effect of Mg^{2+} is electrostatic. This has two consequences. One is that it lowers electrostatic repulsion to facilitate the binding of anionic Bax (pI 5.1) to anionic membranes. In addition, there are effects of the cations on the material properties of the membrane interface. Both Mg^{2+} and Ca^{2+} are known to affect cardiolipin in model systems [37] as well as in intact mitochondria [38]. Mg^{2+} is also known to promote the release of cytochrome *c* from mitochondria [2]. It is known that Mg^{2+} sensitizes phosphatidylserine liposomes to fusion promoted by Ca^{2+} [39]. By analogy with the effects of Ca^{2+} and Mg^{2+} on membrane fusion, there is a synergistic effect of these two cations on the interfacial properties of the membrane, with Mg^{2+} acting largely to diminish electrostatic repulsion between adjacent lipids, allowing Ca^{2+} to be active at a lower concentration.

We have demonstrated directly that the binding of Bax Δ C to membranes is promoted by Ca^{2+} alone (Figure 3), and much lower Ca^{2+} concentrations are required when Mg^{2+} is also present. At very low concentrations of Ca^{2+} it is important that the free Ca^{2+} concentration is buffered in order to maintain a constant concentration of unbound Ca^{2+} . It is necessary to provide a reserve of Ca^{2+} for this purpose, since some Ca^{2+} will bind to the anionic lipids that are present. Using calcium buffers in the presence of Mg^{2+} , we have been able to bring the required concentrations of bivalent cations to physiological levels, adding meaning to the relevance of Ca^{2+} in the physiological action of Bax.

Phosphate ions have been observed to enhance the action of Ca^{2+} in promoting apoptosis [26,40]. We found that phosphate further promoted permeabilization, allowing it to be observed at low concentrations of Ca^{2+} with vesicles composed of lipid mimicking the mitochondrial outer membrane (Figure 2A). The ion concentrations used in this assay were not far from those that would be encountered physiologically. The increased activity in the presence of phosphate may be a consequence of the formation of complexes between calcium and phosphate that have less solubility in water and show greater partitioning into membranes.

We have established an *in vitro* model system that more closely resembles physiological conditions with regard to the concentration of cations required for Bax-induced membrane permeabilization. It has allowed us to lower the concentration of Ca^{2+} required to promote Bax action by orders of magnitude compared with that used in our earlier studies. Cytosolic Ca^{2+} levels rise during apoptosis, and there is transfer of Ca^{2+} from the endoplasmic reticulum to the mitochondria [28,29,35,36,41]. Even in the absence of apoptosis, Ca^{2+} is found at higher concentrations in the mitochondria than in the cytosol [42]. The intracellular level of total magnesium is estimated to be 6.2 mM, but the free Mg^{2+} concentration is only 0.37 mM [43]. The concentration of phosphate in the cytosol is approx. 5.3 mM and is present in various forms [44]. There is thus some quantitative

uncertainty as to how accurately the free ion concentrations correspond to physiological levels, but the amounts used in the present study are not physiologically unreasonable, and we can apply this system with more confidence to study the role of other modulators of apoptosis.

There is considerable evidence to indicate that fatty acids promote apoptosis. This may occur by several alternative pathways, depending on the nature of the fatty acid chain, the cell type and the concentrations of other metabolites. The results of the present study demonstrate directly that fatty acids dramatically enhance the action of Bax in increasing membrane permeabilization when cations are present. One reason why fatty acids increase the leakage caused by Bax is that they enhance the binding of this apoptotic protein to membranes (Figure 7). Binding is increased by bivalent cations, but, at least for palmitic acid, Mg^{2+} in the absence of Ca^{2+} also promotes considerable binding of the protein.

When fatty acids are produced by the action of phospholipase A_2 , another product that is formed is lysophosphatidylcholine. Both fatty acids and lysophosphatidylcholine are micelle-forming lipids, and hence promote positive membrane curvature. It has been suggested that this physical property correlates with an increased activity of Bax [4], although we saw little effect of lysophosphatidylcholine in the specific lipid systems and at the concentrations employed in the present studies. When fatty acids and lysophosphatidylcholine are combined, they form bilayers [45], rather than structures with overall positive curvature. Possibly as a consequence of the loss of the detergent-like properties, this combination of lipids also does not promote Bax-induced leakage.

Another lysolipid that is found in mitochondria, both as an intermediate in cardiolipin synthesis and as a product of cardiolipin degradation, is monolysocardiolipin. In addition, a small amount of dilylcardiolipin is found in the mitochondria [21]. The monolysocardiolipin is reacylated, largely with linoleoyl chains, resulting in a high content of linoleoyl chains in cardiolipin [46]. Monolysocardiolipin has recently been suggested to augment the action of t-Bid and to increase cytochrome *c* release from mitochondria [21]. Little is known about the physical properties of lysocardiolipin and its mixtures with other lipids. From their chemical structure, one would expect them to have properties similar to those of lysophosphatidylcholine, especially in the case of dilylcardiolipin. However, unlike lysophosphatidylcholine, dilylcardiolipin was very potent in inhibiting Bax-induced liposomal permeabilization, in either the presence or the absence of fatty acids (Figure 5). Dilylcardiolipin also inhibited the binding of Bax to membranes (Figure 8A). It has been shown recently that another pro-apoptotic Bcl-2 protein, t-Bid, binds to monolysocardiolipin [21]. It was suggested that this lipid promotes apoptosis by facilitating the action of t-Bid in inducing the release of proteins from the intermitochondrial space [21]. The pro-apoptotic action of monolysocardiolipin can explain the apparent contradiction that cardiolipin promotes the action of Bcl-2 proteins on mitochondria [3,7,32,47–50], but at the same time the lipid composition of mitochondria is depleted of cardiolipin during apoptosis promoted by a variety of stimuli [49,51–53]. It was suggested that the breakdown of cardiolipin during apoptosis results in the formation of monolysocardiolipin, which promotes apoptosis [21]. When considering the actions of Bax, our results are not in agreement with this suggestion. In palmitate-induced apoptosis, it is primarily the build-up of dipalmitoylphosphatidylglycerol, a poor substrate for cardiolipin synthase, that results in decreased synthesis of cardiolipin [20], rather than an increase in the rate of degradation of cardiolipin. The replacement of cardiolipin with phosphatidylglycerol would

be expected to decrease the sensitivity of mitochondria to Bax [5] and to t-Bid [54]. Phosphatidylglycerol has also been found to protect against apoptosis [55]. Thus the mechanism by which fatty acids promote apoptosis is not likely to be explained by the build-up of monolysocardiolipin. Furthermore, we found that dilylcardiolipin inhibited Bax-induced leakage. We suggest that the loss of cardiolipin that does occur during apoptosis is a later event, and is not sufficient to halt the process that is initiated, in the case of fatty acid-induced apoptosis, by sensitizing the permeabilization of the outer membrane of mitochondria to Bax.

In summary, the present study demonstrates that Bax-induced membrane leakage is enhanced by bivalent cations within a concentration range found physiologically. Certain lipids also modulate the efficiency of this process. Lipids that are normally present at low concentrations in the membrane may modulate apoptosis when their concentrations are increased. The effects of these lipids on Bax are not predicted by their bulk physical properties, as has been found for the modulation of the action of t-Bid by membrane curvature [54]. In the present work we have shown that fatty acids have a direct effect on the ability of Bax to promote membrane permeabilization.

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