

Bax-type Apoptotic Proteins Porate Pure Lipid Bilayers through a Mechanism Sensitive to Intrinsic Monolayer Curvature*

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Gorka Basañez‡§, Juanita C. Sharpe¶, Jennifer Galanis‡¶, Teresa B. Brandt**,
J. Marie Hardwick**‡§§, and Joshua Zimmerberg‡¶¶

From the ‡Laboratory of Cellular and Molecular Biophysics, NICHD, the ¶Surgical Neurology Branch, NINDS, and the General Institute of Medical Sciences, National Institutes of Health, Bethesda, Maryland 20892 and the Departments of **Molecular Microbiology and Immunology, ‡‡Pharmacology and Molecular Sciences, and §§Neurology, John Hopkins Schools of Public Health and Medicine, Baltimore, Maryland 21205

During apoptosis, Bax-type proteins permeabilize the outer mitochondrial membrane to release intermembrane apoptogenic factors into the cytosol via a poorly understood mechanism. We have proposed that Bax and $\Delta N76Bcl-x_L$ (the Bax-like cleavage fragment of $Bcl-x_L$) function by forming pores that are at least partially composed of lipids (lipidic pore formation). Since the membrane monolayer must bend during lipidic pore formation, we here explore the effect of intrinsic membrane monolayer curvature on pore formation. Nonlamellar lipids with positive intrinsic curvature such as lysophospholipids promoted membrane permeabilization, whereas nonlamellar lipids with negative intrinsic curvature such as diacylglycerol and phosphatidylethanolamine inhibited membrane permeabilization. The differential effects of nonlamellar lipids on membrane permeabilization were not correlated with lipid-induced changes in membrane binding or insertion of Bax or $\Delta N76Bcl-x_L$. Altogether, these results are consistent with a model whereby Bax-type proteins change the bending propensity of the membrane to form pores comprised at least in part of lipids in a structure of net positive monolayer curvature.

While a variety of triggers initiate apoptosis, many signaling pathways converge at the mitochondria to release cytochrome *c* and other apoptogenic proteins needed to start a cascade that ultimately leads to cellular demise (1). During apoptosis, Bax-type proteins localizing to the outer mitochondrial membrane elicit the release of cytochrome *c* and other intermembrane proteins that promote cell death (2). However, the underlying mechanism(s) of this release are yet to be fully elucidated (1–4). Bax shows structural similarities with pore-forming bacterial toxins (5), suggesting that Bax also functions as a pore former. However, when incorporated into purely lipidic planar phospholipid bilayer membranes, Bax (and homologous pro-

apoptotic molecules) induce highly fluctuating changes in membrane conductance and decrease membrane stability (6–8). Since there are no constant, unitary conductance steps in these recordings, they do not resemble recordings from typical ionic channels where the conduit from one side of the membrane to the other is exclusively lined by protein. Instead, these results reveal striking similarities to lipidic pores, in which the pore is lined by lipid headgroups (9, 10).

We proposed that Bax-type molecules alter the properties of the lipid bilayer so that lipids are able to rearrange and form a lipidic pore (6, 8, 11). A similar mechanism of action has been suggested for a number of viral peptides and bacterial toxins (12–17). One test of the hypothesis that pores formed by Bax-type proteins are at least partially lipidic is the prediction that lipidic pores by their nature should depend more strongly upon the lipid composition of the membrane than purely proteinaceous pores. Lipid pore formation is thought to involve a bending of the lipid monolayer of the membrane (9). The energy for monolayer bending is a function of the intrinsic curvature of its lipid constituents (18). Roughly, the relation between the effective cross-sectional areas of hydrophilic and hydrophobic parts of a lipid molecule determines its intrinsic curvature (19). Major lipid components of biological membranes including phosphatidylcholine (PC)¹ and phosphatidylglycerol (PG) hold similar cross-sectional areas in hydrophilic and hydrophobic parts and thereby organize themselves into flat lamellar structures (lamellar lipids). On the other hand, certain naturally occurring lipid species display an imbalance between the effective cross-sectional areas of hydrophilic and hydrophobic parts; such lipids spontaneously self-organize into curved morphologies (nonlamellar lipids). Nonlamellar lipids with a large effective hydrophilic part relative to the effective hydrophobic part, exemplified by lysophosphatidylcholine (LPC), possess positive intrinsic curvature, whereas nonlamellar lipids with a small effective hydrophilic part relative to the effective hydrophobic part such as unsaturated species of phosphatidylethanolamine (PE) and diacylglycerol possess negative intrinsic curvature (20). To test if the pore formed by Bax-type proteins is sensitive to the tendency of the membrane monolayer for bending, nonlamellar lipids were incorporated into phospholipid bilayer membranes, and then the pore-forming activity of proapoptotic

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§ Present address: Unidad de Biofísica (Centro Superior de Investigaciones Científicas-Universidad del País Vasco), and Departamento de Bioquímica, Universidad del País Vasco, Apartado 644, 48080-Bilbao, Spain

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¶¶ To whom correspondence should be addressed: Laboratory of Cellular and Molecular Biophysics, NICHD, National Institutes of Health, 10 Center Dr., Bethesda, MD 20892-1855. Tel.: 301-496-6571; Fax: 301-594-0813; E-mail: joshz@helix.nih.gov.

¹ The abbreviations used are: PC, phosphatidylcholine; PG, phosphatidylglycerol; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; DPX, *p*-xylenebis pyridinium bromide; LUV, large unilamellar vesicles; DOPC, dioleoylphosphatidylcholine; O-LPC, oleoyl-lysophosphatidylcholine; O-LPE, oleoyl-lysophosphatidylethanolamine; DOG, dioleoylglycerol; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; CL, cardiolipin.

proteins was measured. Our results show that lipids of positive intrinsic curvature favored membrane permeabilization, whereas lipids of negative intrinsic curvature inhibited membrane permeabilization, in agreement with the idea that Bax-type proteins form pores together with lipid molecules.

EXPERIMENTAL PROCEDURES

Materials—All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). KCl, Hepes, EDTA, Triton X-100, and horse heart cytochrome *c* were from Sigma. 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) and *p*-xylenebis(piridinium bromide) (DPX) were from Molecular Probes (Eugene, OR).

Protein Expression and Purification—Full-length Bax was produced in *Escherichia coli* as a chitin fusion-binding protein using the pTYB1 plasmid and was purified by affinity chromatography followed by ion-exchange chromatography (5). The peak protein fraction was concentrated, aliquoted, and stored at -80°C . Bax oligomerization was induced by a 1-h incubation of the protein with 1% octylglucoside (21). Control experiments showed that, at the concentrations used in the liposome assays, octylglucoside alone did not significantly increase membrane permeability. Oligomeric full-length Bcl-x_L and oligomeric $\Delta\text{N76Bcl-x}_L$ were obtained as described previously (8).

Liposome Preparation—In all cases, lipid mixtures at indicated ratios were co-dissolved in chloroform/methanol (2:1). Organic solvents were removed by evaporation under an argon stream, followed by incubation for 2 h in a vacuum. The buffers used for resuspension of dry lipid films were as follows, (i) ANTS release assays: 12.5 mM ANTS, 45 mM DPX, 20 mM KCl, 0.1 mM EDTA, 10 mM Hepes pH 7.0; (ii) cytochrome *c* release assays: 100 mM KCl, 0.1 mM EDTA, 10 mM Hepes, pH 7.0, 0.01 mM cytochrome *c*; (iii) membrane-binding and membrane-insertion assays: 100 mM KCl, 0.1 mM EDTA, 10 mM Hepes, pH 7.0, in D₂O (D₂O-buffer). Large unilamellar vesicles (LUV) were formed by extrusion under N₂ pressure through Nucleopore polycarbonate membranes of 0.1- μm pore size using the method of Mayer *et al.* (22). Untrapped ANTS and cytochrome *c* were removed by gel filtration in Sephadex G-25 and Sephacryl S-400 HR columns, respectively. Dynamic light scattering measurements (Coulter Model N4 Plus) showed that the different LUV populations used in this study had unimodal size distributions with mean diameters (\pm S.D.) ranging from 124 ± 27 nm to $142 \text{ nm} \pm 33$ nm.

Release of LUV-entrapped Markers—Release of ANTS from LUV was monitored in an SLM-2 Aminco-Bowman luminescence spectrometer (Spectronic Instruments), in a thermostatted 1-cm path length cuvette with constant stirring, at 37°C . λ_{ex} was 350 nm, and λ_{em} was 520 nm (slits = 4 nm). The extent of ANTS release was quantified on a percentage basis according to the equation

$$\% \text{ ANTS release} = (F_f - F_0/F_{100} - F_0) \times 100 \quad (\text{Eq. 1})$$

where F_f is the measured fluorescence of protein-treated LUV after a fully stable signal (plateau) was achieved, F_0 is the initial fluorescence of the LUV suspension before protein addition, and F_{100} is the fluorescence value after complete disruption of LUV by addition of TX-100 (final concentration, 0.2% w/v). Release of cytochrome *c* from LUV was measured as described previously (8). Unless otherwise stated, protein and lipid concentrations were 50 nM and 50 μM , respectively.

Assays of Protein Binding to and Insertion into the Membrane—To measure the total amount of protein bound to membrane, a method was used based on the fact that lipid-associated protein, but not free protein, floats in D₂O-buffer (23). Briefly, LUV in D₂O-buffer were incubated with protein for 30 min, followed by ultracentrifugation of the mixture (2 h, $100,000 \times g$) and determination of the protein contents in lipid-associated and lipid-free fractions on the basis of their fluorescence intensities at λ_{ex} 280 nm and λ_{em} 345 nm after addition of the detergent dodecyl octaethyleneglycol mono ether (final concentration, 300 μM). To discriminate between protein inserted into and adsorbed to the membrane, the same protocol was followed except that a second incubation was performed at pH 11.5 for 30 min, the alkaline pH being maintained during sample centrifugation. Upon alkaline pH incubation, the fraction of protein inserted to some degree into the membrane hydrophobic matrix remains associated to LUV, whereas the fraction of protein only peripherally associated with the membrane is detached from the vesicles.

Planar Lipid Bilayer Membrane Assays—Membrane lifetime experiments were done in decane-containing planar lipid bilayers formed by the Mueller-Rudin technique across a 550- μm hole in a lucite chamber (Warner Inst., Hamden, CO). Prior to voltage application across the

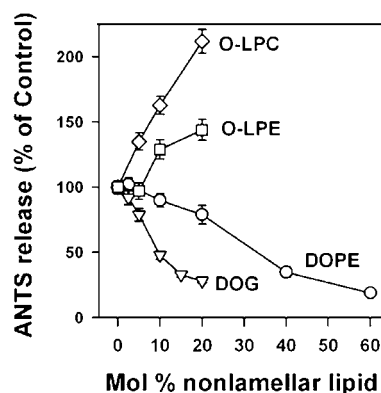


FIG. 1. Effect of membrane-incorporated nonlamellar lipids on Bax-induced vesicular ANTS release. LUV containing aqueous ANTS were prepared of DOPC/dioleoylphosphatidylglycerol (DOPG) (60:40 mole ratio) (Control LUV) and in which the lamellar lipid DOPC was replaced by increasing proportions of O-LPC (diamonds), O-LPE (squares), DOPE (circles), and DOG (triangles). The value of 100% ANTS release corresponds to that induced by Bax in control LUV. Mean \pm S.E. of four to seven independent experiments are depicted.

TABLE I

Dose dependence of Bax-elicited ANTS release in LUV of different lipid compositions

Values given as mean \pm S.E. of three to five independent experiments. Lipid concentration was 30 μM .

Lipid composition (mol/mol)	Bax		
	15	45	150
	% ANTS release		
	nM	nM	nM
DOPC/DOPG (6/4)	8 \pm 0	26 \pm 1	62 \pm 4
DOPC/DOPG/O-LPC (5/4/1)	14 \pm 1	47 \pm 4	91 \pm 6
DOPC/DOPG/DOG (5/4/1)	2 \pm 0	12 \pm 2	27 \pm 3
DOPC/CL (6/4)	9 \pm 1	22 \pm 2	67 \pm 5
DOPC/CL/O-LPC (5/4/1)	16 \pm 2	39 \pm 3	88 \pm 7
DOPC/CL/DOG (5/4/1)	7 \pm 0	16 \pm 2	43 \pm 4

membrane, purified proteins were added to the aqueous subphase, and the solution was stirred for 5 min to ensure good mixing. A software program (BROWSE, available upon request) was modified to apply voltage pulses and facilitate membrane lifetime measurements.

RESULTS

Nonlamellar Lipids Affect Liposome Permeabilization Induced by Bax-type Proapoptotic Proteins—If the pore formed by Bax-type proteins is even partially lipidic, its properties should be affected by the lipid composition of the membrane and thus, by the physicochemical properties of the membrane. Classically, the membrane monolayer bends during lipidic pore formation (9). A key determinant of the propensity of the membrane monolayer to bend is the lipid intrinsic curvature. To test whether lipid intrinsic curvature makes an important contribution to pore formation by proapoptotic proteins, nonlamellar lipids with known intrinsic curvatures were chosen. Specifically, LUV were prepared in which the lamellar lipid dioleoylphosphatidylcholine (DOPC) was gradually replaced by the nonlamellar lipids of positive intrinsic curvature oleoyllysophosphatidylcholine (O-LPC) and oleoyllysophosphatidylethanolamine (O-LPE) (24) or by the nonlamellar lipids of negative intrinsic curvature dioleoylglycerol (DOG) (25, 26) and dioleoylphosphatidylethanolamine (DOPE) (24, 25). Then, the ability of Bax to release the fluorescent probe ANTS encapsulated within those vesicles was studied. The larger the proportion of O-LPC and O-LPE incorporated in the membrane the higher the extent of vesicular ANTS released by Bax (Fig. 1). Conversely, the larger the proportion either of membrane-in-

TABLE II

Dose dependence of $\Delta N76Bcl-x_L$ - and $Bcl-x_L$ -mediated ANTS release in LUV of different lipid compositions

Control, DOPC/DOPG (60:40); O-LPE, DOPC/DOPG/O-LPE (40:40:20); O-LPC, DOPC/DOPG/O-LPC (40:40:20); DOPE, DOPC/DOPG/DOPE (40:40:20); DOG, DOPC/DOPG/DOG (40/40/20). Values given as mean \pm S.E. of two to four independent experiments. ANTS release data obtained using cardiolipin instead of DOPG showed the same trend.

Protein in nM	Lipid composition				
	Control	O-LPE	O-LPC	DOPE	DOG
	% ANTS release				
$\Delta N76Bcl-x_L$ (10 nM)	12 \pm 1	18 \pm 1	27 \pm 3	9 \pm 1	5 \pm 0
$\Delta N76Bcl-x_L$ (50 nM)	55 \pm 5	69 \pm 5	88 \pm 7	38 \pm 4	14 \pm 2
$\Delta N76Bcl-x_L$ (250 nM)	96 \pm 8	95 \pm 8	93 \pm 7	69 \pm 4	47 \pm 4
$Bcl-x_L$ (10 nM)	4 \pm 0	4 \pm 0	6 \pm 0	3 \pm 1	7 \pm 0
$Bcl-x_L$ (50 nM)	13 \pm 2	15 \pm 1	19 \pm 2	12 \pm 1	17 \pm 2
$Bcl-x_L$ (250 nM)	19 \pm 1	22 \pm 2	26 \pm 3	17 \pm 3	24 \pm 3

incorporated DOPE or DOG, the smaller the Bax-induced ANTS release. Hence, positive and negative membrane monolayer curvature promoted and inhibited, respectively, the release of LUV-encapsulated ANTS by Bax. Moreover, O-LPC, having an intrinsic curvature more positive than O-LPE (24), promoted Bax-mediated vesicular ANTS release more effectively than did LPE; and DOG, having an intrinsic curvature more negative than DOPE (25), inhibited Bax-mediated vesicular ANTS release more effectively than did DOPE. A similar pattern of results was obtained at a wide range of Bax concentrations in LUV containing DOPG or tetraoleoylphosphatidylglycerol (cardiolipin, CL) (Table I). Likewise, membrane-incorporated O-LPC and O-LPE promoted and DOG and DOPE inhibited the dose-dependent release of vesicular ANTS induced by $\Delta N76Bcl-x_L$, the proapoptotic Bax-like caspase cleavage fragment of $Bcl-x_L$ (8, 27) (Table II). In the case of the full-length, antiapoptotic $Bcl-x_L$, however, nonlamellar lipids had a more modest effect on ANTS release, and neither DOPE nor DOG inhibited this process (Table II). It is noteworthy that none of these proteins changed the overall structure of the LUV as deduced from dynamic light scattering measurements before and after treatment (data not shown), indicating that they did not induce ANTS release through vesicle fragmentation or aggregation.

Efflux of cytochrome *c* across the outer mitochondrial membrane into the cytosol is a pivotal event during apoptosis, and multiple lines of evidence indicate that Bax-type proteins are actively involved in this process (1–4). We have recently shown that $\Delta N76Bcl-x_L$, but not $Bcl-x_L$, can form pores in pure lipid vesicles capable of releasing intravesicular cytochrome *c* (8). To examine the effect of membrane curvature on this system, cytochrome *c*-encapsulated LUV of different lipid compositions were incubated with $\Delta N76Bcl-x_L$, treated samples were filtered through a 100-kDa microconcentrator, and the cytochrome *c* contents of the retentate and the filtrate were analyzed by immunoblotting. As with ANTS release, the release of vesicle-entrapped cytochrome *c* induced by $\Delta N76Bcl-x_L$ was decreased by incorporation in the membrane of negative curvature-inducing lipids DOG and DOPE, whereas positive curvature-inducing lipids O-LPC and O-LPE had the opposite effect (Fig. 2).

Next, we wished to examine whether O-LPC and O-LPE canceled the effect of DOG and DOPE on LUV permeabilization by proapoptotic proteins since the curvature of O-LPC and O-LPE is opposite to that of DOG and DOPE (24). Inclusion of increasing amounts of lysolipid in the membrane led to a progressive reversal of the inhibitory action of DOG and DOPE on the release of LUV-encapsulated ANTS and cytochrome *c* (Fig. 3). O-LPC was a more potent antagonist of DOG- and DOPE-induced inhibition than O-LPE (Fig. 3A), as expected from the fact that the intrinsic curvature of O-LPC is more positive than that of O-LPE (24). Taken together, these results argue against a specific pharmacological mode of action of DOG and DOPE,

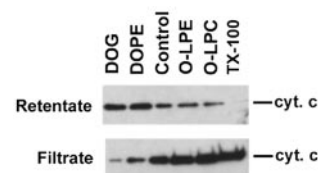


FIG. 2. Effect of membrane-incorporated nonlamellar lipids on cytochrome *c* release from LUV elicited by $\Delta N76Bcl-x_L$. Immunoblot showing the release of cytochrome *c* induced by $\Delta N76Bcl-x_L$ in LUV of DOPC/DOPG/DOG (40:40:20) (DOG), DOPE/DOPG (60:40) (DOPE), DOPC/DOPG (60:40) (Control), DOPC/DOPG/O-LPE (40:40:20) (O-LPE), DOPC/DOPG/O-LPC (40:40:20) (O-LPC), and Control LUV with 0.1% Triton X-100 (TX-100). Results are representative of three independent experiments.

i.e. the possibility that some specific molecular recognition between nonlamellar lipids and proapoptotic proteins is responsible for the changes on vesicular ANTS release. Rather, they are consistent with the idea that nonlamellar lipids affect release of LUV contents through modulation of the intrinsic membrane monolayer curvature of the liposome.

Nonlamellar Lipids Affect Planar Membrane Destabilization by Apoptotic Proteins—Bax-type proteins porate and destabilize planar phospholipid bilayer membranes (6–9). Nonlamellar lipids were used to test the effect of curvature on poration in this system as well. Substitution of egg PC by egg PE inhibited the destabilization of planar membranes induced by Bax and $\Delta N76Bcl-x_L$, whereas egg LPC increased planar membrane susceptibility to destabilization by proapoptotic proteins (Fig. 4). Interestingly, while the membrane-destabilizing effects of Bax and egg LPC were additive, those of $\Delta N76Bcl-x_L$ and egg LPC were synergistic. A closer inspection revealed that the synergism of egg LPC and $\Delta N76Bcl-x_L$ on planar membrane destabilization was voltage-dependent (Fig. 4B). On the other hand, $Bcl-x_L$ exerted no significant effect on membrane stability in the absence or in the presence of egg LPC (Fig. 4B). Therefore, the bending propensity of the membrane monolayer directed not only the release of vesicle contents elicited by Bax and $\Delta N76Bcl-x_L$ but also the reduction of planar membrane stability elicited by Bax and $\Delta N76Bcl-x_L$.

Membrane-binding or Membrane-insertion of Apoptotic Proteins Do Not Appear to Account for the Effect of Nonlamellar Lipids on Membrane Permeabilization—Membrane curvature has been shown to influence membrane binding and insertion of a number of proteins and peptides (28–32). Therefore, we decided to check whether the changes in lipid composition discussed above affected the membrane-binding and/or the membrane-insertion ability of apoptotic proteins. To quantify the total amount of protein bound to LUV we took advantage of the fact that free, but not vesicle-bound protein, sediments when centrifuged in D_2O -buffer (Ref. 23 and data not shown). Of the total protein population, the membrane-inserted protein

FIG. 3. Reversal of DOG- and DOPE-exerted inhibition of ANTS release by lysolipids. *A*, LUV of DOPE/DOPG (60:40) (circles) and DOPC/DOPG/DOG (40:40:20) (triangles) were doped with increasing proportions of O-LPC (filled symbols) and O-LPE (open symbols). Mean \pm S.E. correspond to four independent experiments. *B*, immunoblot showing the release of cytochrome *c* induced by Δ N76Bcl-x_L in LUV of DOPC/DOPG/DOG (40:40:20), in which DOPC is substituted by indicated proportions of O-LPC. Results are representative of two separate experiments.

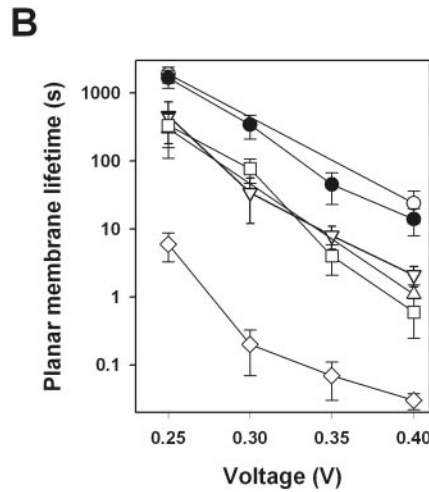
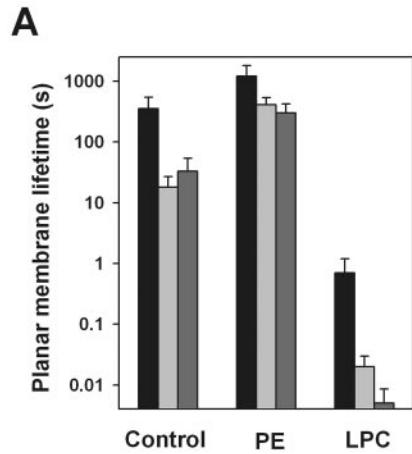
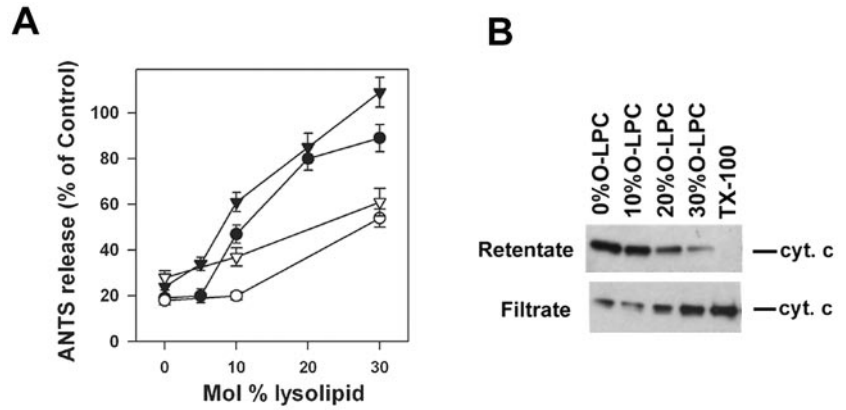
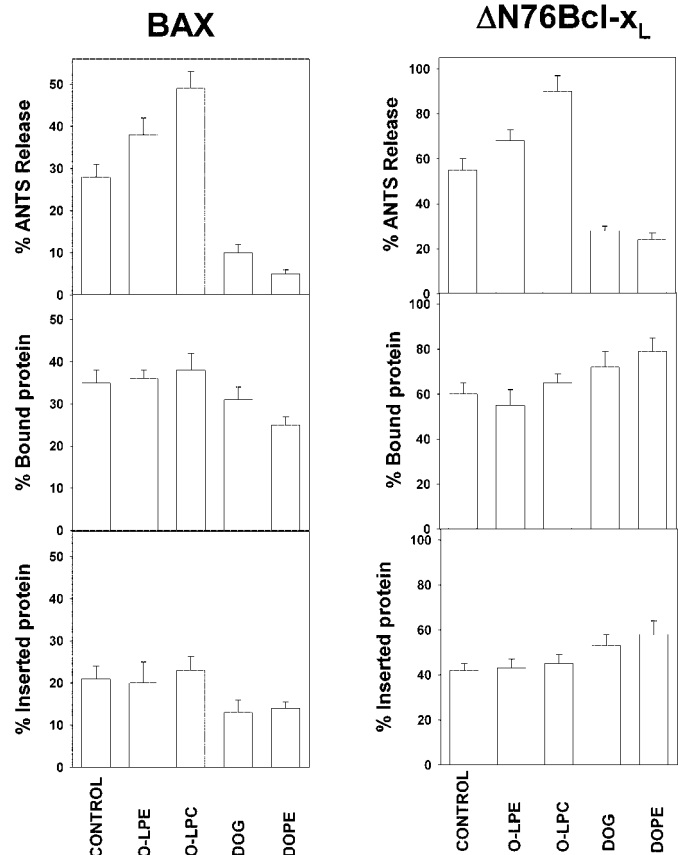


FIG. 4. Effect of nonlamellar lipids on planar membrane lifetime. *A*, lifetime of planar membranes composed of egg PC/egg PG (60:40) (Control), egg PE/egg PG (60:40) (PE), and egg PC/egg PG plus 1 μ M egg LPC (LPC) for 1 min, treated with no protein (black bars), 30 nM Bax (light gray bars), or 10 nM Δ N76Bcl-x_L (dark gray bars). Voltage was 300 mV. *B*, voltage-dependence of PC membrane lifetime in the presence of no additives (filled circles), 3 nM Bcl-x_L (empty circles), 3 nM Δ N76Bcl-x_L (empty squares), 0.3 μ M egg LPC (empty downward triangles), 3 nM Bcl-x_L plus 0.3 μ M egg LPC (empty upward triangles), and 3 nM Δ N76Bcl-x_L plus 0.3 μ M egg LPC (empty diamonds). In all cases, means \pm S.E. of 10–18 independent experiments are shown.

FIG. 5. Comparison of the competency for induction of vesicular ANTS release, membrane binding, and membrane insertion of Bax and Δ N76Bcl-x_L in LUV of different lipid compositions. Lipid compositions were DOPC/DOPG (60:40) (Control); DOPC/DOPG/O-LPE (50:40:10) (O-LPE); DOPC/DOPG/O-LPC (50:40:10) (O-LPC); DOPC/DOPG/DOG (50:40:10) (DOG), and DOPE/DOPG (60:40) (DOPE). Average values and standard errors correspond to three to eight independent experiments. Concentrations of lipid and protein were 150 μ M and 250 nM, respectively.



fraction was determined by measuring the susceptibility of LUV-associated protein to alkaline pH extraction. We caution, however, that this method does not allow discrimination between proteins inserted at different depths of the membrane hydrophobic interior. As shown in Fig. 5, even if differences were found depending on vesicle lipid composition in both the amount of protein bound to and inserted into the membrane, these effects alone could not explain the effect of nonlamellar lipids on Bax and $\Delta N76Bcl-x_L$ -induced vesicular ANTS release. For example, O-LPC and O-LPE favored Bax- and $\Delta N76Bcl-x_L$ -induced vesicular ANTS release, yet lysolipids did not generally potentiate membrane association or insertion of Bax and $\Delta N76Bcl-x_L$. On the other hand, although the proportion of membrane-bound and membrane-inserted Bax diminished by incorporation of DOPE or DOG, both nonlamellar lipids reduced Bax-induced ANTS release to a much larger extent. Most significantly, DOPE and DOG did not decrease but actually *increased* membrane binding and insertion of $\Delta N76Bcl-x_L$. In short, these observations suggest that the effects of curvature on pore formation are unrelated to changes in the incorporation of the active proteins into the membrane.

DISCUSSION

This study demonstrates that the capability of Bax and $\Delta N76Bcl-x_L$ to permeabilize purely lipidic phospholipid bilayer membranes depends upon intrinsic monolayer curvature. In all experiments, increasing intrinsic monolayer curvature toward positive values facilitated membrane permeabilization, whereas increasing intrinsic monolayer curvature toward negative values diminished membrane permeabilization. Such effects did not correlate well with changes in the membrane incorporation of Bax and $\Delta N76Bcl-x_L$, suggesting that the tendency of the membrane monolayer for curvature is an important contributor of pore formation itself.

Regulation of membrane permeabilization by intrinsic monolayer curvature is not unprecedented. A well known example is the alamethicin channel (33, 34). However, alamethicin channels are much more uniform in size and dynamic behavior than Bax and $\Delta N76Bcl-x_L$ pores, and alamethicin does not affect the stability of planar phospholipid bilayer membranes as do proapoptotic proteins. In addition, alamethicin channel formation is favored by nonlamellar lipids with negative intrinsic curvature (33, 34), contrary to what is observed in the case of Bax and $\Delta N76Bcl-x_L$. Also, nonlamellar lipids are known to influence the avidity of alamethicin for lipid membranes, and this has been related to the effects of curvature on alamethicin channel formation (28). This is in contrast with the situation found with Bax and $\Delta N76Bcl-x_L$, for which the effects of curvature-inducing lipids on pore formation could not be explained by changes in protein membrane binding affinity. Alternatively, the tendency of the membrane monolayer to curl may be an important determinant for the adoption of an active, pore-forming conformation by Bax-type proteins within the lipid milieu. For example, the quaternary status of membrane-incorporated Bax and $\Delta N76Bcl-x_L$ may depend upon intrinsic membrane monolayer curvature, with positive and negative curvature stabilizing and destabilizing, respectively, the assembly of active oligomers of Bax and $\Delta N76Bcl-x_L$. Another possibility is that curvature influences the depth and/or mode of insertion of Bax-type proteins into the membrane interior which, in turn, may control their capacity to form proteinaceous channels. Similar explanations have been proposed to account for the effects induced by nonlamellar lipids on the activity of a variety of peptides and proteins (28–32, 35–37). Once again, however, in all those systems nonlamellar lipids with negative intrinsic curvature *facilitated* maximum protein

activity, whereas the opposite trend was obtained here with Bax and $\Delta N76Bcl-x_L$.

On the other hand, a number of membrane-active antimicrobial peptides exemplified by magainin (15), as well as the syringomycin E channel (17) display sensitivities for intrinsic membrane monolayer curvature analogous to those observed with Bax and $\Delta N76Bcl-x_L$. Both the magainin pore and the syringomycin E pore have been modeled as supramolecular assemblies of lipids and peptides with the shape of a torus having net positive curvature (15–17). Interestingly, planar membrane destabilization is also thought to occur through expansion of positively curved toroidal-type lipidic pores (9). These observations speak in favor of the idea that Bax-type proteins function by increasing the tendency of the membrane to form lipid-containing toroidal pores. However, further work is required to unambiguously determine the mechanism by which intrinsic membrane monolayer curvature affects the pore-forming activity of Bax-type proteins, as well as the exact structure and composition of the Bax and $\Delta N76Bcl-x_L$ pores in phospholipid bilayer membranes.

Essential biological processes such as fusion pore formation (38), synaptic vesicle endocytosis (39), Golgi fission (40), and cytokinesis (41) depend upon membrane monolayer curvature. If the apoptotic pore that forms in the outer mitochondrial membrane is the same as the pore formed by Bax and its homologues in phospholipid bilayer membranes, then the permeabilization of the outer mitochondrial membrane elicited by Bax-type proteins during apoptosis will also be governed by membrane monolayer curvature. Alternatively, if Bax-type proteins induce mitochondrial membrane permeabilization through modulation of endogenous mitochondrial channels, an effect of curvature on mitochondrial membrane permeabilization is not expected. Experimental maneuvers similar to the ones shown here but adapted for biological systems may thus be used to gain more insight into the molecular mechanism underpinning mitochondrial membrane permeabilization by Bax-type apoptotic proteins.

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