Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2

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ABSTRACT The BCL-2 family of proteins is composed of both pro- and antiapoptotic regulators, although its most critical biochemical functions remain uncertain. The structural similarity between the BCL-X_I monomer and several ion-pore-forming bacterial toxins has prompted electrophysio-

logy studies. Both BAX and BCL-2 insert into KC1-loaded vesicles in a pH-dependent fashion and demonstrate macroscopic ion efflux. Release is maximum at a pH 4.0 for both proteins; however, BAX demonstrates a broader pH range of activity. Both purified proteins also insert into planar lipid bilayers at pH 4.0. Single-channel recordings revealed a minimal channel conductance for BAX of 22 pS that evolved to channel currents with at least three subconductance levels. The final, apparently stable BAX channel had a conductance of 0.731 nS at pH 4.0 that changed to 0.329 nS when shifted to pH 7.0 but remained mildly CI− selective and predominantly open. When BAX-incorporated lipid vesicles were fused to planar lipid bilayers at pH 7.0, a Cl−-selective (PK/PCI = 0.3) 1.5-nS channel displaying mild inward rectification was noted. In contrast, BCL-2 formed mildly K+ selective (PK/PCI = 3.9) channels with a most prominent initial conductance of 80 pS that increased to 1.90 nS. Fusion of BCL-2-incorporated lipid vesicles into planar bilayers at pH 7.0 also revealed mild K+ selectivity (PK/PCI = 2.4) with a maximum conductance of 1.08 nS. BAX and BCL-2 each form channels in artificial membranes that have distinct characteristics including ion selectivity, conductance, voltage dependence, and rectification. Thus, one role of these molecules may include pore activity at selected membrane sites.

The BCL-2 family of proteins is composed of both antiapoptotic and proapoptotic members that function in a distal apoptotic pathway common to all multicellular organisms. The ratio of death antagonists (BAX, BCL-X_I, MCL-1, A1) to agonists (BAX, BCL-X_S, BAK, BAD, BIK, BID) determines the response to an apoptotic stimulus (1, 2). Family members with a hydrophobic C-terminal signal anchor sequence are intracellular integral membrane proteins most convincingly localized to mitochondria, endoplasmic reticulum, and nuclear membrane (3–6). BCL-2 appears to function upstream of a family of caspases (cysteine proteases with a P1 specificity for aspartic acid) in that BCL-2 expression prevents the activation of proteases such as caspase-3 (7–9). Moreover, the BCL-2 family to form both homodimers and heterodimers, the latter often between anti- and proapoptotic molecules such as BCL-2/ BAX (1). Mutational analysis of BCL-2 and BCL-X_I identified key residues within BH1 and BH2 domains required for both heterodimerization with BAX and repression of cell death (12, 13). However, other mutants that lost dimerization with BAX still retain death-repressor activity, suggesting these two functions are separable (14). Another domain, BH3, has proven essential for the proapoptotic molecules such as BAK, BAX, BID, and BAD to bind antagonists like BCL-2 or BCL-X_I and to function in promoting death (15–18). A genetic approach utilizing gain- and loss-of-function murine models to assess whether death agonists (BAX) or antagonists (Bcl-2) were dominant in regulating apoptosis. Despite the evidence for in vivo competition between the molecules, BCL-2 and BAX each proved capable of regulating apoptosis independent of the other product (19).

The multidimensional NMR and x-ray crystallographic structure of a BCL-X_I monomer indicated that BH1–4 domains corresponded to α helices 1–7. The α helices contributed by BH1–3 are closely juxtaposed to form a hydrophobic pocket (20). A detailed NMR analysis of wild-type and mutant peptides of the BH3 amphipathic α2-helix of BAK indicated it formed critical interactions with this pocket (21). Moreover, the α helical structure of BCL-X_L proved similar to the ion pore-forming toxins of colicin and diphtheria toxin B fragment. Of particular note are the two central hydrophobic helices α5 and α6 of 30-Å length reminiscent of membrane insertion domains in the bacterial toxins. This observation has prompted a series of electrophysiologic studies by others (22–24) and ourselves to examine the capacity of BCL-2 family members to form ion channels in artificial lipid membranes. Here we compare the distinct ion conductive channels of proapoptotic BAX with antiapoptotic BCL-2.

MATERIALS AND METHODS

Plasmid Preparation and Protein Purification. Murine BAX and BCL-2 lacking the C-terminal hydrophobic region (BAX3C19, amino acids 1–173; BCL-2ΔC21, amino acids 1–218) were cloned into pGEX-KG (2, 3). GST-BAX3C19 and GST-BCL-2ΔC21 fusion proteins were induced in XL-1 by 0.1 mM IPTG. The bacterial pellets were resuspended in lysis buffer (0.5 mM EDTA/1 mM DTT/1% Triton X-100/0.1 mg/ml PMSF/2 μg/ml aprotinin/2 μg/ml leupeptine/1 μg/ml pepstatin A in PBS) and sonicated. After centrifugation at 20,000 × g for 20 min, the supernatant was applied to glutathione-agarose beads (Sigma). The beads were washed with buffer and treated with 10 units of thrombin per original liter. Cleaved BAX3C19 and BCL-2ΔC21 were eluted from beads and the cleavage reaction was terminated by adding 80 μg of N-acetyl-L-lysine chloromethyl ketone (TLCK). The

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cleared eluent was dialyzed against buffer (20 mM Tris pH 8.5/5 mM EDTA/1 mM DTT/0.1% Triton X-100). To remove the GST protein and incompletely cleaved fusion proteins, the dialyzed preparation was further purified on a monoQ column and the proteins were eluted with a NaCl gradient. Experiments utilized three independent protein preparations of BAX and BCL-2.

**Release of KCl from Synthetic Lipid Vesicles.** Unilamellar vesicles composed of 40% 1,2-dioleoyl phosphatidyl glycerol and 60% 1,2-dioleoyl phosphatidyl choline (Avanti Polar Lipids) were prepared in 100 mM KCl/2 mM CaNO$_3$/10 mM dimethylglutarate, pH 5.0, as previously described (25). The resulting liposomes were diluted 200-fold to a concentration of 0.05 mg/ml in 10 mM dimethylglutarate, pH 5.0, which subsequently shifted to pH 4.0 (vertical arrow). The preparation of Azolec-19 and BCL-2C21 were added at a concentration of 500 ng/ml. Triton X-100 (0.1%) was added to release the total encapsulated Cl$^{-}$. The total amount of Cl$^{-}$ released was quantitated by a calibration curve produced by successive additions of 25 μM KCl. Cl$^{-}$ efflux was measured with a Cl$^{-}$ combination ion-selective electrode ( Accumet, Hudson, MA).

**Low pH Insertion of BAX and BCL-2 into Planar Lipid Bilayers.** Planar lipid bilayers were prepared from soybean lipids by chloroform extraction of Azolec-19 (10–20% choline, 80–90% negatively charged lipids) (Sigma). The chloroform was removed with a stream of nitrogen, and the lipids were stored under N$_2$ until dissolved in decane at 30 mg/ml. This preparation was then stored under nitrogen. The 0.25-mm orifice of a polystyrene cuvette ( Warner Instruments, Hamden, CT) was pretreated with 2 mM CaNO$_3$ and the solvent was allowed to evaporate. The cuvette was then placed into a bilayer chamber and connected to a Bilayer Clamp BC525-a ( Warner Instruments) by Ag/AgCl electrodes via agar bridges. Data were collected using AXOSCOPE (Axon Instruments, Foster City, CA), archived on videotape using a Neurocorder DR-484 ( Neuro Data Instruments, Delewar Gap, PA), and analyzed using ORIGIN ( Microlab, Amherst, MA) and PCLAMP ( Axon Instruments). Slope conductance was calculated by the method of least squares, and the variance is given. Ion selectivities were calculated using the reversal potential and the Goldman equation.

**RESULTS**

**pH Dependence of BAX- and BCL-2-Mediated Release of Ions from Lipid Vesicles.** Recombinant BCL-2 and BAX that

![Fig. 1](https://example.com/fig1.png)
of internal targeting sequences were removed to better assess the capacity of target proteins. Western blot analysis (not shown) or Coomassie blue purification to homogeneity and verified by SDS-PAGE followed by C-terminal signal-anchor membrane-targeting sequences were removed to better assess the capacity of internal α helices to mediate insertion (5).

were lacking the C-terminal hydrophobic region (BCL-2ΔC21, amino acids 1–218; BAXΔC19, amino acids 1–173) were purified to homogeneity and verified by Western blot analysis (not shown) or Coomassie blue staining (Fig. 1A). The C-terminal signal-anchor membrane-targeting sequences were removed to better assess the capacity of internal α helices to mediate insertion (5). The pH dependence of BAX and BCL-2-mediated release of Cl− from KCl-loaded lipid vesicles was compared (Fig. 1B–E). The maximum release of Cl− by BAX occurs at pH 4.0–4.5, decreasing to 50% at pH 3.5 or 5.0 and to less than 10% at a pH of 5.5 (Fig. 1B). When BAX added to vesicles at pH 6.0 was subsequently shifted to pH 4.0, a rapid release of Cl− resulted, indicating the reversibility of the pH influence (Fig. 1C). BCL-2 displays a more narrow pH dependence of Cl− release, with complete inactivation occurring at pH 5.0 (Fig. 1D). Once again, shifting from pH 5.0 to 4.0 activated the release of Cl− by BCL-2 (Fig. 1E). Thus, both purified BAX and BCL-2 proteins are capable of pH-dependent macroscopic ion release that requires activity of the bulk population of BCL-2 and BAX proteins.

Low pH Formation of BAX Channels in Planar Lipid Bilayers. One microgram of purified, soluble BAX protein was added to the cis chamber of an established planar lipid bilayer in a 450-/150-mM (cis-to-trans) KCl gradient at pH 4.0. The initial, inward current appeared spontaneously (O1), reflecting

Cl− moving down the KCl gradient (Fig. 2A). In multiple experiments the initial current was always inward, usually appeared within 10 min following BAX addition, and had a conductance of 22 (SD 5, n = 5) pS.

Characteristic patterns of BAX currents were observed ranging from O5 to a large, open pore. Fig. 2 shows the initial spontaneous current (O5) (Fig. 2A), a complex multiconductance state (Fig. 2B), and a simple open pore (Fig. 2C). The multiconductance state was most clear at ±40 mV. At ±40 mV four current levels were noted: 0 pA (C and O5), 2.36 pA (O1), 6.4 pA (O2), and 8.81 pA (O1+2) (Fig. 2B Upper). The largest current appears to be the sum of the two smaller levels (O1 and O2). Direct transitions between these levels, which occur in both directions, suggest a random mechanism for movement between the open states (Fig. 2B). At −40 mV there were also four levels: 0 pA (C), −2.14 pA (O5), −10.9 pA (O1), and −30.5 pA (O1+2), but no transitions from O5 to O2 were observed (Fig. 2B Lower). Hyperpolarization to −40 mV shifted the most common state to O1, demonstrating voltage-dependent behavior. Based on reversal potential the Ek for each of these current levels gives a substantial Cl− selectivity that averages Pk/PCl = 0.10. The third state of the BAX channel at pH 4.0 was Cl− selective, with a slope conductance of 0.731 ± 0.01 nS (Fig. 2C Left), and was characteristically open. Typically, BAX currents progressed through these states after the addition of protein to the cis chamber. Increasing the pH to 7.0 altered the conductance of BAX to 0.329 ± 0.002 nS in a 450-/150-mM KCl gradient and 0.302 ± 0.008 nS when
both chambers were 150 mM KCl (Fig. 2C Right). The BAX channel was observed in this activity state at pH 7.0 for prolonged periods. At pH 7.0 the BAX pore had linear voltage relationship and retained a mild selectivity for Cl⁻ \((P_{K^+}/P_{Cl^-} = 0.5)\). 

**Incorporation of BAX into Planar Lipid Bilayers at pH 7.0 by Fusion of Reconstituted Proteoliposomes.** Purified BAX protein incorporated into lipid vesicles was added to the cis chamber of an established lipid bilayer with a 450-/150-mM KCl gradient to permit identification of initial currents. The inward \((P_{K^+} < P_{Cl^-})\) current observed when liposomes containing reconstituted BAX fused to the planar lipid bilayers at pH 7.0 had a large open time \((P_O > 0.95)\) and a large conductance \((1.5 \pm 0.3 \text{ nS}, n = 3, \text{Fig. 3B})\). Transitions between current levels \((O \rightarrow O_2, \text{Fig. 3A})\) that were identical in amplitude suggested that O represented the single-channel conductance \((\text{Fig. 3A})\). The voltage dependence of the current was determined when one channel was present \((\text{Fig. 3B Left})\). Mild outward rectification is evident in symmetrical KCl concentrations. A mild Cl⁻ selectivity \((P_{K^+}/P_{Cl^-} = 0.32)\) was calculated from the reversal potential when cis-to-trans KCl was 450/150 mM. At positive voltages \((+70 \text{ mV})\) the rapid flickering \((\text{Fig. 3C})\) was consistent with channel block as a mechanism for the rectification seen in B. At \(-70 \text{ mV}\) there was no flickering, but periodic closing of 20- to 30-msec duration was observed \((\text{Fig. 3C})\), which is similar to that reported for porin-type channels \((26)\). 

**Low pH Formation of BCL-2 Channels in Planar Lipid Bilayers.** Purified soluble BCL-2 protein was added to the cis chamber of an established bilayer with a 450-/150-mM KCl gradient to permit identification of initial currents. \(P_{K^+}/P_{Cl^-}\) current observed when liposomes containing reconstituted BCL-2 fused to the planar lipid bilayers at pH 7.0 had a large open time \((P_O > 0.95)\) and a large conductance \((0.85 \pm 0.06 \text{ pA}, \text{which flickered open and shut in the first few seconds and subsequently remained open. Under these conditions the BCL-2 channel had a conductance of } 80.3 \pm 0.6 \text{ pS} \text{(Fig. 4C)}\). Subsequently this channel remained open for long periods \((5-10 \text{ sec})\) with brief closures \((\text{Fig. 4B})\). This initial state of the BCL-2 channel was present for only 2-5 min under these conditions. BCL-2 progressed to a stable open pore at pH 4.0 with a \(1.90 \pm 0.06 \text{ nS}\) conductance \((\text{Fig. 4D})\). Shifting the pH to 7.0 maintained a large pore with a \(2.14 \pm 0.04 \text{ nS}\) conductance and \(K^+\) selectivity of \(P_{K^+}/P_{Cl^-} = 6.5\) \((\text{Fig. 4D})\). 

**Incorporation of BCL-2 into Planar Lipid Bilayers by Fusion of Reconstituted Proteoliposomes.** Purified BCL-2 protein incorporated into lipid vesicles was added to the cis chamber of an established bilayer with a 450-/150-mM KCl gradient. In multiple experiments reconstituted BCL-2 always resulted in an outward \((K^+)\) current \((\text{Fig. 5A})\). Fig. 5A shows a channel that initially flickers between multiple levels \((O, O_2)\). When a series of voltage steps were applied to a single channel established in the bilayer, channel closures were observed at voltages greater than \(\pm 50 \text{ mV}\) \((\text{Fig. 5B})\). These closures became more frequent as the voltage increased. The two plots in Fig. 5C show the voltage dependence of the open channel current in symmetrical 150-mM KCl as linear with a slope conductance of 1.08 \((\text{SD 0.10, } n = 5)\) nS. However, with hyperpolarization to more than \(-70 \text{ mV}\), partial channel closers occur \((\text{Fig. 5C Left})\). The reversal potential in KCl
gradients indicated a mild $K^+$ selectivity ($P_K/P_{Cl} = 2.4$). The small channels and time-dependent changes noted when soluble BCL-2 was inserted into planar lipid bilayers were not observed when reconstituted proteoliposomes containing BCL-2 were fused to planar lipid bilayers.

**DISCUSSION**

The structure of BCL-X$_L$, in which two central hydrophobic helices, α5 and α6, are surrounded by four amphipathic helices, is similar to the T8 and T9 helices of diphtheria toxin fragment B, which is important for insertion into membranes at low pH (20, 27, 28). Mutational studies that replace acidic with basic residues in T8/T9 prevented the insertion at low pH, which is consistent with a role for their protonation in membrane fusion (29). The analogy with pore-forming toxins was extended by the demonstration that acid pH facilitates the insertion of BCL-X$_L$ into lipid bilayers (22). We found that both BAX and BCL-2 initiate rapid release of ions from liposomes when added at low pH. However, BAX demonstrated a broader pH optimum, retaining activity as high as pH 5.5. This might reflect the higher α5-helix pI of 10.64 for BAX versus 4.55 for BCL-2 (Fig. 6). The observation that deletion of the α5 and α6 helices of BCL-2 altered its characteristics emphasizes their importance (23). If the insertion of the putative transmembrane α5 and α6 helices of these apoptotic regulators benefits from charge reduction, the lower pH requirement for BCL-2 may reflect glutamic acid residues that would be prone to ionization with increasing pH in contrast to the presence of lysine and arginine residues in BAX (Fig. 6).

The ion channels formed by BAX and BCL-2 in planar lipid bilayers have characteristics that depend in part on the method of incorporation. When soluble BAX or BCL-2 was inserted into bilayers at low pH the initial currents were small with conductances of 22 and 80 pS, respectively. Like BCL-X$_L$ (22) and other observations on BCL-2 (23), we also observed a mild cation selectivity for antiapoptotic BCL-2; however, we noted that the proapoptotic molecule BAX has a consistent anion selectivity. If the α5 and α6 helices contribute to the channel these selectivities may reflect the positively charged residues of BAX and negatively charged residues of BCL-2 (Fig. 6A). Although modest differences in ion selectivity are unlikely to be the sole explanation for opposite influences on apoptosis, these charge reversals appear to be consistent in the α5 and α6 helices of anti-versus proapoptotic members (Fig. 6B). BAX channels respond to shifting the pH to 7.0 after insertion at pH 4.0 consistent with previous observations on toxins and porins (30, 31). The changes we noted could also relate to pH dependent ionization of charged residues in these channels.

A striking progression of the BAX channel in planar bilayers occurred within 2–4 min of its initial appearance. This included (i) an early $Cl^-$-selective small channel, (ii) a transition phase with multiple subconductance levels and moderate $Cl^-$ selectivity, and (iii) an apparently stable ohmic pore of large conductance that is mildly $Cl^-$-selective and open continuously (Fig. 2). The BCL-2 channel activity also progressed from an early $K^+$-selective small channel that opened and closed spontaneously to a large ohmic pore (Fig. 4). Removal of protein from the chamber and alteration of salt concentrations did not prevent this transition, which may represent intramembranous organization of BCL-2 or BAX into its mature form. Of note, shifting from pH 4.0 to 7.0 altered the conductance and selectivity of BAX but not BCL-2. In contrast, the overnight reconstitution of BAX or BCL-2 into lipid vesicles that were subsequently fused to planar bilayers resulted immediately in large, open pores (Figs. 3 B and 5C). These
observations support the mutational and genetic analyses that argue that BCL-2 and BAX can each function independently. However, it remains uncertain as to whether monomers, dimers, or higher-order structures of these molecules are the active subunit. We cannot firmly exclude the possibility that the progression of BCL-2 and BAX currents reflects a non-physiologic aggregation of these molecules. Alternatively, the consistency of these transitions indicates they may result from oligomerization, perhaps dimerization, that might also occur in vivo. The large-conductance pores noted for the BCL-2 family share some characteristics with the porins of bacteria, yeast, and mammals (26). The closest structural homologs, diphtheria toxin and colicin, are also capable of forming large ion-conductive pores (27, 28).

How might ion-conductive channels regulate apoptosis? The selective targeting of the BCL-2 family to the outer mitochondrial membrane, nuclear membrane, and endoplasmic reticulum appears important (32–35). Although the differences in ion selectivity are moderate for BCL-2 and BAX, these channels do display other unique characteristics including conductance, voltage dependence, and rectification. BCL-2 and BAX might regulate an electrochemical gradient, osmotic balance, or transport critical substrates residing in the intermembrane space including cytochrome c (36). Upon subcellular fractionation, BCL-2 was retained in mitoplasts (3) and, by immunoelectron microscopy, was clustered (6), indicating that, in part, it localizes to contact points between inner and outer membranes. Apoptosis is accompanied by a permeability transition pore (PTP) that ensures an electrochemical collapse of mitochondria (37, 38). Patch-clamp studies of mitoplasts identified a 1.3-nS megachannel (MMC) that was considered to represent the PTP (39). BCL-2 and BAX become candidates for regulating or being components of the PTP. Alternatively, the BCL-2 family could modify other ion channels or transport molecules not yet tested. BCL-2 apoptotic regulators might interact with other ion channels or transporters. The large-conductance pores noted for the BCL-2 family also share some characteristics with the porins of bacteria, yeast, and mammals (36).

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