An Interaction between Bcl-x\textsubscript{L} and the Voltage-dependent Anion Channel (VDAC) Promotes Mitochondrial Ca\textsuperscript{2+} Uptake*

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Background: Ca\textsuperscript{2+} moves across the outer mitochondrial membrane (OMM) through the voltage-dependent anion channel (VDAC).

Results: Disrupting the interaction between VDAC and the antiapoptotic protein Bcl-x\textsubscript{L} reduces mitochondrial Ca\textsuperscript{2+} uptake.

Conclusion: Bcl-x\textsubscript{L}/VDAC interactions promote Ca\textsuperscript{2+} uptake by increasing transfer across the OMM.

Significance: Mitochondrial matrix Ca\textsuperscript{2+} is tightly regulated at the OMM by the modulation of VDAC.

The role of the antiapoptotic protein Bcl-x\textsubscript{L} in regulating mitochondrial Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{mito}) handling was examined in wild-type (WT) and Bcl-x\textsubscript{L} knock-out (Bcl-x\textsubscript{L}-KO) mouse embryonic fibroblast cells. Inositol 1,4,5-trisphosphate-generating agonist evoked cytosolic Ca\textsuperscript{2+} transients that produced a larger [Ca\textsuperscript{2+}]\textsubscript{mito} uptake in WT cells compared with Bcl-x\textsubscript{L}-KO. In permeabilized cells, stepping external [Ca\textsuperscript{2+}] from 0 to 3 μM also produced a larger [Ca\textsuperscript{2+}]\textsubscript{mito} uptake in WT; moreover, the [Ca\textsuperscript{2+}]\textsubscript{mito} uptake capacity of Bcl-x\textsubscript{L}-KO cells was restored by re-expression of mitochondrially targeted Bcl-x\textsubscript{L}. Bcl-x\textsubscript{L} enhancement of [Ca\textsuperscript{2+}]\textsubscript{mito} uptake persisted after dissipation of the mitochondrial membrane potential but was absent in mitochondria lacking an outer mitochondrial membrane. The outer membrane-localized voltage-dependent anion channel (VDAC) is a known Ca\textsuperscript{2+} permeability pathway that directly interacts with Bcl-x\textsubscript{L}. Bcl-x\textsubscript{L} interacted with VDAC1 and -3 isoforms, and peptides based on the VDAC sequence disrupted Bcl-x\textsubscript{L} binding. Peptides reduced [Ca\textsuperscript{2+}]\textsubscript{mito} uptake in WT but were without effect in Bcl-x\textsubscript{L}-KO cells. In addition, peptides reduced [Ca\textsuperscript{2+}]\textsubscript{mito} uptake in VDAC1 and VDAC3 knock-out but not VDAC1 and -3 double knock-out mouse embryonic fibroblast cells, confirming that Bcl-x\textsubscript{L} interacts functionally with VDAC1 and -3 but not VDAC2. Thus, an interaction between Bcl-x\textsubscript{L} and VDAC promotes matrix Ca\textsuperscript{2+} accumulation by increasing Ca\textsuperscript{2+} transfer across the outer mitochondrial membrane.

The Bcl-2 proteins are a family of apoptosis regulators that contain both pro- and antiapoptotic members. Apoptotic stimuli cause activation of proapoptotic members that converge on the mitochondria to increase their membrane permeability. The subsequent release of apoptogenic factors, such as cytochrome c, represents a critical step in the cell death signaling cascade. This process is held in check by the antiapoptotic Bcl-2 proteins, which preserve mitochondrial membrane integrity and inhibit cell death by directly binding and sequestering the proapoptotic members (1).

In addition to influencing mitochondrial permeability directly, Bcl-2 proteins also impinge on apoptotic signaling through their ability to regulate Ca\textsuperscript{2+} homeostasis (2). We and others have shown that this can be mediated through direct interactions between the endoplasmic reticulum (ER)-localized inositol 1,4,5-trisphosphate receptor (InsP\textsubscript{3}R) Ca\textsuperscript{2+} release channel and antiapoptotic Bcl-2 members, including Bcl-2 itself, Bcl-x\textsubscript{L} and Mcl-1 (3–7). The functional consequences of these interactions are complex with both inhibition and stimulation of Ca\textsuperscript{2+} signals being reported. This apparent paradox likely reflects differences among Bcl-2 family members in their binding determinants and abilities to allosterically modulate the InsP\textsubscript{3}R (8). Collectively, these data have given rise to a generalized model in which antiapoptotic Bcl-2 proteins confer protection either by limiting Ca\textsuperscript{2+} overload during apoptotic stimuli or by priming cellular resistance through enhanced mitochondrial metabolism (2).

Our previous studies have demonstrated that Bcl-x\textsubscript{L} binds to all three InsP\textsubscript{3}R isoforms to increase their sensitivity to low levels of InsP\textsubscript{3} stimulation (4–6). This results in increased Ca\textsuperscript{2+} signaling and bioenergetics, a requirement for Bcl-x\textsubscript{L} to be maximally effective as an antiapoptotic mediator. More recently, we used mouse embryonic fibroblast (MEF) cell lines derived from Bcl-x\textsubscript{L} knock-out (Bcl-x\textsubscript{L}-KO) embryos to examine the effect of targeting Bcl-x\textsubscript{L} to specific organelles (9). When

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§ The abbreviations used are: ER, endoplasmic reticulum; [Ca\textsuperscript{2+}]\textsubscript{mito}, mitochondrial Ca\textsuperscript{2+}; MEF, mouse embryonic fibroblast; InsP\textsubscript{3}R, inositol 1,4,5-trisphosphate; InsP\textsubscript{3}R, inositol 1,4,5-trisphosphate receptor; [Ca\textsuperscript{2+}]\textsubscript{cyto}, cytosolic Ca\textsuperscript{2+}; VDAC, voltage-dependent anion channel; ICN, intracellular-like medium; HEDTA, N-(2-hydroxyethyl)ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; TMRE, tetramethylrhodamine; N-ter, N-terminal; Δ\textsubscript{m}ψ, mitochondrial membrane potential; ANOVA, analysis of variance; MPTP, mitochondrial permeability transition pore.

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expressed exclusively at the ER, Bcl-xL had predictable effects on Ca\(^{2+}\) handling but surprisingly did not confer any apoptosis protection. In contrast, targeting to the mitochondria alone was protective; however, the maximal antiapoptotic activity of Bcl-xL was only seen when it was present at both the ER and mitochondria. One interpretation of these data would be that localization of Bcl-xL at the mitochondria is required to sense Bcl-xL-regulated InsP\(_3\)/R-dependent Ca\(^{2+}\) signals.

Uptake of Ca\(^{2+}\) into the mitochondrial matrix requires transport across two membranes. Across the inner membrane, this is governed by the calcium uniporter, a channel whose properties have been characterized (10) but whose molecular identity has only recently been described (11, 12). Movement across the outer membrane is mediated largely by the voltage-dependent anion channel (VDAC), a porin channel that serves as the major diffusion pathway for ions and metabolites (13). Of the three VDAC isoforms, VDAC1 is the most abundantly expressed (14) and extensively characterized (13). Indeed, due to the abundance and large pore size of VDAC1, the outer membrane was originally thought to be freely permeable to Ca\(^{2+}\), although recent evidence suggests that it may function as a more regulated Ca\(^{2+}\) permeability (15–17).

Intriguingly, Bcl-xL binds directly to VDAC1; however, there is a lack of consensus as to whether it serves to promote (18) or inhibit (19) the function of the channel as a permeability pathway for metabolites and large anions. Similarly, in electrophysiological studies, Bcl-xL has been reported to both increase (18) and decrease (20) conductance of VDAC1 reconstituted in artificial membranes. It is not known whether Bcl-xL regulates mitochondrial Ca\(^{2+}\) uptake by interacting with VDAC1 or other VDAC isoforms. The purpose of the current study was to define how the interaction between Bcl-xL and VDAC shapes mitochondrial Ca\(^{2+}\) signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The generation of MEF cells from wild-type and Bcl-xL-KO embryos and selection of cell lines stably expressing Bcl-xL targeted to the ER or mitochondria in the KO background have been described previously (9). MEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Gemini, West Sacramento, CA), 100 units ml\(^{-1}\) penicillin (Mediatech), and 100 \(\mu\)g ml\(^{-1}\) streptomycin (Mediatech) in an incubator with 95% humidity and 5% CO\(_2\) at 37 °C.

**Solutions and Reagents**—Hanks’ balanced salt solution contained 137.9 mM NaCl, 5.33 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 0.34 mM Na\(_2\)HPO\(_4\), 5.56 mM glucose, 4.17 mM NaHCO\(_3\), 1.8 mM CaCl\(_2\), 0.49 mM MgCl\(_2\), 0.41 mM MgSO\(_4\), 10 mM HEPES, pH 7.4 with NaOH. To prepare Ca\(^{2+}\)-free Hanks’ balanced salt solution, CaCl\(_2\) was substituted with MgCl\(_2\), and 1 mM EGTA was added. Intracellular-like medium (ICM) contained 120 mM KCl, 10 mM NaCl, 1 mM KH\(_2\)PO\(_4\), 20 mM HEPES, 2 mM sodium succinate, 1 mM EGTA, pH 7.1 with KOH. The free [Ca\(^{2+}\)] was adjusted to the desired level by varying the ratio of Ca\(^{2+}\)/HEDTA, calculated using Maxchelator (C. Patton, Stanford University, CA). The following pharmacological reagents were obtained from the indicated sources: digitonin, oligomycin, and FCCP (Sigma-Aldrich); rotenone (MP Biomedicals, Santa Ana, CA); cyclosporin A (Enzo Life Sciences Inc., Farmingdale, NY); di-thiothreitol (DTT) (Fisher Scientific); Sarkosyl (IBI Scientific, Peosta, IA); CHAPS (Calbiochem); ruthenium red and valinomycin (EMD Millipore, Billerica, MA); Rhod-2 AM and tetramethylrhodamine (TMRE) (Invitrogen), and Fluo-2 AM (TEFLabs, Inc., Austin, TX). The following peptides based on the human VDAC1 sequence were synthesized by Biomatik (Wilmington, DE): control (LVLGYEGWLA), N-terminal (GLGKSARDVFTKGYGFG), and L14-15 (LAWTAGNSNTR). Cell-permeant versions were tagged with antinapenidae homodomain-derived antenapenidae (Antp; RQIKIWFQNRRMKWKK) at the C terminus of each peptide. Anti-VDAC1 mAb was purchased from EMD Millipore.

**GST Pulldown Assay and Western Blot**—Mouse VDAC1, -2, and -3 cDNAs were cloned into pGEX-6P-1 (GE Healthcare). Recombinant GST fusion proteins were expressed in *Escherichia coli* and purified using glutathione-Sepharose beads (GE Healthcare) as described (6). Beads were then incubated with cell lysates or recombinant Bcl-xL (2 \(\mu\)g) as described (6). Samples were analyzed by Western blot using antibodies against Bcl-xL (BD Biosciences) and GST (ViroGen Corp., Watertown, MA) with anti-β-actin (Sigma-Aldrich) as a loading control.

**Isolation of Mitochondrial and Mitoplast Preparations**—Mitochondria were isolated by following established protocols (21). Briefly, MEF cells were harvested and homogenized in mitochondrial isolation buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, 0.05% BSA, protease inhibitor mixture, pH 7.4 with KOH). Following centrifugation, the mitochondrial fraction was resuspended in ICM buffer prior to experimentation. Mitoplasts were generated by incubating isolated mitochondria in 4 volumes of hypotonic solution (5 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4 with KOH) for 20 min and then equilibrated on ice with 1 volume of hypertonic solution (750 mM KCl, 80 mM HEPES, 1 mM EGTA, pH 7.4 with KOH) before centrifugation. For Western blot analysis of isolated mitochondria and mitoplasts, samples were lysed and analyzed using standard approaches and blotted with anti-uncoupling protein 3 (Alpha Diagnostic International Inc., San Antonio, TX) and anti-cytochrome c (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

**Bcl-xL Knockdown Mediated by Lentivirally Encoded shRNA**—Lentiviral transduction particles (Sigma-Aldrich) carrying Bcl-xL shRNA or empty vector controls were added to cells in culture, and selection antibiotic (puromycin; 2 \(\mu\)g ml\(^{-1}\)) was added after 3 days. Single colonies were then isolated by limited dilution, and Bcl-xL knockdown was confirmed by Western blot.

**Cytoplasmic and Mitochondrial [Ca\(^{2+}\)] Measurement**—For simultaneous measurement of cytoplasmic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyto}\)) and mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{mito}\)), cells cultured on glass coverslips were loaded with 3 \(\mu\)M Rhod-2 AM by incubation at 37 °C for 30 min followed by 10 \(\mu\)M Fluo-2 AM at room temperature for a further 40 min. In experiments requiring the flash photolysis of caged Ca\(^{2+}\), the membrane-permeable caged EGTA compound α-nitrophenyl EGTA AM (5 \(\mu\)M) was added along with Fluo-2 AM, and Ca\(^{2+}\) was photoreleased by brief
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pulses (150–550 ms) of UV light delivered uniformly throughout the image field. Coverslips were then mounted in a recording chamber positioned on the stage of an inverted microscope (IX71, Olympus America Inc., Center Valley, PA). Cells were visualized using a PlanApo 60×, 1.42 numerical aperture oil immersion objective, and confocal images were acquired using a VT-Infinity 3 (VisiTech International, Sunderland, UK). Fluo-2 and Rhod-2 were alternately excited using the 488- and 568-nm lines, respectively, of a krypton-argon laser. The emitted fluorescence was filtered using a dual bandpass filter set (VisiTech International) and collected and analyzed using HCl-image software (Hamamatsu Corp., Sewickley, PA). The chamber was continuously perfused with Hanks' balanced salt solution at room temperature, and a rapid solution changer was used to switch the composition of the solution bathing the cells under study. For isolated mitochondria and mitoplasts, samples were prepared from MEF cells, loaded with Rhod-2, and imaged as described above. Wide field fluorescence microscopy was used to measure [Ca<sup>2+</sup>]<sub>mito</sub> in plasma membrane-permeabilized preparations. Rhod-2-loaded cells were mounted in a recording chamber on the stage of an inverted IX71 microscope (Olympus America Inc.) and excited at 548 nm. Emitted fluorescence was filtered at 605 nm and collected using a charge-coupled device-based imaging system running SimplePCI software (Hamamatsu Corp.). The solution perfusion and exchange system was similar to that described above. Cells were permeabilized by 3–4-min exposure to digitonin (25 μg ml<sup>−1</sup>) applied in Ca<sup>2+</sup>-free ICM. The permeabilized preparation was then allowed to equilibrate in regular Ca<sup>2+</sup>-free ICM for 15 min prior to experimental recording.

**Confocal and Electron Microscopy**—To image ER and mitochondria confocally, cells were first transsected with eYFP-ER plasmid (Clontech) using an Amaxa Nucleofector device (Lonza, Allendale, NJ) and seeded onto 35-mm glass coverslips. After 16–24 h, the cells were labeled with CellLight<sup>®</sup> Mitochondria-RFP (Invitrogen) according to the manufacturer's instructions. After a further 24 h in culture, coverslips were mounted in a chamber, and confocal images were acquired using a VT-Infinity 3 system as described above. To image ER and mitochondria using electron microscopy, cells were plated onto ACLAR film disks (Jed Pella, Redding, CA) in a 12-well tissue culture plate with 20,000 cells in each well. After incubation for 24 h, cells were fixed with 3% glutaraldehyde in cacodylate buffer (0.1 M cacodylate in 1× PBS) at 4 °C overnight. Prior to embedding, cells were treated with 2% osmium tetroxide followed by dehydration in graded ethanol (60, 90, and 100%). Cells were then embedded in LX-112 epoxy plastic (Ladd Research, Williston, VT). Ultrathin sections of 80 nm were cut, mounted on uncoated copper grids, and stained with lead citrate and saturated aqueous uranyl acetate. Images were obtained with a Philips CM12 transmission electron microscope (Philips, Andover, MA) at 80 kV.

**Mitochondrial Membrane Potential (∆Ψ<sub>m</sub>) Measurement**—Cells were permeabilized as described above and perfused with ICM containing TMRE (20 nM) for 15 min. Images were acquired using wide field fluorescence microscopy, and TMRE was present throughout the recording period. To monitor ∆Ψ<sub>m</sub> in isolated mitochondria and mitoplasts, 30 μl of the mitochondrial or the mitoplast preparation was added to the recording chamber with 20 nM TMRE, and measurements were made using confocal microscopy as described above.

**Data Collection and Analysis**—In confocal microscopy experiments using a 60× objective, data from three to six intact cells or ~100 isolated mitochondria/mitoplasts were acquired per image field. When using wide field fluorescence microscopy, data were collected using a 20× objective, enabling capture of ~30 cells per image field. For all imaging experiments, multiple fields were acquired from each coverslip, and the data pooled from three to four independent coverslips were acquired on at least two different days. When comparison of different cell lines was required, the cells were cultured at the same density and passaged in parallel, and data were acquired on the same day. Fluorescence intensity changes were background-subtracted and normalized to the initial fluorescence value F<sub>0</sub> and expressed as F/F<sub>0</sub>. Data were summarized as mean ± S.E., and differences between means were assessed using the Student’s t test for unpaired comparisons. A one-way ANOVA with Fisher’s least significant difference post hoc analysis was used for multiple comparisons. For all tests, the differences between means were accepted as statistically significant at the 95% level (p < 0.05).

**RESULTS**

**Mitochondrially Localized Bcl-x<sub>L</sub> Enhances Mitochondrial Ca<sup>2+</sup> Uptake in Intact and Permeabilized MEF Cells**—To investigate the role of Bcl-x<sub>L</sub> in regulating [Ca<sup>2+</sup>]<sub>mito</sub> uptake in response to ER Ca<sup>2+</sup> release, wild-type (WT) and Bcl-x<sub>L</sub>-KO cells were co-loaded with Rhod-2 and Fluo-2 to simultaneously monitor [Ca<sup>2+</sup>]<sub>mito</sub> and [Ca<sup>2+</sup>]<sub>cyto</sub> respectively, and imaged using confocal microscopy (Fig. 1A). To evoke an InsP<sub>3</sub>R-dependent Ca<sup>2+</sup> release, cells were challenged with 1 mM ATP applied in zero Ca<sup>2+</sup>-containing buffer. Consistent with our previous observations (9), a larger amplitude InsP<sub>3</sub>R-dependent ER Ca<sup>2+</sup> release was observed in Bcl-x<sub>L</sub>-KO cells compared with WT (Fig. 1, A and B). A concomitant rise in [Ca<sup>2+</sup>]<sub>mito</sub> was observed in both cell types, but surprisingly the magnitude of [Ca<sup>2+</sup>]<sub>mito</sub> uptake in Bcl-x<sub>L</sub>-KO cells was smaller than that in WT despite the larger ER Ca<sup>2+</sup> release (Fig. 1B). These data suggest that deletion of Bcl-x<sub>L</sub> impinges on the ability of mitochondria to accumulate Ca<sup>2+</sup>. However, this was not due to structural or morphological changes because neither the mitochondrial biomass nor the architecture of ER-mitochondrial contact sites was different in Bcl-x<sub>L</sub>-KO cells (Fig. 1, C–F).

To examine [Ca<sup>2+</sup>]<sub>mito</sub> uptake independently of ER Ca<sup>2+</sup> release, Rhod-2-loaded WT and Bcl-x<sub>L</sub>-KO cells were digitonin-permeabilized and bathed in Ca<sup>2+</sup>-free ICM containing mitochondrial substrates. After equilibration, cells were exposed to Ca<sup>2+</sup>-containing medium, and [Ca<sup>2+</sup>]<sub>mito</sub> uptake was monitored in response to a range of physiologically relevant Ca<sup>2+</sup> concentrations (Fig. 2A). As expected, addition of the [Ca<sup>2+</sup>]<sub>mito</sub> uptake blocker ruthenium red (10 μM) completely inhibited [Ca<sup>2+</sup>]<sub>mito</sub> uptake (22). Compared with WT, the magnitude of [Ca<sup>2+</sup>]<sub>mito</sub> uptake was markedly smaller in Bcl-x<sub>L</sub>-KO cells (Fig. 2B), suggesting that enhanced [Ca<sup>2+</sup>]<sub>mito</sub> uptake in Bcl-x<sub>L</sub>-expressing cells is independent of physiological coupling between the ER and mitochondria. A similar con-
clusion was drawn from a second series of experiments in intact cells. Here, a step increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) evoked by photoreleasing caged \([\text{Ca}^{2+}]_{\text{cyt}}\) resulted in more \([\text{Ca}^{2+}]_{\text{mito}}\) uptake in WT compared with Bcl-xL-KO cells (Fig. 2, C and D).

We next asked whether re-expressing Bcl-xL could restore \([\text{Ca}^{2+}]_{\text{mito}}\) uptake to Bcl-xL-KO cells. Cell lines were generated in the Bcl-xL-KO background that stably expressed Bcl-xL specifically targeted to either ER (Bcl-xL-ER) or mitochondria (Bcl-xL-mito). These cells were expanded from single clones expressing Bcl-xL at levels similar to that detected in WT and extensively characterized in our previous study (9). Permeabilized cells loaded with Rhod-2 and equilibrated in zero \([\text{Ca}^{2+}]_{\text{cyt}}\) medium were exposed to 3 μM \([\text{Ca}^{2+}]_{\text{cyt}}\) to stimulate \([\text{Ca}^{2+}]_{\text{mito}}\) uptake (Fig. 2E). The peak amplitude of the \([\text{Ca}^{2+}]_{\text{mito}}\) response and the maximum \([\text{Ca}^{2+}]_{\text{mito}}\) uptake rate were measured (Fig. 2F). Targeting Bcl-xL to ER in Bcl-xL-KO cells had no effect on \([\text{Ca}^{2+}]_{\text{mito}}\) uptake; in contrast, uptake was promoted by re-expressing Bcl-xL at the mitochondria (Fig. 2F). These findings demonstrate that localization of Bcl-xL to the mitochondria, but not ER, restores \([\text{Ca}^{2+}]_{\text{mito}}\) uptake, recapitulating the WT.

Modulation of Mitochondrial Membrane Potential Is Not a Requirement for Bcl-xL to Promote Mitochondrial \([\text{Ca}^{2+}]\) Uptake—The electrochemical driving force for \([\text{Ca}^{2+}]_{\text{mito}}\) uptake depends on both the \(\Delta \Psi_m\) and the cytosolic-mitochondrial \([\text{Ca}^{2+}]\) gradient. Interestingly, antiapoptotic Bcl-2 proteins, including Bcl-xL, have been shown to help maintain a hyperpolarized potential during cellular stress (23, 24). Given that \([\text{Ca}^{2+}]_{\text{mito}}\) uptake will drive depolarization and thus limit further \([\text{Ca}^{2+}]\) accumulation (25), it is possible that Bcl-xL promotes \([\text{Ca}^{2+}]_{\text{mito}}\) uptake by stabilizing \(\Delta \Psi_m\) and maintaining the driving force for \([\text{Ca}^{2+}]\).
**Bcl-x<sub>L</sub> Modulates Mitochondrial [Ca<sup>2+</sup>]**

![Diagram of experimental setup](image)

**FIGURE 2.** Bcl-x<sub>L</sub> promotes mitochondrial [Ca<sup>2+</sup>] uptake in permeabilized cells and under different [Ca<sup>2+</sup>]<sub>cyto</sub>. A, representative traces showing [Ca<sup>2+</sup>]<sub>mito</sub> in permeabilized WT and Bcl-x<sub>L</sub>-KO cells during step increases in external [Ca<sup>2+</sup>] from 0 to 1.25, 3.4, or 10.2 μM in the presence or absence of ruthenium red (RR; 10 μM). B, summary of Δ[Ca<sup>2+</sup>]<sub>mito</sub> in response to physiological changes in external [Ca<sup>2+</sup>]<sub>mito</sub> (mean ± S.E.; *p < 0.05; ANOVA). C, intact WT and Bcl-x<sub>L</sub>-KO cells were co-loaded with Rhod-2, Fluo-2, and α-nitrophenyl EGTA, and Ca<sup>2+</sup> was uncaged by pulses of UV light ranging from 150 to 550 ms. Representative time course plots of [Ca<sup>2+</sup>]<sub>mito</sub> and [Ca<sup>2+</sup>]<sub>cyclo</sub> are shown. D, the amplitude of the change in [Ca<sup>2+</sup>]<sub>mito</sub> with respect to the amplitude of the evoked [Ca<sup>2+</sup>]<sub>cyto</sub> change in WT and Bcl-x<sub>L</sub>-KO cells. Data from individual cells were pooled and binned according to the amplitude of [Ca<sup>2+</sup>]<sub>mito</sub> (mean ± S.E.; **p < 0.001; ANOVA). Error bars represent S.E.

Resting ΔΨ<sub>m</sub> was measured in WT and Bcl-x<sub>L</sub>-KO cells after permeabilization and equilibration in ICM containing mitochondrial substrates and the membrane potential probe TMRE (20 nm). TMRE fluorescence was normalized to the fluorescence acquired after complete ΔΨ<sub>m</sub> dissipation by the photophoretic FCCP (10 μM). There was no significant difference in resting ΔΨ<sub>m</sub> between WT and Bcl-x<sub>L</sub>-KO cells (Fig. 3A). We next measured dynamic changes in ΔΨ<sub>m</sub> by monitoring TMRE fluorescence in permeabilized WT and Bcl-x<sub>L</sub>-KO cells. As expected, switching the external bathing medium from 0 to 3 μM Ca<sup>2+</sup> evoked a depolarization that was reversed upon Ca<sup>2+</sup> removal (Fig. 3B). The magnitude of depolarization evoked by 3 μM Ca<sup>2+</sup>, however, was larger in WT, presumably due to more Ca<sup>2+</sup> accumulation in these cells. Thus, Bcl-x<sub>L</sub> does not promote [Ca<sup>2+</sup>]<sub>mito</sub> uptake by maintaining a hyperpolarized ΔΨ<sub>m</sub> and strong Ca<sup>2+</sup> driving force. Interestingly, upon Ca<sup>2+</sup> removal, ΔΨ<sub>m</sub> recovered much faster in WT compared with Bcl-x<sub>L</sub>-KO when quantified as the halftime for recovery to baseline (Fig. 3C), consistent with the hypothesis that Bcl-x<sub>L</sub> stabilizes ΔΨ<sub>m</sub> (see “Discussion”).

To further confirm that Bcl-x<sub>L</sub> does not affect [Ca<sup>2+</sup>]<sub>mito</sub> uptake by influencing ΔΨ<sub>m</sub>, [Ca<sup>2+</sup>]<sub>mito</sub> uptake was measured under conditions where the driving force was exclusively due to the Ca<sup>2+</sup> gradient. Cells loaded with Rhod-2 were permeabilized and perfused in Ca<sup>2+</sup>-free ICM. To completely dissipate ΔΨ<sub>m</sub>, the solution was switched to ICM without mitochondrial substrates and containing 5 μM FCCP, 8 μg ml<sup>-1</sup> oligomycin, 10 μM rotenone (26). After 2 min, the [Ca<sup>2+</sup>] in the bathing medium was increased to 3 μM (Fig. 3D). The amplitude and maximum rate of Ca<sup>2+</sup> uptake, however, were larger in the WT group (Fig. 3E), indicating that Bcl-x<sub>L</sub> enhances Ca<sup>2+</sup> uptake when the driving force is only due to the [Ca<sup>2+</sup>] gradient. In addition, [Ca<sup>2+</sup>]<sub>mito</sub> uptake was monitored under conditions in which ΔΨ<sub>m</sub> was generated artificially in WT and Bcl-x<sub>L</sub>-KO cells. Here, WT and Bcl-x<sub>L</sub>-KO cells were permeabilized, and the ΔΨ<sub>m</sub> was dissipated as described. The bathing [K<sup>+</sup>] was
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**FIGURE 3.** Bcl-xL promotes mitochondrial Ca²⁺ uptake independently of mitochondrial membrane potential. A, ΔΨᵢ in WT and Bcl-xL-KO cells assessed by TMRE fluorescence normalized to fluorescence after addition of FCCP (10 μM). Data represent mean ± S.E. (p > 0.05; Student’s t test). B, representative traces of ΔΨᵢ in response to the addition and removal of 3 μM [Ca²⁺]. The amplitude of the Ca²⁺-induced depolarization (ΔFₐ/F₀, TMRE) in WT and Bcl-xL-KO cells was 0.80 ± 0.01 and 0.75 ± 0.01, respectively (p < 0.001; Student’s t test). C, the half-times for the ΔΨᵢ recovery upon Ca²⁺ removal are summarized. Data represent mean ± S.E. (**p < 0.01; Student’s t test). D, permeabilized cells were treated with FCCP, oligomycin, rotenone, and valinomycin to collapse ΔΨᵢ. Traces are depicted showing the Ca²⁺ gradient-driven [Ca²⁺]ᵢ/mito uptake and efflux during application and removal of 3 μM Ca²⁺. E, the summary bar graphs show the peak [Ca²⁺]ᵢ/mito amplitude and the maximal rate of [Ca²⁺]ᵢ/mito uptake in 0 ΔΨᵢ (mean ± S.E., *p < 0.05, **p < 0.01, ***p < 0.001; Student’s t test). F, typical records showing ΔΨᵢ hyperpolarization measured with TMRE in response to stepping [K⁺] from 140 to 0.1 mM in permeabilized WT and Bcl-xL-KO cells incubated without mitochondria substrates in the presence of FCCP, oligomycin, rotenone, and valinomycin. The amplitude of the hyperpolarization (ΔFₐ/F₀, TMRE) in WT and Bcl-xL-KO cells was 2.61 ± 0.06 and 2.71 ± 0.04, respectively (mean ± S.E., p < 0.05; Student’s t test). G, representative traces showing [Ca²⁺]ᵢ/mito accumulation during a step increase in bathing [Ca²⁺] from 0 to 3 μM when ΔΨᵢ was [K⁺] gradient-driven. Under these conditions, the mean ± S.E. amplitude (ΔFₐ/F₀, TMRE) recorded in WT and Bcl-xL-KO cells was 10.96 ± 0.19 and 8.20 ± 0.16 (p < 0.001; Student’s t test), and the maximum uptake rate (ΔFₐ/F₀Δt) was 1.12 ± 0.02 in WT compared with 0.89 ± 0.02 in Bcl-xL-KO (p < 0.001; Student’s t test). Error bars represent S.E.

The effect of Bcl-xL on [Ca²⁺]ᵢ/mito uptake persisted (Fig. 3G). These data strongly support a model in which Bcl-xL regulation of Ca²⁺ uptake into the matrix is largely independent of any influence it may have on ΔΨᵢ. 

Promotion of [Ca²⁺]ᵢ/mito Uptake by Bcl-xL Is Dependent on the Integrity of the Outer Mitochondrial Membrane—The mitochondrial permeability transition pore (mPTP) is a molecular complex spanning the outer and inner membranes whose formation collapses ΔΨᵢ (27). The mPTP is normally associated with pathological states, but transient mPTP formation under physiological conditions can function as a Ca²⁺ efflux pathway. Importantly, inhibiting this efflux pathway by blocking mPTP has been shown to promote [Ca²⁺]ᵢ/mito uptake (28). Because Bcl-xL also has an inhibitory effect on mPTP (29), we tested the hypothesis that Bcl-xL mediates its effect on [Ca²⁺]ᵢ/mito through mPTP inhibition. Isolated mitochondria were prepared from WT and Bcl-xL-KO cells, suspended in zero Ca²⁺ medium, loaded with Rhod-2, and imaged confocally. Consistent with the data collected using intact and permeabilized cells, the peak amplitude of the [Ca²⁺]ᵢ/mito response and the maximum [Ca²⁺]ᵢ/mito uptake rate were significantly larger in WT compared with Bcl-xL-KO (Fig. 4A, control traces). Treatment with the mPTP inhibitor cyclosporine A, however, had no effect on Ca²⁺ uptake in either WT or Bcl-xL-KO mitochondria (Fig. 4A and B), indicating that mPTP modulation cannot account for the effect of Bcl-xL on [Ca²⁺]ᵢ/mito handling.

It is widely accepted that Bcl-xL resides primarily on the outer mitochondrial membrane; however, an important role for its localization at the inner membrane has recently been identified (30, 31). We therefore asked whether the effect of Bcl-xL on matrix Ca²⁺ accumulation persisted after removal of the outer membrane. Mitoplasts were prepared, and disruption of the outer mitochondrial membrane was confirmed by the loss of the intermembrane protein cytochrome c (Fig. 4C). The inner mitochondrial membrane remained viable as indicated by the presence of the inner membrane protein uncoupling protein 3 along with an intact ΔΨᵢ measured with TMRE (Fig. 4C and D). With respect to the magnitude and maximum rate of Ca²⁺ uptake, there was no statistically significant difference between mitoplasts prepared from WT or Bcl-xL-KO cells (Fig. 4E). These data indicate that localization to the outer mitochondrial membrane is required for Bcl-xL to promote [Ca²⁺]ᵢ/mito uptake.

The Effect of Bcl-xL on [Ca²⁺]ᵢ/mito Uptake Is Dependent on an Interaction with VDAC—The VDAC is the major permeability pathway for Ca²⁺ transfer across the outer mitochondrial membrane (27). This channel is known to directly interact with several members of the Bcl-2 family, including Bcl-xL (19, 32–34). Based on an analysis of the binding determinants, peptides representing sections of the VDAC1 sequence were shown previously to be highly effective in disrupting the interaction between either Bcl-2 or Bcl-xL and VDAC1 (20, 35). We adopted this approach to study the effect of VDAC1/Bcl-xL interactions on [Ca²⁺]ᵢ/mito uptake. Two peptides (N-terminal (N-ter) and L14-15) were synthesized based on previously published sequences shown to block binding between VDAC1 and Bcl-2 (35). In lysates from WT MEF cells, the interaction between VDAC1 and Bcl-xL (detected using a GST pulldown then decreased to 0.1 mM (KCl was substituted with choline chloride) in the presence of the K⁺ ionophore valinomycin (50 ng ml⁻¹). The ensuing outward K⁺ current rapidly hyperpolarized the ΔΨᵢ as measured with TMRE, and the K⁺-driven ΔΨᵢ attained a new steady state in about 50 s and remained stable for ~30 s (Fig. 3F). Under these conditions, it would be extremely unlikely for Bcl-xL to influence ΔΨᵢ; however, the
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**Assay** was eliminated by pretreating cells with cell-permeant N-ter and L14-15 peptides but not by pretreatment with control peptide (see Fig. 6C). In imaging studies, permeabilized cells were incubated with peptides (2 μM for 2 min) prior to stimulating [Ca<sup>2+</sup>]<sub>mito</sub> uptake and remained present throughout the experiment. This effectively reduced the amplitude of [Ca<sup>2+</sup>]<sub>mito</sub> uptake in WT to levels normally observed in Bcl-x<sub>L</sub>-KO cells but had no effect on Bcl-x<sub>L</sub>-KO cells (Fig. 5, A and B). These results strongly indicate that Bcl-x<sub>L</sub> facilitates [Ca<sup>2+</sup>]<sub>mito</sub> uptake by interacting with VDAC. To support the hypothesis that this interaction is likely to be ubiquitous, the effect of cell-permeant peptides on [Ca<sup>2+</sup>]<sub>mito</sub> uptake were assessed in HeLa cells and found to have qualitatively similar effects (Fig. 5, C and D). The fact that peptides are effective in other cell types also increases confidence that observations made in the Bcl-x<sub>L</sub>-KO MEF cells are not due to artifacts or adaptations to gene knock-out. In our previous study, we performed microarray analysis of WT and Bcl-x<sub>L</sub>-KO cell lines and adaptations to gene knock-out. In our previous study, we performed microarray analysis of WT and Bcl-x<sub>L</sub>-KO cell lines and adaptations to gene knock-out. In our previous study, we performed microarray analysis of WT and Bcl-x<sub>L</sub>-KO cell lines and adaptations to gene knock-out.

**Bcl-x<sub>L</sub> Interacts with VDAC1 and VDAC3 to Regulate [Ca<sup>2+</sup>]<sub>mito</sub> Uptake—**To determine whether Bcl-x<sub>L</sub> interacts with all three VDAC isoforms, GST fusion proteins of VDAC1, -2, and -3 were generated, and the binding of purified recombinant Bcl-x<sub>L</sub> was assessed. GST-VDAC1 and -3 bound robustly to purified recombinant Bcl-x<sub>L</sub> (Fig. 6A). In addition, GST-VDAC3 and -3 effectively pulled down endogenous Bcl-x<sub>L</sub> from WT MEF cell lysates (Fig. 6C). Pretreating WT MEF cells with cell-permeant N-ter and L14-15 peptides had no effect on Bcl-x<sub>L</sub> expression levels (Fig. 6D). Collectively, these biochemical data suggest that Bcl-x<sub>L</sub> interacts with VDAC1 and -3 and that binding is effectively disrupted by peptides based on the VDAC1 sequence.

Next we investigated the effect of Bcl-x<sub>L</sub> and VDAC isoform interactions on [Ca<sup>2+</sup>]<sub>mito</sub> uptake. WT and VDAC knock-out MEF cells, including VDAC1, VDAC2, VDAC3 single knock-outs and a VDAC1 and -3 double knock-out cell line, were loaded with Rhod-2 and permeabilized, and [Ca<sup>2+</sup>]<sub>mito</sub> uptake was measured as described earlier. The presence of N-ter and L14-15 peptides decreased the [Ca<sup>2+</sup>]<sub>mito</sub> uptake in all cell lines except the VDAC1 and -3 double KO in which only VDAC2 was expressed. Consistent with the binding studies, these data suggest that Bcl-x<sub>L</sub> interacts with both VDAC1 and -3 to promote [Ca<sup>2+</sup>]<sub>mito</sub> uptake and that this is inhibited by disrupting

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**FIGURE 4.** Promotion of [Ca<sup>2+</sup>]<sub>mito</sub> uptake by Bcl-x<sub>L</sub> is dependent on the integrity of the outer mitochondrial membrane but not on mPTP opening. A, typical traces showing [Ca<sup>2+</sup>]<sub>mito</sub> uptake monitored confocally in Rhod-2-loaded mitochondria isolated from WT and Bcl-x<sub>L</sub>-KO cells. Extracellular [Ca<sup>2+</sup>] was stepped from 0 to 3 μM in the absence or presence of cyclosporine A (CAs1 μM). B, bar graphs summarizing (mean ± S.E.) the peak [Ca<sup>2+</sup>]<sub>mito</sub> uptake and maximal uptake rate (***, p < 0.001; ANOVA). C, representative Western blot detecting uncoupling protein 3 (UCP3) and cytochrome c (cyto c) in isolated mitochondrial and mitoplast preparations. D, representative confocal sections of mitoplasts in the presence of TMRE (20 nM) before and after the addition of FCCP (10 μM). Scale bar, 2 μm. E, summary data (mean ± S.E.) depicting peak [Ca<sup>2+</sup>]<sub>mito</sub> uptake and uptake rate monitored in response to a step increase in extracellular [Ca<sup>2+</sup>] from 0 to 3 μM in mitoplasts prepared from WT and Bcl-x<sub>L</sub>-KO cells (p > 0.05; Student’s t test). Error bars represent S.E.
the interaction using peptides based on the VDAC1 sequence. Of note, deletion of VDAC isoforms either singly or in combination did not appear to reduce $[\text{Ca}^{2+}]_{\text{mito}}$ uptake. This was surprising and suggests some form of adaptation (see “Discussion”).

To further verify the effect of Bcl-x<sub>L</sub> on VDAC isoforms, particularly VDAC2, we generated stable Bcl-x<sub>L</sub> knockdown cell lines in the MEF WT, VDAC1, and VDAC1 and -3 double KO background. Bcl-x<sub>L</sub> knockdown was achieved by infecting cells with lentiviral particles carrying Bcl-x<sub>L</sub> shRNA and verified by Western blot (Fig. 6E). When compared with cells generated using control shRNA, WT and VDAC1 KO cells generated with Bcl-x<sub>L</sub> shRNA showed less $[\text{Ca}^{2+}]_{\text{mito}}$ uptake when permeabilized and challenged with 3 μM $\text{Ca}^{2+}$ (Fig. 6F). As
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**DISCUSSION**

It is well established that VDAC is a major permeability pathway for the transfer of Ca$^{2+}$ across the outer mitochondrial membrane (15–17). The major finding of the current study is that this permeability pathway is tightly regulated by a protein/protein interaction between Bcl-xL and VDAC isoforms 1 and 3. Moreover, endogenous levels of mitochondrially localized Bcl-xL under basal conditions in the absence of apoptotic stimuli facilitates Ca$^{2+}$ uptake into the mitochondrial matrix in response to physiological Ca$^{2+}$ elevations.

**Morphological and Structural Properties of Mitochondria Are Unaffected by the Loss of Bcl-xL.—** There are several scenarios in which previously reported functions of Bcl-xL could be invoked to explain the observed data. Bcl-xL has been shown to regulate mitochondrial fusion, fission, and biomass (36), and mitochondrial morphology can be an important factor in shaping the Ca$^{2+}$ signal (37). Could Bcl-xL-dependent structural changes in mitochondria be at play in the current study? This does not seem to be the case because imaging experiments at both confocal and electron microscopic resolutions did not reveal any apparent difference between mitochondria in WT and Bcl-xL-KO cells (Fig. 1, C–F). Moreover, in both cell types, the expression levels of key mitochondrial proteins were the same, indicating that there is no difference in the total number of mitochondria.

Regions of close contact between mitochondria and ER represent specific microdomains that enable Ca$^{2+}$ released from ER channels to be effectively sensed by closely apposed mitochondria either by virtue of proximity or through protein/protein interactions that bridge the organelles (15, 38). Considering that Bcl-xL interacts with both VDAC and InsP$_3$R, it is possible that all three proteins exist in a tertiary complex at the ER-mitochondria interface to facilitate ER-mitochondrial Ca$^{2+}$ transfer. We failed to find any difference in the number or distance between ER-mitochondrial contact sites in WT and Bcl-xL-KO cells, arguing against such a model; however, redistribution of proteins to the contact sites remains a possibility. Nevertheless, increased Bcl-xL-dependent [Ca$^{2+}]_{\text{mito}}$ uptake persisted in permeabilized cells and isolated mitochondria but not in mitoplasts, strongly implicating a mechanism intrinsic to the outer mitochondrial membrane.

**Bcl-xL Does Not Promote Mitochondrial Ca$^{2+}$ Uptake by Influencing mPTP or $\Delta\Psi_m$.—** The observations that antiapoptotic Bcl-2 members regulate the sensitivity to mPTP opening (24, 39, 40) and that physiological mPTP activation may be important in mitochondrial Ca$^{2+}$ homeostasis (28) raise the possibility that Bcl-xL increases [Ca$^{2+}]_{\text{mito}}$ uptake by inhibiting mPTP activity. In this model, [Ca$^{2+}]_{\text{mito}}$ uptake transiently induces the formation of the mPTP, which functions as a Ca$^{2+}$ removal pathway; Bcl-xL would be expected to inhibit mPTP and hence removal, thereby promoting greater matrix Ca$^{2+}$ accumulation. However, this was not the case in the current study because pharmacological inhibition of mPTP with cyclosporin A had no effect on [Ca$^{2+}]_{\text{mito}}$ uptake in mitochondria isolated from either WT or Bcl-xL-KO cells (Fig. 4).

Both Bcl-2 and Bcl-xL have long been known to regulate $\Delta\Psi_m$ by helping to maintain hyperpolarization during mito-
clonal stress (39, 41, 42). Presumably, this enables a greater, more tolerable \([\text{Ca}^{2+}]_{\text{mito}}\) load and would be antiapoptotic because \(\Delta \Psi_{m}\) loss is a prerequisite for cytochrome c release. More recently, a series of elegant studies in neurons demonstrated that Bcl-x<sub>L</sub> when localized to the inner mitochondrial membrane functions to inhibit an ion leak pathway that is essential to its role in \(\Delta \Psi_{m}\) stabilization (30, 31). Interestingly, Bcl-x<sub>L</sub> knockdown in these cells was associated with increased \(\Delta \Psi_{m}\) (31). In our study, resting \(\Delta \Psi_{m}\) was similar in WT and Bcl-x<sub>L</sub>-KO cells, although faster repolarization was observed in WT after a \([\text{Ca}^{2+}]_{\text{mito}}\) increase, consistent with the role of Bcl-x<sub>L</sub> in \(\Delta \Psi_{m}\) stabilization. The function of Bcl-x<sub>L</sub> as a \(\Delta \Psi_{m}\) modulator, however, cannot account for the majority of its effects on \([\text{Ca}^{2+}]_{\text{mito}}\) because increased \([\text{Ca}^{2+}]_{\text{mito}}\) uptake persisted under conditions where Bcl-x<sub>L</sub> cannot impinge on \(\Delta \Psi_{m}\); either when \(\Delta \Psi_{m}\) was completely collapsed (Fig. 3D) or when \(\Delta \Psi_{m}\) was generated artificially by a \(K^+\) gradient (Fig. 3G).

**Outer Membrane-localized Bcl-x<sub>L</sub> Facilitates \([\text{Ca}^{2+}]_{\text{mito}}\) Uptake into the Mitochondrial Matrix**—There are two lines of evidence to support the idea that Bcl-x<sub>L</sub> exerts the majority of its effects on \([\text{Ca}^{2+}]_{\text{mito}}\) uptake by acting at the outer, rather than the inner, mitochondrial membrane. First, expressing mitochondrially targeted Bcl-x<sub>L</sub> in the Bcl-x<sub>L</sub>-KO background recapitulated the \([\text{Ca}^{2+}]_{\text{mito}}\) uptake phenotype of WT cells (Fig. 2E). In these studies, Bcl-x<sub>L</sub> was tagged with the membrane localization sequence of the listerial protein ActA, a well-established approach that targets proteins to the outer membrane with cytoplasmic orientation (9, 43). Second, the increase in \([\text{Ca}^{2+}]_{\text{mito}}\) uptake conferred by Bcl-x<sub>L</sub> was lost in mitoplasts prepared from mitochondria isolated from Bcl-x<sub>L</sub>-expressing cells (Fig. 4E), effectively ruling out the possibility that Bcl-x<sub>L</sub> functions by interacting with inner membrane proteins.

**The Effect of Bcl-x<sub>L</sub> on Mitochondrial \([\text{Ca}^{2+}]_{\text{mito}}\) Uptake Requires an Interaction with VDAC1 or VDAC3**—Traditionally, the outer mitochondrial membrane was not thought to offer a significant permeability barrier to \([\text{Ca}^{2+}]_{\text{mito}}\); however, this paradigm has been challenged by studies demonstrating that it not only limits \([\text{Ca}^{2+}]_{\text{mito}}\) delivery to the unipporter (44) but can do so in a regulated fashion (16, 17, 26). Our study provides a novel physiological mechanism whereby the magnitude of \([\text{Ca}^{2+}]_{\text{mito}}\) transfer to the mitochondrial matrix is regulated by protein/protein interactions between Bcl-x<sub>L</sub> and VDAC isoforms 1 and 3 (Figs. 5 and 6). To our knowledge, ours is also the first study to demonstrate that Bcl-x<sub>L</sub> binds to VDAC1 and -3 but not to VDAC2. The lack of binding to VDAC2 is particularly interesting because the proapoptotic Bcl-2 protein BAK is known to interact only with VDAC2 and functions to recruit BAK to the outer mitochondrial membrane (45, 46). Our data therefore raise the possibility that the binding exclusivity and functional outcomes of the Bak/VDAC2 interaction could be facilitated by the inability of VDAC2 to interact with Bcl-x<sub>L</sub>.

We have focused exclusively on Bcl-x<sub>L</sub>; however, Bcl-2 is also known to bind VDAC1 (35). Despite a large degree of structural and functional homology between these two antiapoptotic proteins, it cannot be assumed that they both affect VDAC function in the same way. In discussion of the current data, therefore, it is only meaningful to consider the literature regarding the Bcl-x<sub>L</sub>/VDAC1 interaction. VDAC gating involves voltage-dependent transitions between high and low conductance states that are associated with dramatic changes in selectivity. Open conformations are high conductance, anion-selective states permissive to the transfer of metabolites; closed conformations are lower conductance states favoring cation selectivity (13). Biochemical and structural studies have defined the Bcl-x<sub>L</sub>/VDAC1 interaction (33, 34, 47); however, the functional implications are still unclear. Initial studies suggested that Bcl-x<sub>L</sub> closed the channel as evidenced by the inhibition of radiolabeled sucrose uptake into VDAC1-containing liposomes (47). More recent electrophysiological experiments, however, have not produced a consistent set of observations with Bcl-x<sub>L</sub> being shown to promote both the open (18) and closed (20) conformations across similar voltage ranges. Because the closed state is cation-selective and more \([\text{Ca}^{2+}]_{\text{mito}}\)-permeable (17), our observation that Bcl-x<sub>L</sub> facilitated \([\text{Ca}^{2+}]_{\text{mito}}\) uptake is consistent with a model in which Bcl-x<sub>L</sub> promotes VDAC closing. On the other hand, Bcl-x<sub>L</sub>-induced VDAC opening cannot be ruled out because a large conductance, cation-selective open state has been described (17, 48), and it is possible that Bcl-x<sub>L</sub> could increase the frequency of transitions to this state. Future studies will address exactly how Bcl-x<sub>L</sub> binding affects VDAC1 \([\text{Ca}^{2+}]_{\text{mito}}\) permeability.

In addition to direct effects on VDAC cation permeability, there are several other ways in which Bcl-x<sub>L</sub> could affect \([\text{Ca}^{2+}]_{\text{mito}}\) uptake. The interaction between VDAC and the adenine nucleotide translocase of the inner membrane (49) provides precedent that VDAC exists in complexes spanning the outer and inner membranes. It remains to be determined whether or not VDAC directly interacts with the \([\text{Ca}^{2+}]_{\text{mito}}\) transport machinery on the inner membrane in a similar way, but it is an intriguing possibility. Another factor to consider is that Bcl-x<sub>L</sub> regulates the oligomeric state of the channel. In the absence of Bcl-x<sub>L</sub>, VDAC self-associates to form dimers, trimers, and tetramers (50), whereas binding of Bcl-x<sub>L</sub> promotes the formation and stabilization of VDAC dimers (34). The potential physiological importance of VDAC self-association was illustrated by bilayer experiments showing that VDAC cross-linking had profound effects on channel conductance (50). Intriguingly, when compared with WT controls, we observed increased \([\text{Ca}^{2+}]_{\text{mito}}\) uptake in VDAC1 and VDAC3 knockouts (Fig. 6D). This is somewhat counterintuitive because it is already known that deletion of any given VDAC isoform does not result in compensatory up-regulation of the remaining isoforms in these knock-out MEF cells (51). This opens up the possibility that knocking down a specific isoform alters \([\text{Ca}^{2+}]_{\text{mito}}\) permeation through the remaining isoforms. Perhaps such functional compensation is driven by changes in the heterooligomeric makeup of VDAC multimers. However, this is pure speculation, and additional studies will be required to define intermembrane complexes and assess the effect of VDAC oligomerization and the role of individual isoforms on the \([\text{Ca}^{2+}]_{\text{mito}}\) permeability of the outer membrane.

**The Physiological Implications for Bcl-x<sub>L</sub>-regulated \([\text{Ca}^{2+}]_{\text{mito}}\)**—We showed previously that Bcl-x<sub>L</sub> functions at the ER by allosterically modulating the InsP<sub>3</sub>R to increase the frequency of oscillating \([\text{Ca}^{2+}]_{\text{mito}}\) signals under resting conditions (5, 6). We now demonstrate that through its interactions with...
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VDAC1 and VDAC3 Bcl-x<sub>L</sub> also enables the ER-released Ca<sup>2+</sup> to be more effectively taken up by mitochondria. This is physiologically significant because constitutive Ca<sup>2+</sup> signaling to the mitochondrial matrix is essential for the maintenance of cellular bioenergetics (52). Moreover, the ability to maintain bioenergetic competence in the face of apoptotic stimuli is strongly antiapoptotic, and this is now recognized to be one of the mechanisms by which Bcl-x<sub>L</sub> is protective (18, 31, 53, 54). Thus, our data suggest a role for mitochondrial Ca<sup>2+</sup> signaling in bioenergetics regulated by outer membrane-localized Bcl-x<sub>L</sub>.

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