The BH4 Domain of Anti-apoptotic Bcl-XL, but Not That of the Related Bcl-2, Limits the Voltage-dependent Anion Channel 1 (VDAC1)-mediated Transfer of Pro-apoptotic Ca\(^{2+}\) Signals to Mitochondria*

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Excessive Ca\(^{2+}\) fluxes from the endoplasmic reticulum to the mitochondria result in apoptotic cell death. Bcl-2 and Bcl-XL proteins exert part of their anti-apoptotic function by directly targeting Ca\(^{2+}\)-transport systems, like the endoplasmic reticulum-localized inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) and the voltage-dependent anion channel 1 (VDAC1) at the outer mitochondrial membranes. We previously demonstrated that the Bcl-2 homology 4 (BH4) domain of Bcl-2 protects against Ca\(^{2+}\)-dependent apoptosis by binding and inhibiting IP\(_3\)Rs, although the BH4 domain of Bcl-XL was protective independently of binding IP\(_3\)Rs. Here, we report that in contrast to the BH4 domain of Bcl-2, the BH4 domain of Bcl-XL binds and inhibits VDAC1. In intact cells, delivery of the BH4-Bcl-XL peptide via electroporation limits agonist-induced mitochondrial Ca\(^{2+}\) uptake and protects against staurosporine-induced apoptosis. Moreover, delivery of the N-terminal domain of VDAC1 as a synthetic peptide (VDAC1-NP) abolishes the ability of BH4-Bcl-XL to suppress mitochondrial Ca\(^{2+}\) uptake and to protect against apoptosis. Importantly, VDAC1-NP did not affect the ability of BH4-Bcl-2 to suppress agonist-induced Ca\(^{2+}\) release in the cytosol or to prevent apoptosis, as done instead by an IP\(_3\)-R-derived peptide. In conclusion, our data indicate that the BH4 domain of Bcl-XL, but not that of Bcl-2, selectively targets VDAC1 and inhibits apoptosis by decreasing VDAC1-mediated Ca\(^{2+}\) uptake into the mitochondria.

Intracellular Ca\(^{2+}\) signals originating from the endoplasmic reticulum (ER)\(^6\) critically control mitochondrial functions ranging from pro-survival mitochondrial bio-energetics and autophagy to pro-death mitochondrial outer membrane permeabilization (MOMP) and apoptosis (1–3). ER mitochondria Ca\(^{2+}\) cross-talk is mainly due to two features as follows: 1) the close proximity between the ER and mitochondria at specific contact sites called mitochondrion-associated ER membranes (MAMs) (4–6); and 2) the high driving force for Ca\(^{2+}\) accumu-

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6 The abbreviations used are: ER, endoplasmic reticulum; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetracetic acid; BH domain, Bcl-2 homology domain; IDP, IP\(_3\)-R-derived peptide; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_R\), IP\(_3\) receptor; MAM, mitochondrion-associated ER membranes; HBSS, Hanks’ balanced salt solution; MEF, mouse embryonic fibroblast; MOM, mitochondrial outer membrane; MOMP, mitochondrial outer membrane permeabilization; STS, staurosporine; VDAC, voltage-dependent anion channel; VDAC1-NP, VDAC1 N-terminal peptide; OMM, outer mitochondrial membrane; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.
loration into the matrix of the mitochondria, due to an existing membrane potential, usually between −150 and −180 mV, across the inner mitochondrial membrane (7). MAMs are hotspots for the Ca\(^{2+}\) transfer mediated by ER and mitochondrial Ca\(^{2+}\)-transport mechanisms that are physically linked. Inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (IP\(_3\)Rs), ER-resident intracellular Ca\(^{2+}\)-release channels, are indirectly linked to the Ca\(^{2+}\)-permeable voltage-dependent anion channels (VDACs) (8, 9). Further studies revealed that particular isoforms may be preferentially located in the MAMs. For instance, in HeLa cells IP\(_3\)R3 and VDAC1 have been specifically involved in mediating the transfer of pro-apoptotic Ca\(^{2+}\) signals from the ER into the mitochondria (10, 11).

Given the central role of the IP\(_3\)R and VDAC1 in apoptosis (12, 13), it is not surprising that anti- and pro-apoptotic proteins target these channels, thereby controlling the cellular sensitivity to Ca\(^{2+}\)-dependent apoptosis. In particular, different anti-apoptotic members of the Bcl-2 protein family, including Bcl-2, Bcl-XL, and Mcl-1, have attributed important roles in regulating IP\(_3\)R and VDAC1 channel activities, thereby exerting part of their anti-apoptotic function via the control of Ca\(^{2+}\) fluxes across ER and mitochondrial membranes (14–17). Furthermore, selective roles for distinct anti-apoptotic Bcl-2 family members are emerging. Bcl-2 mainly suppresses pro-apoptotic Ca\(^{2+}\) transients by dampening Ca\(^{2+}\) flux through IP\(_3\)R channels (18), although Bcl-XL promotes pro-survival Ca\(^{2+}\) oscillations by sensitizing IP\(_3\)Rs to low levels of IP\(_3\) signaling (19). These distinct effects of Bcl-2 and Bcl-XL on IP\(_3\)R function reflect differences in the molecular determinants underlying their interaction with the IP\(_3\)R. Bcl-2 through its BH4 domain targets a site located in the center of the modulatory and regulatory domain of the IP\(_3\)R, although Bcl-XL, through a yet unidentified domain, preferentially targets the 6th transmembrane domain located in the proximity of the C-terminal part of the IP\(_3\)R’s Ca\(^{2+}\) channel pore (20).

We recently found a critical and conserved difference between the BH4 domain of Bcl-2 (Lys-17) and Bcl-XL (Asp-11), being responsible for the privileged binding and inhibition of IP\(_3\)R channels by BH4-Bcl-2 but not by BH4-Bcl-XL (21). Moreover, although both BH4-Bcl-2 and BH4-Bcl-XL were able to protect against apoptosis upstream of MOMP, only BH4-Bcl-2-mediated protection was abolished by the co-addition of a peptide corresponding to the Bcl-2-binding site on IP\(_3\)Rs (IP\(_3\)R-derived peptide; IDP). In addition, the co-administration of both BH4-Bcl-2 and BH4-Bcl-XL peptides did not provide any additive protective effect against apoptosis, and neither BH4-Bcl-2 nor BH4-Bcl-XL protected against cell death induced by procaspase-activating compound 1, a post-MOMP caspase (cysteine-dependent aspartate-specific protease) activator (21, 22). Hence, we hypothesized that the protective effect of BH4-Bcl-XL was due to its action on a target downstream of IP\(_3\)R signaling but upstream of caspases, e.g. at the cross-road between ER and mitochondria. Considering the preferential distribution of Bcl-XL to the OMM (23, 24), we therefore anticipated the possible involvement of a mitochondrial target. In this respect, earlier studies (25) showed that the BH4 domain of Bcl-XL was more potent than the one of Bcl-2 in suppressing VDAC1-mediated mitochondrial swelling. Later studies also revealed that Bcl-2 and Bcl-XL proteins directly bind to VDAC1 and modulate its conductance, with the VDAC1 N-terminal region being an important region for its function (26–30).

Driven by these previous studies and observations, we hypothesized that the anti-apoptotic effect of the BH4 domain of Bcl-XL could be due to its targeting of VDAC1 and inhibition of VDAC1-mediated Ca\(^{2+}\) transfer into the mitochondria. To test this assumption, we first re-examined the role of VDAC1 as a mitochondrial Ca\(^{2+}\) entry mechanism and simultaneously profiled the molecular interaction of VDAC1 with both Bcl-2 and Bcl-XL. We compared the alleged ability of Bcl-2 and Bcl-XL BH4 peptides to bind VDAC1, to control its single channel activity, and to protect against Ca\(^{2+}\)-mediated apoptosis. Our results propose a novel role for the BH4 domain of Bcl-XL in apoptosis and in mitochondrial Ca\(^{2+}\) entry by controlling VDAC1 channel conductance, while the BH4 domain of Bcl-2 would mainly act at the level of the IP\(_3\)R channels.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Peptides—**Both wild-type (WT) and VDAC1 knock-out (VDAC1(−/−)) mouse embryonic fibroblast cells (MEFs) were kindly provided by Dr. W. J. Craigen, Baylor College of Medicine, Houston, TX (31). MEFs were maintained at 37 °C and 5% CO\(_2\) in DMEM medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma), 2 mM L-glutamine (GlutaMAX, Life Technologies), 1 mM pyruvate, 7.5 mM KCl, 25 mM HEPES, 1% penicillin/streptomycin. Rat C6 glioma cells and COS-1 cells were cultured as described previously (21). Peptides used in this study were obtained from Thermo Electron (Germany) or from Lifetein when biotinylation was necessary. All peptides in this study were obtained from Thermo Electron (Germany) or from Lifetein when biotinylation was necessary. All peptides were more than 80% pure, and their identity was confirmed via mass spectrometry. Their respective amino acid sequences are given in Table 1.

**SDS-PAGE, Western Blotting, and Antibodies—**COS-1 and MEFs were lysed in a lysis buffer containing 10 mM Hepes, pH 7.5, 0.25% Nonidet P-40, 140 mM KCl, 5 mM MgCl\(_2\), 2 mM EDTA, 1 mM EGTA and containing protease inhibitor mixture (Roche Applied Science). The Bradford assay (Sigma) was used to determine sample protein concentrations relative to the standard curve of bovine serum albumin (BSA). Samples for SDS-PAGE were prepared by adding NuPAGE LDS Sample Buffer (Life Technologies, 1.6-fold final concentration) and 5 min of incubation at 70 °C. Proteins samples (10–20 μg) were

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**TABLE 1**

| Peptide name | Amino acid sequence |
|--------------|---------------------|
| BH4-Bcl-2    | RTGYDNREIVMKYIHYKLSQGYSW |
| Biotin-BH4-Bcl-2 | Biotin-RTGYDNREIVMKYIHYKLSQGYSW |
| Biotin-BH4-Bcl-XL | Biotin-MSQSNRELVDLSSYKQYSGYW |
| Biotin-BH4-Bcl-2 scramble | Biotin-WVERQSLGIVYMIEEDRTKGYR |
| Biotin-BH4-Bcl-XL scramble | Biotin-WSKQRSLGGLMVYLEDKNSQFS |
| IDP          | NVYTEIKNSLLLPLDDIVKVR |
| VDAC1-NP     | MVAPPYDAGKSSARVPTFKGYPPL |
| VDAC1-NP scramble | DEVPAAKTYLMVPSDQFGYPAFF |
separated by NuPAGE 4–12% or 10% BisTris SDS-polyacrylamide gels using MOPS/SDS-running buffer (Life Technologies). When needed, gels were stained with GelCode™ blue stain reagent (Pierce) following the manufacturer’s recommendations. Alternatively, gels were transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween and 5% nonfat dry milk, membranes were incubated with the primary antibody overnight as appropriate, i.e., anti-FLAG M2 (clone M2, Sigma, 1:10,000); anti-VDAC1 (clone D73D12, Cell Signaling, 1:1000, or polyclonal anti-C-terminal, Antibodies Online, 1:1000); anti-β-actin (clone AC-15, Sigma, 1:20,000); Rbt475 (in-house developed pan-IP3R antibody (32), 1:2000); anti-calnexin (Enzo Life Science, 1:1000); anti-Bcl-XL (clone YTH-2H12, Trevigen, 1:1000); anti-Bcl-2 (clone C-2, Santa Cruz Biotechnology, 1:1000); and anti-cytochrome c (BD Biosciences, 1:1000). Next, membranes were incubated for 1 h with a secondary horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling Technology, dilution 1:2000 in 0.1% Tween/TBS). Immunoreactive proteins were detected using the ECL Western blotting Substrate (Thermo Fisher Scientific, Belgium). The resulting bands were quantified using ImageJ software (rsweb.nih.gov).

Isolation of MAMs—Isolations of MAMs were performed by an adaptation of the protocol described in Ref. 33. Approximately 100 × 10^6 MEFs were collected after culture and homogenized in isolation buffer 1 (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, and 30 mM Tris-HCl, pH 7.4). The homogenate was spun at 600 × g for 10 min to remove entire cells and nuclei; the supernatant was recovered and further centrifuged for 10 min at 7000 × g. Supernatant was removed, and the pellet was washed in isolation buffer 1 before being centrifuged at 10,000 × g for 10 min. Pellet was then resuspended in isolation buffer 2 (225 mM mannitol, 75 mM sucrose, and 30 mM Tris-HCl, pH 7.4). The homogenate was purified by centrifugation at 95,000 × g for 30 min on a Percoll™ density gradient in isolation buffer 1 (a 15% Percoll gradient on a 30% layer). The low density bands (denoted as MAMs) and the high density bands (denoted as pure mitochondria) were collected. The obtained mitochondrial layer was washed free of Percoll and resuspended in isolation buffer 2. MAMs were centrifuged for 10 min at 7000 × g three times to remove mitochondrial contamination and finally centrifuged at 100,000 × g for 1 h. The MAM fraction pellet was collected and concentrated using Pierce Protein Concentrators, 9K MWCO (Thermo Fisher Scientific Inc.) before Western blotting.

FLAG Immunoprecipitation Assay—For immunoprecipitation, 300 µg of protein lysate from COS-1 cells overexpressing Bcl-2 or Bcl-XL in the 3×FLAG pCMV-24 vector (Sigma) was added to 30 µl of anti-FLAG M2-agarose affinity gel (Sigma) previously washed according to the manufacturer’s protocol. Successively, the samples were incubated in lysis buffer (see lysis buffer under “SDS-PAGE, Western Blotting, and Antibodies”) at 4 °C. After 2 h, the beads were washed twice in spin columns (Pierce) with washing buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). Protein complexes containing FLAG fusion proteins were eluted by incubating the beads for 30 min with 35 µg of 3×FLAG peptide (Sigma, final concentration 150 ng/µl), and the eluate was subjected to Western blot analysis.

Biotin Pulldowns—Neutavidin-agarose resin (Pierce) (25 µl) was added to the tested sample and washed according to the manufacturer’s guidelines. Next, 30 µg of each biotinylated peptide was bound to the resin in interaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl; 0.1% Nonidet P-40, protease inhibitor mixture (Pierce)) that contains 500 µg of COS-1 total cell lysate expressing endogenous VDAC1 or an ectopic version of VDAC1 N-terminally truncated (Δ(1–26)-VDAC1, pcDNA4/TO vector (28)). After overnight mixing, beads were washed three times with interaction buffer in spin columns before proceeding with SDS-PAGE and Western blotting.

VDAC1 Single Channel Current Recordings—VDAC1 purified from rat liver mitochondria was solubilized with N,N-dimethyldodecylamine-N-oxide and purified using hydroxyapatite resin, as described previously (26). Purified VDAC1 was used for channel reconstitution into a planar lipid bilayer prepared from soybean asolectin dissolved in n-decane (50 mg/ml). Purified VDAC1 was added to the cis chamber containing 1 M NaCl and 10 mM Hepes, pH 7.4. After one or more channels were inserted into the planar lipid bilayer, currents were recorded by voltage clamping using a Bilayer ClampBC-525B amplifier (Warner Instruments). Current was measured with respect to the trans side of the membrane (ground). The current was digitized online using a Digidata 1200 interface board and pCLAMP10.2 software (Axon Instruments). Three independent recordings have been collected.

Electroporation Loading—In situ electroporation of adherent C6 cell monolayer cultures was performed, as described previously (21, 34), following a procedure optimized for cell death studies (35). In brief, C6 cell monolayers were placed 400 µm underneath a two-wire Pt-Ir electrode on the microscopic stage and electroporated in the presence of solution (10 µl) containing peptides (20 µM) or vehicle (DMSO, final 0.2%) dissolved in electroporation buffer (4.02 mM KH2PO4, 10.8mM K2HPO4, 1.0 mM MgCl2, 300 mM sorbitol, 2.0mM Hepes, pH 7.4) (10 µl). Electroporation was done with 50 kHz bipolar pulses applied as trains of 10 pulses of 2 ms duration each and repeated 15 times. Cells were electroporated in the presence of 100 µM dextran Texas red (Life Technologies) to define the electroporated zone. After electroporation, cells were thoroughly washed with HBSS/Hepes and left for 5 min to recover before proceeding with Ca2+ imaging or apoptosis assay.

Mitochondrial and Cytosolic Ca2+ Measurements—Changes in [Ca2+]im and [Ca2+]cyt in MEFs or C6 cells were triggered by 200 or 2 µM ATP, respectively, and monitored as described previously (34, 36). Briefly, C6 or MEFs cells were seeded on 18-mm diameter glass coverslips, and experiments were performed the next day after loading cells for 30 min with 10 µM Fluo3-AM (cytosol) or 5 µM Rhod-FF-AM (mitochondria). Subsequently, cells were subjected to de-esterification over 15 min and loaded with 100 µM dextran Texas red and 20 µM BH4 peptides using the in situ electroporation technique as described above. Ca2+ imaging was performed with an inverted Nikon Eclipse TE300 fluorescence microscope (Nikon, Belgium) equipped with a ×40 oil immersion objective (Plan Fluor, NA 1.3) and an EM-CCD camera (QuantEM 512SC, Photomet-
Cells were perfused for 1 min with HBSS-Hepes followed by 4 min (MEFs) or 9 min (C6 cells) with ATP (Sigma) in HBSS-Hepes. Alternatively, cells were kept in 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, 3 mM; Sigma) to chelate extracellular Ca\(^{2+}\) before (1 min) and during the ATP addition images (1/s) were generated with custom-developed software written in Microsoft Visual C++ 6.0. Fluorescence-intensity changes in all cells/mitochondria were analyzed. For each individual trace, the relative change of fluorescence (\(\Delta F/F\)) was calculated. \(\Delta F/F\) equals \(F_t - F_o/F_o\), with \(F_o\) denoting the fluorescence before stimulation with ATP and \(F_t\), the fluorescence at different time points after ATP stimulation. Subsequently, relative [Ca\(^{2+}\)]\(_{cyt}\) or [Ca\(^{2+}\)]\(_{mit}\) changes were quantified as the area under the curve of the various Ca\(^{2+}\) traces. Data were normalized to the control condition (DMSO, 0.2%), which was set as 100%. A minimum of three dishes has been used for each condition.

**Apopotosis Assay**—MEFs and C6 cells were kept in 200 µl of culture medium containing 2 µM staurosporine (STS) (Sigma). Six hours later, cultures were stained with 10 µM of the CaspA-CE3FITC-VAD-FMK *In situ* Marker (Promega Benelux, The Netherlands) in HBSS-Hepes for 40 min at 37°C. After fixing the cells with 4% paraformaldehyde for 25 min at room temperature, nuclei were additionally stained for 5 min with 1 µg/ml DAPI (Sigma) in PBS supplemented with Ca\(^{2+}\) and Mg\(^{2+}\) (Life Technologies). Cells were then mounted with Vectorshield fluorescent mounting medium (Labconsult, Belgium) on glass slides. Apoptosis in MEFs in the presence or absence of STS was quantified by taking five images in each culture using a Nikon TE300 epifluorescence microscope equipped with a ×10 objective (Plan APO, NA 0.45; Nikon) and a Nikon DS-Ri1 camera (Nikon, Belgium). The number of caspase-positive cells was counted in each image and expressed relative to the number of nuclei present. Analysis was carried out by making use of custom-developed counting software. The STS-induced apoptosis in C6 cultures was quantified as for MEFs but expressed relative to the STS-induced apoptosis outside the electroporated area.

**Data and Statistical Analysis**—Data are expressed as means ± S.E., except for Western blot (mean ± S.D.). Statistically significant differences were considered at \(p < 0.05\) (single symbols) or \(p < 0.01\) (double symbols) after using a two-tailed paired Student’s t test (Excel Microsoft Office) or one-way analysis of variance and a Bonferroni post-test using Origin7.0.

**RESULTS**

**VDAC1 Is Essential for Transferring Ca\(^{2+}\) Signals to the Mitochondria and Determines the Apoptotic Sensitivity toward Staurosporine**—In an initial set of experiments, we established the contribution of VDAC1 in the transfer of Ca\(^{2+}\) signals from the ER to the mitochondria. Therefore, we compared mitochondrial Ca\(^{2+}\) signals in response to extracellular agonists between WT and VDAC1\(^{-/-}\) MEFs loaded with Rhod-FF-AM, a mitochondrial Ca\(^{2+}\) indicator. The lack of VDAC1 expression in the VDAC1\(^{-/-}\) MEFs was confirmed by Western blot analysis (Fig. 1A). Extracellular ATP was used to trigger intracellular IP\(_3\) production that subsequently induces Ca\(^{2+}\) release from the ER (37). In WT MEFs, ATP (200 µM) caused a transient increase in the mitochondrial Ca\(^{2+}\) signal (Fig. 1, B and C). In VDAC1\(^{-/-}\) MEFs, the ATP-triggered increase in mitochondrial Ca\(^{2+}\) was severely compromised. Quantitative analysis of five independent experiments showed that cells lacking VDAC1 displayed a more than 95% reduction in the maximal amplitude of the ATP-triggered mitochondrial Ca\(^{2+}\) transient (Fig. 1C). To verify that the mitochondrial Ca\(^{2+}\) handling was specifically targeted, we also compared the cytosolic Ca\(^{2+}\) signals between Fluo3-AM-loaded WT and VDAC1\(^{-/-}\) MEFs in response to ATP (Fig. 1, D and E). These data show that both cell types exhibit an increase in cytosolic Ca\(^{2+}\) in response to ATP, indicating that the Ca\(^{2+}\) release from the ER is not compromised in VDAC1\(^{-/-}\) MEFs. Moreover, the VDAC1\(^{-/-}\) MEFs show a relatively higher (\(p = 0.11\)) rise of Ca\(^{2+}\) levels in their cytosol after ATP stimulation. This finding is in accordance with the decreased mitochondrial Ca\(^{2+}\) uptake (Fig. 1, B and C).

Next, we examined the influence of VDAC1 knock-out on the cellular sensitivity to STS, a known apoptosis inducer that also leads to mobilization of intracellular Ca\(^{2+}\) (38). Therefore, we exposed WT and VDAC1\(^{-/-}\) MEFs to staurosporine (1 µM; 6 h) and quantified their apoptotic response by counting the number of apoptotic cells in the population using a fluorescent caspase marker (Fig. 1F). We found that VDAC1\(^{-/-}\) MEFs were considerably more resistant toward STS-induced apoptosis than their WT counterparts. These results correlate with previous data showing that VDAC1 is required for mitochondrial Ca\(^{2+}\) loading in response to extracellular agonists and that it dictates the sensitivity toward apoptotic stimuli (11, 39).

**BH4 Domain of Bcl-XL, but Not That of Bcl-2, Specifically Interacts with VDAC1**—Because Bcl-2 and Bcl-XL modulation of the ER-mitochondrial Ca\(^{2+}\) transfer is likely occurring at the close contact sites between these two organelles (11), we therefore assessed whether VDAC1, IP\(_3\)Rs, Bcl-2, and Bcl-XL localize at the MAMs as anticipated or indicated by previous studies (1, 40, 41). The immunoblotting results (Fig. 2A) confirmed the presence of these proteins in our MAM preparations, which appeared pure considering that cytochrome c and calnexin were exclusively enriched in the mitochondrial and MAM fraction, respectively. Following up on the previously reported binding of Bcl-2 and Bcl-XL with VDAC1 (26, 27), we also compared their relative strengths of interaction by performing affinity pulldowns with COS-1 cells ectopically expressing 3×FLAG-tagged Bcl-2 and Bcl-XL (Fig. 2B) and containing high endogenous levels of VDAC1. We found that both proteins bind to VDAC1, although 3×FLAG-Bcl-XL tended to exhibit a higher binding capacity than 3×FLAG-Bcl-2 (Fig. 2C, \(p = 0.08\) in a two-tailed t test). Next, we examined whether the isolated BH4 domains of Bcl-2 and Bcl-XL differ in their VDAC1-binding properties. To investigate this, we performed affinity pulldowns with COS-1 cells using biotinylated BH4-Bcl-2 or BH-Bcl-XL peptides conjugated to NeutrAvidin beads. These experiments showed that biotin-BH4-Bcl-XL strongly interacts with VDAC1 (Fig. 2D, *upper blot*), although this is not the case for biotin-BH4-Bcl-2. We also performed affinity pull-down assays using an ectopically expressed version of VDAC1 lacking its first 26 amino acids (Δ1–26-VDAC1), because the VDAC1 N-terminal region has been previously proposed as a target for Bcl-2 and Bcl-XL (26, 27). However, in these experi-
BH4-Bcl-XL Versus BH4-Bcl-2 in VDAC1 Modulation

**FIGURE 1.** VDAC1<sup>−/−</sup> MEFs show impaired mitochondrial Ca<sup>2+</sup> loading and a manifest reduction of apoptotic response to staurosporine. A, MEF WT or VDAC1<sup>−/−</sup> cells were lysed and subjected to immunoblotting using an anti-VDAC1 antibody (upper panel) and using β-actin as loading control (lower panel, anti-β-actin). B, **top,** illustrative pseudo-colored images of MEF cells loaded with Rhod-FF-AM before (left panels; 1 s) and after (right panels; 100 s) exposure to AT<sub>P</sub> (200 μM). ATP was administered at 60 s. The scale bar measures 10 μm. **bottom,** representative Ca<sup>2+</sup> traces obtained from WT or VDAC1<sup>−/−</sup> MEFs loaded with Rhod-FF-AM. The relative changes in fluorescence with respect to baseline ([F<sub>0</sub> − F<sub>F</sub>]/F<sub>0</sub>) reflect the changes in mitochondrial Ca<sup>2+</sup> levels in response to ATP (arrow). C, quantitative analysis of the area under the curve obtained from at least five independent experiments. D, representative increases in Fluo3 fluorescence ([F<sub>0</sub> − F<sub>F</sub>]/F<sub>0</sub>) of ATP-stimulated (200 μM, arrow) WT or VDAC1<sup>−/−</sup> MEFs. Increases in fluorescence normalized to baseline reflect the extent of the cytosolic Ca<sup>2+</sup> transients. E, quantitative analysis of the area under the curve obtained from at least three independent experiments. Data were normalized to the WT condition of MEFs, which was set as 100% for each experiment, and were plotted as means ± S.E. F, quantification of the percentage of MEFs (WT and VDAC1<sup>−/−</sup>) stained positive for active caspases in response to STS (1 μM, 6 h). Data points represent mean ± S.E. Statistically significant differences between the two examined cell lines are indicated by *p* < 0.01 (**).
the cells suppressed ATP-induced mitochondrial Ca\(^{2+}\) transients (Fig. 4, A–C, see also Fig. 1H of Ref. 21).

Along with the BH4-Bcl-2 or BH4-Bcl-XL peptide, cells were co-electroporated either with the IP\(_3\)R-derived peptide (IDP) or the VDAC1 N-terminal peptide (VDAC1-NP). The former corresponds to the Bcl-2-binding site on IP\(_3\)Rs (21, 42, 43), and the latter mimics the N terminus of VDAC1, a presumed target of Bcl-XL (26, 27). BH4-Bcl-2-mediated inhibition of mitochondrial Ca\(^{2+}\) transients was completely reversed by co-loading IDP but was not altered by the presence of VDAC1-NP (Fig. 4, B and C). In contrast, BH4-Bcl-XL-mediated inhibition of mitochondrial Ca\(^{2+}\) transients could be reversed by co-loading VDAC1-NP but not IDP (Fig. 4, B and C). Similar results were obtained by stimulating the cells with ATP in the presence of extracellular BAPTA to chelate extracellular Ca\(^{2+}\) (Fig. 4D), indicating that Ca\(^{2+}\) influx did not impinge on the ER-mitochondrial Ca\(^{2+}\) transfer.

In a final step, we examined the relevance of the above results in the context of Ca\(^{2+}\)-mediated apoptosis by exposing C6 cells to STS (Fig. 4E). Both BH4-Bcl-2 and BH4-Bcl-XL protected against STS-induced apoptosis, but BH4-Bcl-2 was significantly more protective than BH4-Bcl-XL, as observed previously (21). The co-loading of VDAC1-NP alleviated BH4-Bcl-XL-mediated protection against apoptosis but not that of BH4-Bcl-2 (Fig. 4E). In contrast, we previously reported that IDP co-electroporation exclusively counteracted BH4-Bcl-2-mediated protection against apoptosis (21). Altogether, these data indicate a selective action in both Ca\(^{2+}\) signaling and Ca\(^{2+}\)-dependent apoptosis of the BH4 domain of Bcl-2 and Bcl-XL by their role on IP\(_3\)Rs and VDAC1, respectively.

**DISCUSSION**

IP\(_3\)Rs and VDAC1 channels, intracellular Ca\(^{2+}\)-transport systems at the interface between ER and mitochondria, play a
critical role in determining cell fate. VDAC1 channels are important players in cell survival versus cell death processes by mediating Ca\(^{2+}\)/H\(^{+}\) transfer across the OMM. Previous work revealed a unique role of the BH4 domain of Bcl-2, but not the BH4 domain of Bcl-XL, to suppress “toxic” Ca\(^{2+}\)/H\(^{+}\) flux originating from the ER, by targeting and inhibiting IP\(_{3}\)R channels (21). We now report that the BH4 domain of Bcl-XL, in contrast to that of Bcl-2, directly binds to and inhibits VDAC1 activity as a Ca\(^{2+}\)/H\(^{+}\)-transport system at the OMM. Notably, although both BH4-Bcl-2 and BH4-Bcl-XL protected against a Ca\(^{2+}\)/H\(^{+}\)-dependent apoptotic stimulus, a peptide corresponding to the N-terminal region of VDAC1 (VDAC1-NP) exclusively alleviated the anti-apoptotic properties of BH4-Bcl-XL but not those of BH4-Bcl-2 (Fig. 4C). Conversely, we showed (21) that the anti-apoptotic properties of BH4-Bcl-2 were abrogated by an IP\(_{3}\)R-derived peptide corresponding to the Bcl-2-binding site (IDP), although it was not the case for BH4-Bcl-XL. Collectively, our results implicate a selective function for the BH4 domains of Bcl-2 and Bcl-XL proteins in targeting the main Ca\(^{2+}\)-transport systems at the ER/mitochondrial interface. The BH4 domain of Bcl-2 would function as an inhibitor of IP\(_{3}\)R channels, but not of VDAC1, by targeting the central modulatory domain of IP\(_{3}\)Rs, whereas the BH4 domain of Bcl-XL would function as an inhibitor of VDAC1, but not of IP\(_{3}\)R channels, likely by acting at the level of its N-terminal region (see Fig. 5 for a model).

The VDAC1 high conductance channel has previously been implicated as the major Ca\(^{2+}\)-transport system mediating Ca\(^{2+}\) flux across the OMM (8, 11, 39–46). Ca\(^{2+}\) is then further transported from the inter-membrane space into the mitochondrial matrix via the recently identified mitochondrial Ca\(^{2+}\) uniporter (47, 48). The expression levels of VDAC1 thereby have a direct impact on the mitochondrial Ca\(^{2+}\) uptake.

FIGURE 3. Differential effect of BH4-Bcl-XL- and BH4-Bcl-2-based peptides on the conductance of bilayer-reconstituted VDAC1. A, VDAC1 was reconstituted into a planar lipid bilayer, and current in response to a voltage step from 0 to −10 mV was recorded before and 10–15 min after the addition of BH4-Bcl-XL or BH4-Bcl-2 peptide (6 μM). B, VDAC1-mediated currents at −60 mV, before and after the addition of BH4-Bcl-XL or BH4-Bcl-2 peptide. The dashed lines indicate the zero and the maximal current levels. C and D show the multichannel recordings as a function of the voltage (4 s at each voltage), and the average steady-state conductance of VDAC1 before (●) and 10 min after (○) the addition of BH4-Bcl-XL peptide (C) or BH4-Bcl-2-peptide (D). Relative conductance was determined as the ratio of the conductance at a given voltage (G) to the maximal conductance (Go). The results are representative of three similar independent experiments.
VDAC1 overexpression enhances mitochondrial Ca\(^{2+}\) uptake, whereas VDAC1 silencing impairs mitochondrial Ca\(^{2+}\) uptake (11, 39). Here, we support these findings using VDAC1 +/- MEFs, which displayed a marked reduction in mitochondrial Ca\(^{2+}\) uptake in response to ATP (Fig. 1, A–C). Consistent with this, ATP-induced Ca\(^{2+}\) signals in the cytosol were higher in VDAC1 +/- MEFs (Fig. 1, D and E). These data are also fully in line with previous work showing that VDAC1 knockdown mainly impacts mitochondrial Ca\(^{2+}\) transfer without affecting the ER Ca\(^{2+}\)-release kinetics (11). Hence, in intact cells, it is anticipated that there is a “quasi-synaptic” Ca\(^{2+}\) transfer between ER and mitochondria, involving both IP\(_3\)Rs at the ER and VDAC1 in the OMM (49, 50). In permeabilized cells, microsomal preparations, or isolated mitochondria, these ER/mitochondrial junctions might be lost (39, 51, 52). In these experimental conditions, mitochondrial Ca\(^{2+}\) uptake might be granted by the activity of Ca\(^{2+}\)-transport systems other than VDAC1 and located outside the MAMs (e.g. the other VDAC isoforms that have been proven to be able to permeate Ca\(^{2+}\))(11)). In particular, the role that VDAC1 plays in mitochondrial Ca\(^{2+}\) transfer makes it the prime route for conveying pro-apoptotic Ca\(^{2+}\) signals to the mitochondria. Silencing of VDAC1, but not of VDAC2 or VDAC3, suppressed H\(_2\)O\(_2\)-induced mitochondrial Ca\(^{2+}\) uptake and rendered cells more resistant to apoptosis triggered by H\(_2\)O\(_2\) or ceramide, also acting via Ca\(^{2+}\) (11, 39). In addition, siRNA-mediated down-regulation of VDAC1 prevented cell death by cisplatin (53) and attenuated endostatin-induced apoptosis (54). This functional role of VDAC1 overexpression in mitochondrial Ca\(^{2+}\) uptake is further supported by the observation that siRNA-mediated down-regulation of VDAC1 prevented cell death by cisplatin (53) and attenuated endostatin-induced apoptosis (54).
VDAC1 was also supported by molecular data showing that it was mainly VDAC1 that formed a complex with IP₃Rs channels (9). In our hands, VDAC1-deficient cells were more resistant than their WT counterparts to STS (Fig. 1F), an apoptotic stimulus known to act via intracellular Ca²⁺ mobilization (38). These data validate the concept that VDAC1 critically contributes to mitochondrial Ca²⁺ overload under apoptotic conditions.

Bcl-2 and Bcl-XL are assumed to counteract apoptosis, in part by limiting VDAC1-mediated Ca²⁺ flux into the mitochondria (55). In this paradigm, both proteins are able to bind VDAC1, with only minimal differences in their binding affinities (Fig. 2, B and C) (26), and to inhibit VDAC1 activity (25–27). The impact of Bcl-2 and Bcl-XL on the ER-mitochondrial Ca²⁺ fluxes is further supported by their presence in the MAM fraction together with VDAC1 and IP₃Rs (Fig. 2A) (9, 40). In this respect, this study is, to the best of our knowledge, the first to prove Bcl-XL's presence at the interface between the two organelles.

Bcl-2 and Bcl-XL directly affect VDAC1 activity presumably by binding different regions of the protein, e.g. the N-terminal domain, some of the cytosol-facing loops (namely LP1, LP2, and LP4) (26–28, 56), or part of VDAC1 transmembrane β-barrels (B18 and B19) (57). For the interaction sites on Bcl-2 and Bcl-XL, a prominent role for their BH4 domains was proposed (25), because VDAC1-mediated mitochondrial swelling could be alleviated by the presence of either the BH4-Bcl-2 or the BH4-Bcl-XL peptide. Although these measurements were not geared toward the Ca²⁺-flux properties of VDAC1, careful analysis of the results nevertheless indicated that BH4-Bcl-XL is much more effective in suppressing VDAC1 activity than BH4-Bcl-2. Our results show for the first time that VDAC1 is BH4-Bcl-XL’s preferential target at the molecular and functional level, as we observed an exclusive ability of the BH4-Bcl-XL peptide to bind VDAC1 (Fig. 2D) and to reduce its conductance (Fig. 3, A–D).

The inability of BH4-Bcl-2 peptides to affect channel conductance, further suggests that the effect of BH4-Bcl-XL is due to a specific interaction with VDAC1 and not to a mere penetration into the VDAC1 pore thereby blocking ion movement. Remarkably, BH4-Bcl-XL’s effect on VDAC1 activity is more pronounced at the low voltage differences (±10 mV), resembling the physiological OMM potentials (58, 59). In addition to this, the inherent ability of BH4-Bcl-XL to accumulate in mitochondrial membranes (24) correlates with its role on VDAC1. Nonetheless, it still remains to be determined whether BH4-Bcl-2 possesses a similar ability to accumulate in mitochondria because it shares with BH4-Bcl-XL common molecular determinants (positively charged residues and β-helical properties) for mitochondrial targeting (Arg-6/Lys-16/Lys-20 on BH4-Bcl-XL; Arg-12/Lys-22/Arg-26 on BH4-Bcl-2).

Additional experiments revealed that both BH4-Bcl-2 and BH4-Bcl-XL suppress the ATP-induced and VDAC1-mediated Ca²⁺ uptake in the mitochondria, protecting the cells against apoptosis (Fig. 4, A–E) (21). However, only the BH4-Bcl-XL effect is alleviated by co-incubation with VDAC1-NP. This indicates that the protective effect of BH4-Bcl-XL is mediated through inhibition of VDAC1 likely by acting at the level of its N terminus. These data are in good agreement with previous reports showing that Bcl-XL binding to VDAC1 was abrogated in VDAC1 lacking its N terminus (26). However, further experiments are required to unequivocally determine the relative

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**FIGURE 5. Proposed role of Bcl-2 and Bcl-XL BH4 domains at the ER-mitochondrial interface.** An excessive Ca²⁺ transfer from ER to the mitochondria, via the IP₃-VDAC1 contact site, promotes MOMP and consequently apoptosis. According to our previous results (21), Bcl-2 protects against Ca²⁺-mediated apoptosis mainly by using its BH4 domain to interact and inhibit IP₃Rs at the ER membranes. The results of this study indicate that BH4-Bcl-XL protection is dependent on its inhibition, downstream from the same signaling pathway, of the Ca²⁺-flux properties of mitochondrial outer membrane-located VDAC1 channel likely by interacting with its N-terminal domain. Therefore, IP₃-derived peptides and VDAC1-derived peptides are unique tools for selective BH4 domain-based antagonism of Bcl-2 and Bcl-XL, respectively.
importance of the N-terminal domains of VDAC1 and of Bcl-XL (i.e. the BH4 domain) in the context of their full-length protein/protein interaction.

Interestingly, the targeting of VDAC1 by BH4-Bcl-XL may not be limited to mitochondrial VDAC1 but likely can be extended to plasmalemmal VDAC1 (pl-VDAC1) (60). pl-VDAC1 seems to open in response to hypotonic conditions, whereas a peptide covering the BH4 domain of Bcl-XL keeps it closed. In the latter study, a mechanism involving the N terminus of pl-VDAC1 was also suggested. Furthermore, additional sites, besides the N terminus of the mitochondrial VDAC1, have been reported to account for the interaction between the full-length Bcl-2/Bcl-XL and mitochondrial VDAC1 (25, 26, 56, 61), emphasizing the possibility that distinct protein domains may underlie the formation of VDAC1-Bcl-XL versus VDAC1-Bcl-2 protein complexes.

Finally, in contrast to our findings and those of others (26, 62), recent reports indicated that anti-apoptotic Bcl-XL and Mcl-1 are both able to enhance VDAC1-mediated Ca\(^{2+}\) transfer into the mitochondria under some conditions (51, 63). Indeed, their overexpression enhanced while their deficiency suppressed mitochondrial Ca\(^{2+}\) uptake into the MOM. In the latter instance, we could include the following: 1) the use of intact cells (this study) versus permeabilized cells (47); 2) the use of protein domains like the BH4 domain (this study) versus full-length proteins (47); and 3) the use of short term methods like electroporation (this study) versus long term methods like knock-out and stable overexpression (47). It is of note that in permeabilized cell systems the majority of the mitochondrial Ca\(^{2+}\) uptake from the external solution will occur via uptake mechanisms (like VDAC1) spread across the MOM. In contrast, in intact cells exposed to agonists the majority of the mitochondrial Ca\(^{2+}\) uptake likely will occur by VDAC1 located at the MAMs. It is therefore possible that regulation of VDAC1 by associated proteins like Bcl-XL in the “bulk” of the MOM is different from the regulation of VDAC1 at the MAMs. Additionally, these apparently conflicting results may reflect the “dual” and opposing role of VDAC1 in cell death and mitochondrial bioenergetics (64–66). Indeed, beyond VDAC1’s role in mitochondrial Ca\(^{2+}\) overload and apoptosis, VDAC1 also has an essential function for the survival of cells by fine-tuning the activity of the Ca\(^{2+}\)-dependent Krebs cycle enzymes as well as by mediating the exchange of mitochondrial metabolites and ATP. Hence, Bcl-XL-enhancing ER-mitochondrial Ca\(^{2+}\) cross-talk would correlate with Bcl-XL driving basal mitochondrial bio-energetics by sensitizing IP\(_{3}\)Rs (19, 67) and/or enhancing VDAC1 activity (51, 68–70), although in other conditions it could suppress the excessive and pro-apoptotic flux of Ca\(^{2+}\) into mitochondria by dampening VDAC1 activity. In this complex scenario, it will be interesting to decipher the functional switch of VDAC1 from a pro-survival to a lethal Ca\(^{2+}\) gate, which may also involve post-translational regulation of VDAC1 (71), Bcl-XL (72), and/or a simultaneous role of other VDAC1-interacting molecules. Per-}

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