Ryanodine receptors are targeted by anti-apoptotic Bcl-X$_L$ involving its BH4 domain and Lys87 from its BH3 domain

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Anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members target several intracellular Ca$^{2+}$-transport systems. Bcl-2, via its N-terminal Bcl-2 homology (BH) 4 domain, inhibits both inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) and ryanoendine receptors (RyRs), while Bcl-X$_L$, likely independently of its BH4 domain, sensitizes IP$_3$Rs. It remains elusive whether Bcl-X$_L$ can also target and modulate RyRs. Here, Bcl-X$_L$ co-immunoprecipitated with RyR3 expressed in HEK293 cells. Mammalian protein-protein interaction trap (MAPPII) and surface plasmon resonance (SPR) showed that Bcl-X$_L$ bound to the central domain of RyR3 via its BH4 domain, although to a lesser extent compared to the BH4 domain of Bcl-2. Consistent with the ability of the BH4 domain of Bcl-X$_L$ to bind to RyRs, loading the BH4-Bcl-X$_L$ peptide into RyR3-overexpressing HEK293 cells or in rat hippocampal neurons suppressed RyR-mediated Ca$^{2+}$ release. In silico superposition of the 3D-structures of Bcl-2 and Bcl-X$_L$ indicated that Lys87 of the BH3 domain of Bcl-X$_L$ could be important for interacting with RyRs. In contrast to Bcl-X$_L$, the Bcl-X$_L$$^{K87D}$ mutant displayed lower binding affinity for RyR3 and a reduced inhibition of RyR-mediated Ca$^{2+}$ release. These data suggest that Bcl-X$_L$ binds to RyR channels via its BH4 domain, but also its BH3 domain, more specific Lys87, contributes to the interaction.

The B-cell lymphoma 2 (Bcl-2) protein family has long been studied with respect to its prominent role in the regulation of apoptosis. Beyond this, it is becoming increasingly clear that both the pro- and anti-apoptotic Bcl-2 family proteins are crucial regulators of intracellular Ca$^{2+}$ signaling. In this way, Bcl-2 proteins affect various targets related to intracellular Ca$^{2+}$ homeostasis. More specific, this protein family was found to regulate the mitochondrial voltage-dependent anion channels, plasma-membrane Ca$^{2+}$-ATPases, sarco/endothelium-remetium Ca$^{2+}$-ATPases (SERCA), Bax inhibitor, inositol 1,4,5-trisphosphate (IP$_3$) receptors (IP$_3$R) and ryanodine receptors (RyRs).

Anti-apoptotic Bcl-2 proteins are characterized by the presence of four Bcl-2 homology (BH) domains important for their biological function. Although their structural organization is very similar, Bcl-2 and Bcl-X$_L$ may act in very different ways on their targets. As such, the BH4 domain of Bcl-2 is critical for binding to a site in the regulatory domain of the IP$_3$R (a.a. 1389–1408 for mouse IP$_3$R1) thereby inhibiting IP$_3$-induced Ca$^{2+}$ release. In contrast, the BH4 domain of Bcl-X$_L$ fails to bind to this IP$_3$R domain and to inhibit IP$_3$R$_{29}$. Moreover, we showed that this difference between the BH4 domains of Bcl-2 and Bcl-X$_L$ can largely be attributed to a single amino acid change (Lys17 in BH4-Bcl-2 corresponding to Asp11 in BH4-Bcl-X$_L$) in the center of their respective BH4 domains. Indeed, the mutated BH4$^{K17D}$ domain of Bcl-2 and mutated full-length Bcl-2$^{K17D}$ are greatly impaired in targeting and regulating the IP$_3$R.

We recently showed that, similar to its interaction with the IP$_3$R, Bcl-2 via its BH4 domain targets a RyR region (a.a. 2263–2688 for mink RyR3) containing a highly conserved regulatory site (a.a. 2309–2330 for mink RyR3), which shows striking resemblance to the known Bcl-2 binding site on the IP$_3$R. The interaction of Bcl-2 and the
RyR via its BH4 domain results in an inhibition of RyR-mediated Ca\(^{2+}\) release. The Bcl-2\(^{K17D}\) mutant does not show a dramatic loss of binding to the RyR and is as potent as wild-type Bcl-2 in inhibiting RyR-mediated Ca\(^{2+}\) release. These results may indicate that in contrast to the IP\(_3\)R, which is differentially targeted by Bcl-2 and Bcl-X\(_L\), RyRs might have a common interaction site for both proteins and do not distinguish between these two proteins for their regulation.

In this paper, we show that similarly to Bcl-2, Bcl-X\(_L\) binds to the RyR via a site located in its central, modulatory domain, thereby inhibiting RyR-mediated Ca\(^{2+}\) release. Although the BH4 domain of Bcl-X\(_L\) was sufficient for inhibiting RyRs, we found that in full-length Bcl-X\(_L\) not only the BH4 domain but also the BH3 domain contributed to Bcl-X\(_L\)/RyR-complex formation. In particular, we identified Lys87, located in the BH3 domain of Bcl-X\(_L\), as an important contributor of Bcl-X\(_L\) binding to the RyR.

**Results**

**Bcl-X\(_L\) binds to RyR3.** Bcl-2\(^{K17D}\) is a Bcl-2 mutant based on a critical difference between the BH4 domains of Bcl-2 and Bcl-X\(_L\) and is impaired in binding to and regulating IP\(_3\)Rs\(^{19}\). However, this mutant still binds to and regulates RyRs with similar efficiencies as wild-type Bcl-2\(^{16}\), suggesting that Bcl-X\(_L\) may also bind to and regulate RyRs. Hence, we performed co-immunoprecipitation studies using lysates from HEK293 cells stably overexpressing RyR3 (HEK RyR3). In these cells, transiently overexpressed 3XFLAG-tagged Bcl-X\(_L\) co-immunoprecipitated with RyR3 indicating the formation of RyR3/Bcl-X\(_L\) complexes (Fig. 1A and Supplementary Fig. 1A for uncorrected Western-blot images).

In our previous work we reported that the interaction between Bcl-2 and the RyR occurred via the BH4 domain of Bcl-2 and a central regulatory domain of the RyR (a.a. 22632268–2688 for mink 2688 for mink RyR3)\(^{16}\). To examine whether a direct interaction between RyRs and the BH4 domain of Bcl-X\(_L\) exists and whether this interaction occurs via the same or similar domains, surface plasmon resonance (SPR) experiments were performed (Fig. 1B). A concentration-dependent binding between biotin-BH4-Bcl-X\(_L\) immobilized to streptavidin coated SPR chips, and the purified GST-RyR3 domain (mink RyR3, a.a. 2263-2688) could be detected. In contrast, but consistent with our previous observations, purified GST-tagged IP\(_3\)R1 domain 3 (mouse IP\(_3\)R1, a.a. 9232263–2688 for mink 1581), which is known to bind to the BH4 domain of Bcl-2, failed to bind to biotin-BH4-Bcl-X\(_L\).\(^{19}\). While biotin-BH4-Bcl-X\(_L\) was able to bind to the GST-RyR3 domain, it seemed to be less effective than biotin-BH4-Bcl-2\(^{16}\). To confirm the proper loading of the biotin-BH4-Bcl-X\(_L\) peptide to the sensor chip, we monitored the binding of an antibody directed against the BH4 domain of Bcl-X\(_L\), which caused a prominent increase in resonance unit (RU) values (Supplementary Fig. 2). Collectively, these results indicate that the interaction of Bcl-X\(_L\) with the RyR is direct and that Bcl-X\(_L\) via its BH4 domain targets the same domain as Bcl-2 on the RyR. However, the BH4 domain of Bcl-X\(_L\) seems to have a lower affinity for the GST-RyR3 domain compared to the BH4 domain of Bcl-2. This could indicate that biontinylation of the BH4 domain of Bcl-X\(_L\) influences its binding capabilities more than is the case for the BH4 domain of Bcl-2. Alternatively, other domains besides Bcl-X\(_L\)’s BH4 domain may be involved in the interaction of full-length Bcl-X\(_L\) with the RyR. Therefore, we wanted to identify if other domains besides the BH4 domain of Bcl-X\(_L\) are important for interacting with the RyR.

**Superposition of the 3D-structures of Bcl-2 and Bcl-X\(_L\) reveals a spatial resemblance of Lys17 in the BH4 domain of Bcl-2 with Lys87 in the BH3 domain of Bcl-X\(_L\).** To identify the contribution and involvement of other Bcl-X\(_L\) domains for targeting RyR channels, an in silico superposition of the Bcl-2 (PDB-entry 4AQ3\(^{20}\)) and Bcl-X\(_L\) (PDB-entry 1R2D\(^{21}\)) structures was performed with the aid of PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.). This superposition allowed the comparison of corresponding residues in the 3D-structures of Bcl-2 and Bcl-X\(_L\) (Fig. 2). This analysis revealed that the positively charged epsilon amino terminus of the side chain of Lys87 in Bcl-X\(_L\), located in the
The BH3 domain, in the same spatial constraints as the positively charged ε-amino terminus of the side chain of Lys17 located in the BH4 domain of Bcl-2. Furthermore, Lys87 did not seem to be part of the hydrophobic cleft of Bcl-X<sub>L</sub>, as it was directed towards the space facing the BH4 domain.

The Bcl-X<sub>L</sub><sup>K87D</sup> mutant is impaired in RyR3 binding. The relevance of Lys87 in Bcl-X<sub>L</sub> for RyR binding was addressed via mammalian protein-protein interaction trap (MAPPIT)<sup>22</sup>, an in cellulo protein-protein interaction assay. MAPPIT is based on the functional complementation of cytokine receptor signaling. To study the possible existence of RyR/Bcl-X<sub>L</sub> complexes, the RyR3 domain was cloned downstream of a chimeric cytokine receptor (RyR3 bait), consisting of the extracellular domain of the erythropoietin (Epo) receptor fused to the transmembrane and cytosolic part of the leptin receptor. In the latter, three tyrosines were mutated to phenylalanine to down regulate receptor signaling. Bcl-X<sub>L</sub><sup>+</sup> or the Bcl-X<sub>L</sub><sup>Δ</sup>,<sup>K87D</sup> mutant were cloned downstream of a part of the glycoprotein 130 receptor (Bcl-X<sub>L</sub> or Bcl-X<sub>L</sub><sup>Δ</sup>,<sup>K87D</sup> prey). If the Bcl-X<sub>L</sub> and Bcl-X<sub>L</sub><sup>Δ</sup>,<sup>K87D</sup> prey constructs interact with the RyR3 bait construct, functional complementation of the chimeric cytokine receptor occurs, leading to ligand-dependent downstream STAT signaling. The latter is monitored via a luciferase reporter assay driven by a STAT-sensitive promoter. We also used the SV40 large antigen T (irrelevant prey) as a prey to monitor the signal representing the non-specific binding to RyR3. As a negative control, binding of the chimeric cytokine receptor without the RyR3 fragment (no bait) to the two Bcl-X<sub>L</sub> preys was also assessed. These MAPPIT results confirmed the data obtained via SPR and co-immunoprecipitation experiments, showing that Bcl-X<sub>L</sub> could interact with the RyR3 domain in a cellular context (Fig. 3A, top). Moreover, the Bcl-X<sub>L</sub><sup>K87D</sup> mutant was severely impaired in interacting with the RyR3 domain without affecting its expression (Fig. 3A, bottom panel and Supplementary Fig. 1B for uncropped Western-blot images). No binding was detected when the RyR3 domain was not present in the bait vector (Fig. 3A, top panel), indicating that the interaction was specific.

The impact of mutating Lys87 into Asp was also examined in the context of the full-length RyR3 protein using co-immunoprecipitation experiments. Consistent with the MAPPIT data, 3XFLAG-tagged Bcl-X<sub>L</sub><sup>K87D</sup> displayed a reduced affinity for full-length RyR3 channels (Fig. 3B and Supplementary Fig. 1C for uncropped Western-blot images). Taken together, these data indicate that Bcl-X<sub>L</sub>, similarly to Bcl-2, binds via its BH4 domain to the same regulatory domain on RyR3. However, whereas for Bcl-2 the BH4 domain appears to be the main determinant for complex formation with RyR channels, it seems that for Bcl-X<sub>L</sub> both the BH4 domain and the BH3 domain, likely via Lys87, contribute to the interaction with RyR channels.

**Bcl-X<sub>L</sub>, but not Bcl-X<sub>L</sub><sup>K87D</sup>, inhibits RyR3-mediated Ca<sup>2+</sup> release.** Driven by the fact that Bcl-X<sub>L</sub> can bind to RyR3, we examined whether Bcl-X<sub>L</sub> could modulate RyR-mediated Ca<sup>2+</sup> release (Fig. 4). Single-cell cytosolic [Ca<sup>2+</sup>]<sup>1</sup> measurements in HEK RyR3 cells loaded with Fura-2-AM were performed (Fig. 4A). An empty vector (pCMV24) control, 3XFLAG-tagged Bcl-X<sub>L</sub> or the 3XFLAG-tagged Bcl-X<sub>L</sub><sup>K87D</sup> mutant were transiently transfected into the HEK RyR3 cells. An mCherry coding plasmid was co-transfected (at a 1:3 ratio) to identify transfected cells. After chelating extracellular Ca<sup>2+</sup> with BAPTA (3 mM), caffeine (1.5 mM) was applied to induce RyR-mediated Ca<sup>2+</sup> release. Overexpression of 3XFLAG-tagged Bcl-X<sub>L</sub> inhibited caffeine-induced Ca<sup>2+</sup> release compared to the empty vector control. The Bcl-X<sub>L</sub><sup>K87D</sup> mutant failed to inhibit caffeine-induced Ca<sup>2+</sup> release (Fig. 4B), correlating with its poor RyR3-binding properties. To exclude that the observed reduction in caffeine-induced Ca<sup>2+</sup> release upon Bcl-X<sub>L</sub> overexpression would have been due to an indirect effect via lowering of the Ca<sup>2+</sup>-filling state of the endoplasmatic reticulum (ER), we determined the amount of thapsigargin (1 μM)-releasable Ca<sup>2+</sup><sup>1</sup>. This irreversible SERCA inhibitor causes a depletion of the ER Ca<sup>2+</sup> stores and provides a good measure for the ER Ca<sup>2+</sup>-store content. The ER Ca<sup>2+</sup>-store content was not affected by overexpression of 3XFLAG-tagged Bcl-X<sub>L</sub> (Fig. 4C). This supports the view that Bcl-X<sub>L</sub>, similarly to Bcl-2, suppresses RyR-mediated Ca<sup>2+</sup> release.

The BH4 domain of Bcl-X<sub>L</sub> by itself seems sufficient to inhibit RyR-mediated Ca<sup>2+</sup> release. In order to assess whether the BH4 domain of Bcl-X<sub>L</sub> is sufficient for inhibiting RyR-mediated Ca<sup>2+</sup> release, Fluo-3-AM loaded HEK RyR3 cells were loaded acutely with the BH4 domain of Bcl-X<sub>L</sub>, a control peptide or the vehicle via electroporation (Fig. 5A). The BH4 domain of Bcl-X<sub>L</sub>, but not a control peptide, suppressed caffeine (1 mM)-induced Ca<sup>2+</sup> release. The BH4 domain of Bcl-X<sub>L</sub> inhibited caffeine-induced Ca<sup>2+</sup> release in a concentration-dependent manner (Fig. 5B). This indicates that the BH4 domain of Bcl-X<sub>L</sub> was sufficient for inhibiting RyR-mediated Ca<sup>2+</sup> release.

We also assessed whether the BH4 domain of Bcl-X<sub>L</sub> could inhibit endogenous RyR channels by using 14- to 18-day-old rat hippocampal cultures known to express different RyR isoforms<sup>23</sup>. The experimental set-up was identical to the one previously used for characterization of the effect of the BH4 domain of Bcl-2 on native RyRs<sup>14</sup>. Cytosolic [Ca<sup>2+</sup>] was monitored in GCaMP3-expressing hippocampal neurons. The BH4 domain of Bcl-X<sub>L</sub>, a control peptide or the vehicle were introduced into the neurons via a patch pipette. After loading the neuron for five minutes with the peptides or
vehicle, cytosolic $[^{25}Ca]$ release was triggered via a local puff of caffeine (10 mM) delivered via a second patch pipette positioned next to the neuron. A time lapse (Fig. 5C) and a $[^{25}Ca]$ trace (Fig. 5D) of a typical experiment are shown for each condition. Loading of the neurons with the BH4 domain of Bcl-XL (20 μM) caused a significant reduction of the caffeine-induced $[^{25}Ca]$ release compared to the control peptide (Fig. 5 D, E). These results indicate that the BH4 domain of Bcl-XL can regulate endogenously expressed RyR channels.

**Bcl-XL and its BH4 domain directly inhibit RyRs at the level of the ER.** Bcl-XL and its isolated BH4 domain as a synthetic peptide inhibit the caffeine-induced [Ca$^{2+}$] rise in the cytosol. Bcl-XL has also been implicated in the control of mitochondrial Ca$^{2+}$ transport at the level of VDAC1. Bcl-XL was shown to inhibit Ca$^{2+}$ uptake into the mitochondria$^{24}$. However, it was also reported that Bcl-XL could stimulate mitochondrial Ca$^{2+}$ uptake$^{25}$. The latter effect could result in a decrease in caffeine-induced [Ca$^{2+}$] rise in the cytosol. Therefore, we set out to document whether the decrease in caffeine-induced Ca$^{2+}$ release in the cytosol by Bcl-XL is due to a decreased Ca$^{2+}$ release from the ER or to an increased Ca$^{2+}$ accumulation into the mitochondria. Direct ER-Ca$^{2+}$ measurements were performed in HEK RyR3 cells utilizing a recently described green fluorescent CEPIA1 protein, that is targeted to the lumen of the ER (G-CEPIA1er$^{26}$). HEK RyR3 cells were transiently transfected with the G-CEPIA1er-encoding vector (at a 3:1 ratio). G-CEPIA1er-positive cells were selected and measurements were performed as in Fig. 4A. A typical average trace of one experiment and the quantification of all performed experiments are shown in Fig. 6A and B, respectively. These results indicate that overexpression of 3XFLAG-Bcl-XL suppressed the caffeine-induced Ca$^{2+}$ release from the ER, supporting a model in which the inhibitory effect of Bcl-XL on RyR-mediated [Ca$^{2+}$] rise in the cytosol occurs at least in part due to inhibition of the Ca$^{2+}$ release from the ER. Finally, we set out to directly measure the effect of the BH4 domain of Bcl-XL on caffeine-induced mitochondrial Ca$^{2+}$ release. Rhod-FF-loaded HEK RyR3 cells were electroporated with either the vehicle (DMSO) or the BH4 domain of Bcl-XL (10 and 20 μM) and then stimulated with caffeine. Caffeine stimulation resulted in an increase in mitochondrial [Ca$^{2+}$] (Fig. 6C). Compared to the vehicle control however, the BH4 domain of Bcl-XL potently inhibited the mitochondrial Ca$^{2+}$ entry (Fig. 6 C, D). Furthermore, the effectiveness of BH4-Bcl-XL to inhibit caffeine-induced [Ca$^{2+}$] rise in the mitochondria seemed higher than for inhibiting the caffeine-induced [Ca$^{2+}$] rise in the cytosol, because 10 μM BH4-Bcl-XL inhibited caffeine-induced Ca$^{2+}$ release in the cytosol by about 50% but inhibited caffeine-induced Ca$^{2+}$ uptake in the mitochondria by about 90%. Taken together these data suggest that BH4-Bcl-XL likely inhibits, rather than stimulates, mitochondrial Ca$^{2+}$ accumulation. This is consistent with our recent findings showing that BH4-Bcl-XL directly interacts with VDAC1 and suppressed VDAC1-mediated Ca$^{2+}$ transfer into the mitochondria$^{27}$. These experiments indicate that Bcl-XL can directly inhibit the caffeine-induced Ca$^{2+}$ release at the level of the ER and potently inhibit mitochondrial Ca$^{2+}$ uptake under these conditions.
experimental settings. We therefore conclude that the observed decrease in caffeine-induced Ca^{2+} release in the cytosol (Fig. 4 and 5) is mainly due to a direct inhibition of RyR3.

**Discussion**

The main conclusion of this paper is that Bcl-X\(_L\) binds to and regulates RyR3 channels. Similarly to Bcl-2, Bcl-X\(_L\) targets the central modulatory domain of the RyR protein, thereby suppressing RyR-mediated Ca^{2+} release. Moreover, the BH4 domain of Bcl-X\(_L\) was sufficient to inhibit both over- and endogenously expressed RyR channels in HEK293 cells or primary rat hippocampal neurons respectively. Consistent with this, the BH4 domain of Bcl-X\(_L\) could bind to the purified RyR3 domain. However, the RyR3-binding efficiency of the BH4 domain of Bcl-X\(_L\) seemed much lower than that of the BH4 domain of Bcl-2. Via an *in silico* superposition of the Bcl-2 and Bcl-X\(_L\) crystal structures, a spatial overlap was observed between Lys17 in the BH4 domain of Bcl-2 and Lys87 in the BH3 domain of Bcl-X\(_L\): the positively charged \(\varepsilon\)-amino groups of their side chains coincide in space. Consistent with the moderate RyR3-binding properties of the isolated BH4 domain of Bcl-X\(_L\), we found that Lys87 from Bcl-X\(_L\) played a prominent role in binding to and regulating RyR3.

**Figure 4** | Bcl-X\(_L\) but not Bcl-X\(_L^{K87D}\) inhibits RyR-mediated Ca^{2+} release. Single-cell cytosolic [Ca^{2+}] measurements were performed in HEK RyR3 cells utilizing Fura-2-AM. (A) Average calibrated [Ca^{2+}] trace of 15 to 20 HEK RyR3 cells transfected (mCherry positive) with an empty vector as control (pCMV24), 3XFLAG-Bcl-X\(_L\) or 3XFLAG-Bcl-X\(_L^{K87D}\). Addition of BAPTA and caffeine is indicated by the arrows. (B) Quantitative analysis of the single-cell cytosolic [Ca^{2+}] measurements. Values indicate averages of all peak values \(\pm\) S.E.M. These experiments were independently performed at least four times (\(>\)120 cells/condition) (\(p=0.008\)). (C) Quantitative analysis of the ER Ca^{2+}-store content. ER-store content was determined by performing similar experiments as in A except that 1 \(\mu\)M thapsigargin was used as the stimulus. The values indicate the average area under the curve (AUC) \(\pm\) S.E.M. of at least three independent experiments (\(>\)80 cells/condition).
The association of Bcl-XL with RyR channels and its functional implications appear to be very similar as the ones observed for Bcl-2, since i) RyR3/Bcl-XL binding is direct; ii) the binding of Bcl-XL to RyR3 occurs, at least in part, via the BH4 domain; iii) Bcl-XL overexpression inhibits RyR-mediated Ca\textsuperscript{2+} release; and iv) the BH4 domain of Bcl-XL is also sufficient to suppress RyR activity. These

Figure 5 | The BH4 domain of Bcl-XL by itself was sufficient to inhibit RyR-mediated Ca\textsuperscript{2+} release. (A) Representative trace of the performed Fluo-3-AM single-cell cytosolic [Ca\textsuperscript{2+}] measurements in HEK RyR3 cells loaded by electroporation with either the vehicle (DMSO), a control peptide or the BH4 domain of Bcl-XL. The addition of caffeine is indicated by the arrow. Traces were normalized to the baseline fluorescence (\textit{(F-F\text{\textsubscript{0}})/F\text{\textsubscript{0}}}). (B) Quantitative analysis of the single-cell cytosolic [Ca\textsuperscript{2+}] measurements with indicated concentrations of the BH4 domain of Bcl-XL. Values indicate caffeine-induced Ca\textsuperscript{2+} release after electroporation loading with different concentrations of the BH4 domain of Bcl-XL relative to the response after electroporation loading with the same concentration of the control peptide. Values depict average ± S.E.M. of at least four independent experiments (p-values were 0.0037, 0.001 and 0.0039 for 10 μM, 20 μM and 40 μM of the BH4 domain of Bcl-XL respectively). (C-E) Single-cell [Ca\textsuperscript{2+}] measurements performed in 14- to 18-day-old hippocampal cultures. GCaMP3, introduced into these neurons via adeno-associated infection, was used as cytosolic Ca\textsuperscript{2+} indicator. Utilizing whole-cell voltage clamp the membrane potential of the neurons was clamped at −60 mV. 20 μM of the BH4 domain of Bcl-XL, a control peptide or the vehicle (DMSO) was introduced into each measured neuron via the patch pipette. All experiments were performed in the presence of 1 μM tetrodotoxin. A 10 mM caffeine puff was locally administered via a second patch pipette positioned 15-25 μm from the soma of the neuron. (C) Time lapse of a typical experiment for each of the tested conditions. Caffeine was administered after 60 sec. The scale bar depicts 5 μm. (D) Typical responses to caffeine after loading the neurons with 20 μM of either the control peptide the BH4 domain of Bcl-XL or the vehicle. Traces were normalized to the baseline fluorescence (\textit{(F-F\text{\textsubscript{0}})/F\text{\textsubscript{0}}}). The arrow indicates when caffeine was administered. (E) Scatter plot showing peak responses of all performed measurements and the median (horizontal line). All values were normalized to the caffeine response after vehicle control treatment (p=0.0037, N=12 and N=15 for the control peptide and BH4-Bcl-XL respectively).
findings correlate with the fact that the Bcl-2K17D mutant and BH4-Bcl-2K17D remain capable of binding to and regulating RyR channels, although this mutation changes the lysine critical for binding to the IP3R into the Asp11 residue in the BH4 domain of Bcl-XL. This lack of selectivity between Bcl-2 and Bcl-XL may illustrate an important difference between IP3R- and RyR-mediated Ca2+ release. However, the binding of Bcl-XL versus Bcl-2 to RyRs in native tissues expressing RyRs ought to be further explored. In particular, it will be important to carefully analyze the Bcl-2- and Bcl-XL-expression levels in the relevant tissues and to determine whether a preferential binding of Bcl-2 to Bcl-XL is observed. Despite these similarities, the molecular determinants underlying RyR/Bcl-XL-complex formation do not seem identical to those of Bcl-2, because the BH4 domain of Bcl-XL by itself displays rather moderate RyR3-binding properties. As a consequence, additional domains seem to be involved in RyR/Bcl-XL-complex formation. Here, we identified Lys87, located in the BH3 domain of Bcl-XL, as a critical determinant contributing to binding to and regulating RyR channels. Despite the importance of Lys87, the BH4 domain of Bcl-XL alone was able to suppress RyR activity.

The BH4 domain of Bcl-XL has been implicated in numerous studies to display strong anti-apoptotic and protective effects against a wide variety of insults and triggers, including in the heart, endothelial cells, blood cells, pancreatic islets, and neurons. Many of the cell types and tissues reported to benefit from the BH4 domain of Bcl-XL for their survival endogenously express RyR channels (cardiomyocytes, lymphocytes, pancreatic islets and neurons). Furthermore, in many apoptotic paradigms, reactive oxygen species

Figure 6 | Bcl-XL and its BH4 domain directly inhibit RyR-mediated Ca2+ release from the ER. (A) Typical average normalized (F/F0) traces of single-cell ER [Ca2+]i measurement performed in HEK RyR3 cells transfected with G-CEPIA1er plasmid. G-CEPIA1er-positive cells transfected with the empty control vector (pCMV24) or 3XFLAG-Bcl-XL were selected for these measurements. After chelating extracellular Ca2+ with BAPTA, caffeine was added to stimulate RyR-mediated Ca2+ release (arrows). (B) Quantitative analysis of the performed experiments. For each trace the caffeine-induced Ca2+ release was determined by subtracting the fluorescence after caffeine addition (during plateau phase) from the fluorescence just before caffeine addition after normalization. Values depict average ± S.E.M. These experiments were independently repeated at least four times (>100 cells/condition) (p=0.0018). (C) Normalized ((F-F0)/F0) representative traces of mitochondrial [Ca2+]i measurements. The vehicle (DMSO) or the BH4 domain of Bcl-XL (10 µM and 20 µM) were introduced into Rhod-FF-loaded HEK RyR3 cells via electroporation loading. Mitochondrial Ca2+ was measured after caffeine (arrow) stimulation. (D) Quantification of the performed experiments. Values show the average caffeine-induced mitochondrial Ca2+ entry as area under the curve (AUC) ± S.E.M. Experiments were independently performed at least three times (p<0.0001).

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(ROS) are implicated. ROS can impact the redox state and activity of the RyR channels (implicated by Ref. 38). Mild increases in ROS moderately increase RyR activity by increasing its sensitivity for Ca²⁺. However, severe ROS production associated with oxidative stress (e.g. in the context of ischemia/reperfusion injury) can lead to a continuously opening of the RyR channels, provoking an excessive Ca²⁺ leak from the ER or sarcoplasmic reticulum. In the context of the heart, ROS has been clearly implicated to cause unzipping of the interdomain interactions critical for RyR2-channel stabilization. During oxidative stress conditions, the BH4 domain of Bcl-Xₐ may thus inhibit excessive RyR-mediated Ca²⁺ release from the intracellular Ca²⁺ stores in addition to exerting its protective effects at the mitochondria, thereby providing additional protection against cell death.

RyRs have important physiological functions in a variety of excitable cells and tissues, including skeletal muscle, cardiac muscle, neurons and pancreatic cells. Furthermore, dysregulation of RyRs, either by somatic mutations or by altered expression levels, has been implicated in a variety of pathophysiological conditions, including malignant hyperthermia and central core disease, cardiac diseases and neurodegenerative diseases like Alzheimer’s disease and Huntington’s disease. At this point, the existence and physiological relevance of RyR/Bcl-2 and RyR/Bcl-Xₐ complex formation in these tissues and their potential disturbance in RyR-associated pathophysiological will require further research.

In conclusion, our data further expand the number of Bcl-2-family members that are able to form protein complexes with RyR channels, thereby underpinning their critical role in regulating intracellular Ca²⁺ dynamics at the level of intracellular Ca²⁺ release channels.

Methods

Chemicals, antibodies and peptides. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were used: mouse monoclonal anti-actin antibody, anti-FLAG M2 antibody and HRP-conjugated anti-FLAG M2 antibody (Sigma-Aldrich), mouse monoclonal anti-RyR antibody 34C (Thermo Scientific, Rockford, IL, USA), or Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) and mouse monoclonal anti-Bcl-Xₐ, antibody YTH-2H12 ( Trevigen, Gaithersburg, WV, USA). The sequences of the peptides used in this study were:

Biotin-BH4-Bcl-Xₐ: Biotin-MSQNRKVEVDLFSLYKSLQKGYSW (also used without the biotin tag)
Biotin-scrambled BH4-Bcl-Xₐ: Biotin-WYSQKRSLSLGVMYLEDKNSQFS Control peptide: WYEKQSRHLGIMYVEEDRTNGTR

These peptides were synthesized by Life Tech (Hilborough, NJ, USA) with a purity of at least 85%.

Plasmids, constructs and protein purifications. The 3XFLAG-Bcl-Xₐ was obtained as previously described. The 3XFLAG-Bcl-Xₐ mutant was obtained by PCR site-directed mutagenesis utilizing the following primers: forward: 5’TGCCTCGCTGAGGACTGATGATGACGCGTGGGAGGAGGAA3; reverse: 5’TGGCTCTCGTCTGGGAGGAGGAGGAGGAGGAGGAA3. The pCMV-G-CEPIA1er containing plasmid was a gift from Dr. Masamitsu Iino (Addgene plasmid # 58215). The GST-IP.R1 domain 3 construct and the GST-RyR3 construct were obtained and purified as described.

Cell culture, transfections and dissociated hippocampal cultures. All media and supplements added to the medium used in this paper were purchased from Life Technologies (Ghent, Belgium). HEK293 cells stably expressing RyR3 were cultured at 37°C in a 5% CO₂ incubator in α-Minimum Essential Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax and 800 μg/ml G418. HEK293 cells were grown in Dulbecco’s Modified Eagle Medium containing 4500 mg/l glucose, 10% fetal bovine serum and 50 μg/ml gentamicin.

24 hours after seeding, the 3XFLAG-Bcl-Xₐ or the 3XFLAG-Bcl-Xₐ mutant constructs were introduced into the HEK RyR3 cells utilizing JETPrime transfection reagent (Polyplus Transfections, Illkirch, France) according to the manufacturer’s protocol. 48 hours after the cells were harvested and lysed utilizing a CHAPS-based lysis buffer (pH 7.5, 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% CHAPS and protease inhibitor tablets (Roche, Basel, Switzerland)). For single-cell cytosolic [Ca²⁺] measurements the same constructs or the empty pCMV24 vector were introduced 48 hours after seeding in the HEK RyR3 cells utilizing X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer’s protocol. A pCDNA 3.1(+) mCherry expressing vector was co-transfected at a 1:3 ratio as a selection marker. For direct ER [Ca²⁺] measurements, the G-CPE1αer construct was co-transfected (ratio 3:1) and used as selection marker instead of the mCherry expressing vector. Dissociated hippocampal cultures were obtained as described previously. All animal experiments were performed according to approved guidelines.

SPR analysis. SPR analysis was performed using a Biacore T200 (GE Healthcare, Dunfermline, UK). Immobilization of the streptavidin-coated sensor chip (BR-1005-31; GE Healthcare) and SPR measurements were performed as described previously. NaOH (50 mM) with 0.0026% SDS was used as a regeneration buffer.

Immunoblot analysis. Samples were prepared and used as previously described. For visualization of RyR3, NuPAGE 3–8% tris-acetate gels were run. Detection was performed using Pierce ECL Western Blotting Substrate (Thermo Scientific) when using the Chemidoc™ MP system (Bio-Rad, Nazareth Eke, Belgium) or an X-OMAT 1000 processor (Kodak, Zaventem, Belgium). When using the Odyssey imager (Westburg, Leuven, The Netherlands) detection was performed using anti-mouse-IRDye680 (green) or anti-rabbit-IRDye700 (red) as secondary antibodies (Thermo Scientific).

Co-immunoprecipitation experiments. Co-immunoprecipitation experiments were performed utilizing a co-immunoprecipitation kit (Thermo Scientific). RyR antibody or mouse IgG control antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was immobilized according to the manufacturer’s protocol. Gelatin was removed from the IgG control antibody utilizing a Pierce Antibody Clean-up Kit (Thermo Scientific). Preclreated HEK RyR3 lysates containing the 3XFLAG-Bcl-Xₐ constructs (150 μg) were added to the resin to which the antibodies were immobilized and allowed to incubate overnight at 4°C. The next day, the resin was washed at least five times utilizing the CHAPS-based lysis buffer. The immune complexes were eluted by boiling (95°C) in 50 μl 2X LDS (Life Technologies) supplemented with 1/200 β-mercaptoethanol for 5 min.

MAPPIT. The RyR3 domain was amplified by PCR using the following primers, forward: 5’TATGGTCGAGGCAAGAGAACATGTCAGGGAG3; and reverse: 5’TATGTCGAGGCAAGAGAACATGTCAGGGAG3. The pCMV-G-CEPIA1er lysates containing the 3XFLAG-Bcl-Xₐ, constructs were cloned into the pCMV24 vector were introduced 48 hours after seeding in the HEK RyR3 cells as described above. A Zeiss Axio Observer Z1 Inverted Microscope equipped with a 20× air objective and a high-speed digital camera (AxioCam Hsm, Zeiss, Jena, Germany) were used for these measurements. Changes in fluorescence were monitored in the GFP channel (480/520 excitation/emission). To chelate extracellular Ca²⁺, 3 mM BAPTA (Alfa Aesar, Ward Hill, MA, USA) was added. One minute later, 1.5 mM caffeine was added to trigger RyR-mediated Ca²⁺ release. All traces were normalized (F₀/F₅₀) when F₅₀ is the starting fluorescence of each trace.

Electroporation loading. Electrical voltage loading of HEK RyR3 cells was performed as previously described.

Single-cell cytosolic Ca²⁺ imaging. Fura-2 AM and FluO-3 AM measurements in HEK RyR3 cells and GCaMP3 single-cell [Ca²⁺] measurements in dissociated hippocampal neurons were performed as described.

Single-cell ER Ca²⁺ imaging. The G-CPE1αer construct was introduced into HEK RyR3 cells as described above. A Zeiss Axio Observer Z1 Inverted Microscope equipped with a 20× air objective and a high-speed digital camera (AxioCam Hsm, Zeiss, Jena, Germany) were used for these measurements. Changes in fluorescence were monitored in the GFP channel (480/520 excitation/emission). To chelate extracellular Ca²⁺, 3 mM BAPTA (Alfa Aesar, Ward Hill, MA, USA) was added. One minute later, 1.5 mM caffeine was added to trigger RyR-mediated Ca²⁺ release. All traces were normalized (F₀/F₅₀) when F₅₀ is the starting fluorescence of each trace.

Single-cell mitochondrial Ca²⁺ imaging. HEK RyR3 cells were loaded for 30 min with 5 μM RhodFF-AM. Subsequently, cells were subjected to de-esterification over 15 min. During this time the BH4 domain peptides were introduced into the cells using the in situ electroporation technique. Fluorescence-intensity changes in mitochondria were analyzed with custom-developed FluoFrames software. For each individual trace, the relative change of fluorescence (ΔF/F) was calculated. ΔF/F equals [F₂-F₁]/F₁, with F₀ denoting the fluorescence before stimulation with caffeine and F₁ the fluorescence at different time points after caffeine stimulation. Subsequently, relative mitochondrial [Ca²⁺] changes were quantified as the area under the curve of the various Ca²⁺ traces.

Statistical analysis. Two-tailed student’s t-tests were performed when two conditions were compared. When comparing three conditions a one-way ANOVA with Bonferroni’s multiple comparison test was performed. * indicates significantly different results (p<0.05). Exact p-values are indicated in the figure legends, where available.

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Author contributions

The study was conceived and originally designed by T.V., H.D.S., J.B.P. and G.B. with additional input from E.V. and L.Ma. for molecular modeling, J.T. and N.N.K. for MAPPIT and hippocampal neurons, respectively. T.V., E.D., I.L., E.L. and G.M. performed the experiments. T.V., E.D., L.Mi., L.L., H.D.S., I.L., E.L., H.I., G.M., J.T., L.Ma., N.N.K., J.B.P. and G.B. analyzed, interpreted and/or discussed the data. T.V. and G.B. drafted the manuscript. All authors critically revised the manuscript and approved the final article.

Additional information

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