Bcl-2 and IP₃ compete for the ligand-binding domain of IP₃Rs modulating Ca²⁺ signaling output

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ABSTRACT

Bcl-2 protein has emerged as a critical regulator of intracellular Ca^{2+} dynamics by directly targeting and inhibiting the IP_3 receptor (IP_3R), a major intracellular Ca^{2+} -release channel. Here, we demonstrate that such inhibition occurs under conditions of basal, but not high IP_3R activity, since overexpressed and purified Bcl-2 (or its BH4 domain) can inhibit IP_3R function provoked by low concentration of agonist or IP_3 , while fails to attenuate against high concentration of agonist or IP_3 . Surprisingly, Bcl-2 remained capable of inhibiting $\text{IP}_3\text{R1}$ channels lacking the residues encompassing the previously identified Bcl-2-binding site (a.a. 1380-1408) located in the ARM2 domain, part of the modulatory region. Using a plethora of computational, biochemical and biophysical methods, we demonstrate that Bcl-2 and more particularly its BH4 domain bind to the ligand-binding domain (LBD) of $\text{IP}_3\text{R1}$. In line with this finding, the interaction between the LBD and Bcl-2 (or its BH4 domain) was sensitive to IP_3 and adenophostin A, ligands of the IP_3R . Consistent with this, the BH4 domain of Bcl-2 counteracted the binding of IP_3 to the LBD. Collectively, our work reveals a novel mechanism by which Bcl-2 influences IP_3R activity at the level of the LBD. This allows for exquisite modulation of Bcl-2's inhibitory properties on IP_3Rs that is tunable to the level of IP_3 signaling in cells.

Key words: inositol 1,4,5-trisphosphate receptor; calcium channels; protein binding; ligand-receptor interaction; ligand-binding domain; inhibition; mechanism of interaction

1 INTRODUCTION

2 B cell lymphoma-2 (Bcl-2) protein is the founding member of the Bcl-2 family of proteins and
3 a well-known inhibitor of apoptosis [1-4]. Bcl-2 harbors all four Bcl-2-homology (BH1-4)
4 domains, which are evolutionary conserved α -helical motifs and a shared feature in the family
5 [5, 6]. Bcl-2 executes its pro-survival functions by a complex binding network, employing the
6 BH domains in various protein-protein interactions. The canonical mechanism of apoptosis
7 inhibition by Bcl-2 involves the neutralization of the pro-apoptotic members of the family,
8 exemplified by the executioners Bax and Bak or the BH3-only proteins such as Bim. This
9 process occurs by sequestration of the BH3 domain of the pro-apoptotic members in the
10 hydrophobic cleft of Bcl-2, which is formed by its BH3-BH1-BH2 domains [4, 7, 8]. In
11 addition, prevention of Bax activation by Bcl-2 involves an interaction between the N-terminus
12 of Bax and the BH4 domain of Bcl-2 [9]. Another mechanism to block apoptosis that is
13 exploited by Bcl-2 is the modulation of intracellular Ca^{2+} signals [10-13]. Bcl-2 was shown to
14 target the main Ca^{2+} -release channel in cells, the inositol 1,4,5-trisphosphate (IP_3) receptor
15 (IP_3R) and to suppress its activity. By decreasing the amount of Ca^{2+} released from the
16 endoplasmic reticulum (ER), Bcl-2 prevents Ca^{2+} -dependent apoptosis triggered by various
17 stimuli [14, 15]. The current model of IP_3R inhibition by Bcl-2 involves multi-region binding
18 interactions. To date, two distinct Bcl-2 binding regions on IP_3R were identified. Bcl-2 targets
19 the modulatory region, particularly the Fragment 3 (a.a. 923-1581, according to mouse $\text{IP}_3\text{R1}$)
20 [16-18], which results from the *in vitro* chymotrypsinization or trypsinization of the receptor
21 [19, 20] and which predominantly consists of the ARM2 domain [21]. Bcl-2 also binds to the
22 C-terminal region, particularly to a fragment containing the cytosolic tail and the 6th
23 transmembrane domain (TMD) of IP_3R (C-terminus, a.a. 2512-2749, according to mouse
24 $\text{IP}_3\text{R1}$) [17, 22]. The binding of Bcl-2 to the Fragment 3 of IP_3R occurs via its BH4 domain,
25 which appeared to target a particular stretch of 20 residues (a.a. 1389-1408, according to the
26 mouse and rat $\text{IP}_3\text{R1}$). This interaction is considered to be the driving force behind the IP_3R
27 inhibition by Bcl-2 as various techniques have demonstrated that the isolated BH4 domain of
28 Bcl-2 was sufficient to bind and inhibit the IP_3R [18, 23, 24]. A more recent study revealed that
29 the C-terminal TMD of Bcl-2 is required for efficient *in cellulo* inhibition of IP_3R activity and
30 prevention of subsequent Ca^{2+} -mediated cell death [25]. The latter finding was an important
31 step in clarifying the mechanism of highly effective regulation of Ca^{2+} -dependent apoptosis by
32 Bcl-2 in intact cells. Nevertheless, the full complexity of the interaction between Bcl-2 and
33 IP_3R is still not completely understood and requires further investigation. In particular,
34 according to the published cryo-electron microscopic structure of full-length $\text{IP}_3\text{R1}$ [21, 26],
35 the binding site in the Fragment 3 and the C-terminus are located a relatively large distance
36 apart, making it difficult to explain how one Bcl-2 molecule could occupy both sites at the same
37 time.

38 Bcl-2 was also reported to distinguish between patterns of distinct cellular Ca^{2+} signals,
39 inhibiting pro-apoptotic high amplitude Ca^{2+} elevations, but not pro-survival Ca^{2+} oscillations
40 [27]. However, the mechanism of this process remains unresolved. Interestingly, it was
41 previously demonstrated that Bcl-2 suppresses intracellular Ca^{2+} signals, triggered by
42 submaximal, but not supramaximal concentrations of agonist [15]. This suggests that anti-
43 apoptotic Bcl-2 may lose its IP_3R -inhibitory effect in conditions of strong IP_3R activation,
44 though the underlying mechanisms responsible for this effect remain unknown. Here, we

demonstrate that high concentrations of IP₃ or of agonists alleviate the inhibitory effect of the BH4 domain of Bcl-2, as well as of the recombinant purified or overexpressed Bcl-2, on IP₃R activity in various experimental settings. Furthermore, the deletion of the targeted by Bcl-2 stretch (a.a. 1389-1408) in the Fragment 3 failed to prevent the Bcl-2-mediated inhibition of IP₃R. These data suggest the presence of an additional region in IP₃R as a target of Bcl-2, whose interaction can be modulated by IP₃ levels. Using a computational modelling approach, we found that (i) Bcl-2's BH4 domain could form a complex with the ligand-binding domain (LBD) and (ii) the conformational changes in the LBD induced by IP₃ binding hindered this interaction. This was supported by different experimental approaches, demonstrating that the LBD (a.a. 1-604), and more particular the IP₃-binding core (IBC, a.a. 226-604) of IP₃R1 could bind full-length Bcl-2 or its BH4 domain, produced as synthetic peptide. Furthermore, IP₃ and adenophostin A (AdA), two IP₃R ligands, interfered with the binding of Bcl-2 and its BH4 domain to the LBD. In contrast, the BH4 domain of Bcl-2 attenuated the binding of IP₃ to the LBD. Thus, the present study represents an important contribution towards revealing the molecular mechanism of interaction between the anti-apoptotic-protein Bcl-2 and IP₃R.

MATERIALS AND METHODS

Peptides

The following peptides, obtained from LifeTein (Hillsborough, NJ, USA) with purity $\geq 85\%$ were used: BH4-Bcl-2: RTGYDNREIVMKYIHYKLSQRGYEW and the control scrambled peptide BH4-Bcl-2-CTR: WYEQRSRLHGIMYYVIEDRNTKGYR.

Plasmids, constructs and protein purification

The pCMV construct containing the genetically encoded Ca²⁺ sensor CEPIA targeted to the ER lumen (G-CEPIA1-ER) was a gift from Dr. Masamitsu Iino (Addgene plasmid # 58215) [28]. pCMV24-3xFLAG-Myc constructs for expression of 3xFLAG-Bcl-2 were obtained as previously described [29]. pcDNA3 construct for expression of rat IP₃R1 lacking the binding site for BH4 of Bcl-2 in Fragment 3 (IP₃R1^{Δ1389-1408}) was developed by using Q5 mutagenesis protocol using the following primers pair:

Forward: GCTGTGTGCACAGAGGGCAAGGTCACATGAAGACTGTATC

Reverse: GATACAGTCTTCATGAGTGACCTTGCCCTCTGTGCACACAGC

BL21(DE3) *Escherichia coli* cells were transformed with pGEX-6p2 constructs containing cDNAs of parental GST, GST-LBD (a.a. 1-604), GST-SD (a.a. 1-225), GST-IBC (a.a. 226-604), GST-Fragment 3 (a.a. 923-1581), GST-C-terminus (a.a. 2512-2749), which were obtained as previously described [17, 30]. The expressed parental GST or GST-fusion proteins were purified as previously described [17, 30].

BL21(DE3) *Escherichia coli* cells were transformed with pET47-Bcl-2 Δ23 plasmid, which was kindly provided by dr. Varda Shoshan-Barmatz (Ben-Gurion University of the Negev, Israel). After growing overnight at 37°C, bacteria were diluted to an A₆₀₀ of 0.2 and incubated at 40°C for 2 h (heat shock). Protein expression was induced by isopropyl-D-1-thiogalactopyranoside (100 μM) at 20 °C for 2 h. Bacteria were harvested by centrifugation at 5000 g for 5 min and lysed in a buffer containing 150 mM NaCl, 10 mM Tris pH 7.4, 20% glycerol, 30 mM imidazole. Samples were sonicated and centrifuged at 35000 rpm for 40 min. Supernatants were collected and incubated with a Ni²⁺-nitrilotriacetic acid resin (Ni-NTA

agarose) for 1 h at 4°C. The recombinant N-terminal His-tagged and C-terminal truncated Bcl-2 (6xHis-Bcl-2) were eluted with lysis buffer containing 500 mM imidazole.

The purified GST- and His-tagged proteins were dialyzed against standard phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Invitrogen, Merelbeke, Belgium) using Slide-A-Lyzer cassettes with a cut-off of 10 kDa (Thermo Fisher Scientific, Pittsburg, PA, USA). The concentration of the purified and dialyzed proteins was determined using the Bradford assay (Sigma-Aldrich, Munich, Germany). Purity and quality were assessed after SDS-PAGE *via* total protein staining using the Imperial Protein Stain (Thermo Fisher Scientific, Pittsburg, PA, USA) following the manufacturer's recommendations.

Cell culture and transfections

All media and supplements used in this paper were purchased from Life Technologies (Ghent, Belgium) unless stated otherwise.

COS-1 cells used for GST-pull downs and single cell measurements were cultured at 37°C, 10% CO_2 in Dulbecco's Modified Eagle's medium (DMEM), containing 10% fetal calf serum (Sigma-Aldrich), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone and 2 mM glutamax. 24 h after seeding COS-1 cells were transiently transfected with empty pCMV24-3xFLAG-Myc (3xFLAGempty) or with 3xFLAG-Bcl-2 plasmids, containing mCherry and the p2a sequence (3xFLAG-p2a-mCherry and 3xFLAG-Bcl-2-p2a-mCherry) [29]. X-tremeGene HP DNA (Roche, Basel, Switzerland) was used as a transfection reagent according to the manufacturer's instructions.

COS-7 cells used for the competitive fluorescent ligand assay were obtained from the RIKEN Cell Bank (Tokyo, Japan) and were cultured in DMEM with 1000 µg/ml glucose (Sigma-Aldrich), and supplemented with 10% fetal calf serum (Gibco BRL, Rockville, MD, U.S.A.), 100 IU/ml penicillin (Gibco BRL) and 100 µg/ml streptomycin (Gibco BRL). Cells were transfected with 5 µg/ml of plasmid using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions.

MEF cells were cultured at 37°C in a 10% CO_2 incubator in DMEM/Ham's F12 medium supplemented with 10% fetal calf serum, 3.8 mM L-glutamine, 85 IU/ml penicillin and 85 µg/ml streptomycin.

DT40-3KO with ectopically expressing $\text{IP}_3\text{R1}$ [31] were used for the nuclear patch-clamp experiments. The cells were cultured at 39°C in a 5% CO_2 incubator in RPMi medium supplemented with 10% fetal calf serum, 1% chicken serum, 2.05 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

The previously described HEK cells lacking all three IP_3R isoforms (HEK-3KO) [32], were used for single-cells Ca^{2+} measurements. HEK-3KO were co-transfected with pcDNA3- IP_3R or pcDNA3- $\text{IP}_3\text{R}^{\Delta 1389-1408}$ and 3xFLAG-p2a-mCherry or 3xFLAG-Bcl-2-p2A-mCherry.

GST-pull down assays

48 h after transfection COS-1 cells overexpressing 3xFLAG-Bcl-2 were harvested and lysed in a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl_2 , 0.5 mM DTT, 1% Triton X-100 and protease inhibitor cocktail tablets (Roche). After 30 min of incubation at 4°C the clear lysates were collected *via* centrifugation for 2 min at 10 000 rpm at 4°C. Parental GST or GST-fused fragments of $\text{IP}_3\text{R1}$ (0.5 µM) were incubated together with 100 µg lysate in the lysing buffer (final volume 500 µl) at 4°C. After 1 h the GST-proteins, used as bait, were immobilized on glutathione-Sepharose 4B beads (GE Healthcare, Diegem, Belgium) for 1.5 h

at 4°C. In order to study the effect of the AdA, 3 µM AdA (Merck Chemicals, Belgium) or the vehicle control MQ water was added during the incubation. The beads were washed 5 times with the Triton X-100 buffer. The GST-complexes were eluted in 40 µl 2×LDS (Life Technologies) supplemented with 1:200 β-mercaptoethanol by boiling for 5 min at 95°C. Samples (10 µl) were analyzed *via* SDS-PAGE, using mouse monoclonal HRP-conjugated anti-FLAG M2 (1:1000; Sigma-Aldrich) and the quantification was performed as previously described.

SPR experiments

SPR analysis was performed using a Biacore T200 (GE Healthcare, Diegem, Belgium). Immobilization to the streptavidin-coated sensor chip (BR-1005-31; GE Healthcare) and SPR measurements were performed as described previously [18]. NaOH (50 mM) with 0.0026% SDS was used as a regeneration buffer. The IP₃ and AdA were added at the indicated concentration to the analytes.

Single-cell cytosolic Ca²⁺ imaging

Fura-2-AM [Ca²⁺] measurements in COS-1 and HEK-3KO cells were performed as previously described [25]. BAPTA (3 mM) was added for 1 min prior to stimulation with ATP or CCH to chelate all free extracellular Ca²⁺. Cytosolic Ca²⁺ rises in response to 0.5 µM and 100 µM ATP or 10 µM CCH were measured in mCherry-positive (excitation 546 nm, emission 610 nm) and Fura-2-loaded cells. Intracellular cytoplasmic Ca²⁺ concentrations were calculated as previously described [18]

Single-cell ER Ca²⁺ imaging

The G-CEPIA1-ER construct was introduced into COS-1 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 min later 0.5 or 100 µM ATP was added. All traces were normalized (F/F₀) where F₀ is the baseline fluorescence of each trace.

Unidirectional ⁴⁵Ca²⁺-flux assay

The unidirectional ⁴⁵Ca²⁺-flux experiments were performed in permeabilized MEFs as previously described [18]. IICR was triggered during the unidirectional ⁴⁵Ca²⁺-efflux phase by the addition of the indicated [IP₃] for 2 min. BH4-Bcl-2 peptide was added 2 min before IP₃ till 2 min after IP₃. IICR was plotted as fractional loss, representing the amount of actively accumulated Ca²⁺ leaving the stores during the sampling period [33].

Electrophysiology

Isolated nuclei from DT40-3KO cells stably transfected with rabbit IP₃R1 were prepared by homogenization as previously described [31]. A 3 µl aliquot of nuclear suspension was placed in 3 ml of bath solution which contained 140 mM KCl, 10 mM Hepes, 500 µM BAPTA and 246 nM free Ca²⁺, pH 7.1. Nuclei were allowed to adhere to a plastic culture dish for 10 min prior to patching. Single IP₃R1 channel potassium currents were measured in the on-nucleus patch clamp configuration using pCLAMP 9 and an Axopatch 200B amplifier (Molecular Devices, Sunnydale, CA, USA). Pipette solution contained 140 mM KCl, 10 mM Hepes, 1-10 µM IP₃, 5 mM ATP, and 200 nM free Ca²⁺ as well as BH4-Bcl-2 peptide or 6xHis-Bcl-2 where noted. Traces were consecutive 3 s sweeps recorded at -100 mV, sampled at 20 kHz and filtered

at 5 kHz. A minimum of 15 s of recordings were considered for data analyses. Pipette resistances were typically 20 M Ω and seal resistances were >5 G Ω . Single channel openings were detected by half-threshold crossing criteria using the event detection protocol in Clampfit 9. We assumed that the number of channels in any particular nuclear patch is represented by the maximum number of discrete stacked events observed during the experiment. Only patches with one apparent channel were considered for analyses. P_o was calculated using Clampfit 9 and Origin 6 software (Origin Lab, Northampton, MA, USA). Error bars are S.E.M.

***In silico* docking study**

The ZDock server version ZD3.0.2 [34] was used to perform a targeted docking experiment with PDB-entry 3UJ4 as template for IP₃R1 in its apo-form [35], and PDB-entry 4LXD as template for Bcl-2 [36]. Seven possible complexes were obtained. Analysis of these complexes reveals that only two complexes did not result in clashes between the LBD of IP₃R and Bcl-2. FiberDock [37] was then used to refine these complexes. The Fiberdock output statistics are given in Supplementary Material. Next, we superimposed the resulting complex with the IP₃-bound structure of IP₃R (PDB-entry 3T8S [38]). PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) was used for visual inspection, superposition and generation of the figures.

Competitive fluorescent ligand assay

COS-7 cells were grown in experimental chambers consisting of 7-mm plastic cylinders and fibronectin-coated cover slips. Permeabilization was performed by exposing cells to intracellular-like medium (ICM: 125 mM KCl, 19 mM NaCl, 10 mM Hepes-KOH, pH 7.3, 1 mM EGTA, and 330 μ M CaCl₂) containing 200 μ g/ml (w/v) saponin (ICN, Cleveland, OH, U.S.A.) for ~ 90 sec. Permeabilized cells were washed with ICM and then exposed to ICM containing various concentrations of IP₃ or other reagents. Fluorescence images were captured using a dual-wavelength ratio imaging system consisting of an EM-CCD camera (C9135-special; Hamamatsu photonics, Shizuoka, Japan) and W-View (Hamamatsu photonics) optics coupled to a Nikon TE2000 inverted fluorescence microscope equipped with a Nikon S Fluor 60 oil immersion objective (NA 1.25). Fluorescence were monitored with excitation at 425nm and dual-emission at 480nm and 535 nm. Data were analyzed with AQUACOSMOS 2.6 software (Hamamatsu photonics). All the experiments were performed at room temperature.

Statistical analysis

Two-tailed unpaired Student's *t*-tests were used to compare two conditions. When comparing three or more conditions a repeated measure ANOVA with Bonferroni post test was performed.

* indicates significantly different results with $p < 0.05$.

RESULTS

Bcl-2 fails to inhibit IP₃R-mediated Ca²⁺ release triggered by high concentrations of agonist in intact cells. Various studies have demonstrated that Bcl-2 overexpression dampens agonist-induced Ca²⁺ release in multiple intact cell models [15, 18, 39]. Nevertheless, most of those experiments were performed using submaximal concentrations of agonist. Here, we compared the impact of Bcl-2 overexpression on IP₃R-mediated Ca²⁺ signaling triggered by a range of ATP concentrations. Using the ratiometric fluorescent Ca²⁺ dye Fura-2, we monitored the cytosolic [Ca²⁺] rises in COS-1 cells transfected either with an empty vector or with 3xFLAG-Bcl-2. As a selection marker, we used mCherry encoded in the same plasmid, but

separated by the p2A sequence to eventually yield two separate expressed proteins, 3xFLAG(-Bcl-2) and mCherry [29]. Thus, all cells that express the mCherry protein also express 3xFLAG-Bcl-2. The free extracellular $[Ca^{2+}]$ was reduced to < 10 nM by the addition of BAPTA (3 mM) ensuring that the ATP-induced $[Ca^{2+}]$ rise is only due to Ca^{2+} release from intracellular stores. As previously shown, 3xFLAG-Bcl-2 decreased the cytosolic $[Ca^{2+}]$ rises in response to a submaximal ATP concentration (0.5 μ M) (**Fig. 1A**). In contrast, when a high concentration (100 μ M) of the agonist was applied, 3xFLAG-Bcl-2 failed to inhibit the Ca^{2+} release (**Fig. 1B, Fig. S1**), in good agreement with a previous report [15]. In addition to the decreased capacity of Bcl-2 to inhibit agonist-induced $[Ca^{2+}]$ rises at high [ATP] (**Fig. 1C**), we observed a decrease in the number of responding cells in that condition (**Fig. 1D**). From our raw data it is clear that the signal obtained by the addition of 100 μ M ATP is much lower than the response to ionomycin (**Fig. S1**), excluding a possible saturation of the Fura-2 signal as a potential cause for our observation.

The overall increase in the cytosolic $[Ca^{2+}]$ triggered by ATP is influenced by the net Ca^{2+} release from the ER into the cytosol and its extrusion from the cytosol to other compartments such as the mitochondria or to the extracellular medium. Furthermore, in living cells, Bcl-2 is well known to modulate other Ca^{2+} -transport systems besides IP₃Rs [11], including the Ca^{2+} flux into the mitochondria [40]. Therefore, to strengthen the contention that the decrease in ATP-induced $[Ca^{2+}]$ rises in the cytosol caused by Bcl-2 overexpression is due to an inhibition of Ca^{2+} flux from the ER and not to altered Ca^{2+} extrusion from the cytosol, we directly monitored the Ca^{2+} dynamics in the ER using the genetically encoded Ca^{2+} sensor G-CEPIA1-ER. We challenged COS-1 cells expressing G-CEPIA1-ER together with either 3xFLAG-vector or 3xFLAG-Bcl-2 with 0.5 or 100 μ M ATP and determined the decrease in the ER Ca^{2+} content. Compared to the control cells, Bcl-2-overexpressing cells displayed a lower decrease in ER Ca^{2+} levels when evoked by 0.5 μ M ATP, but not by 100 μ M ATP (**Fig. 1E-G**). Similar results were obtained by analyzing the % of responding cells (**Fig. 1H**), which indicated that in living cells Bcl-2 inhibits Ca^{2+} flux from the ER triggered by low, but not by high [ATP]. Nevertheless, the cytosolic $[Ca^{2+}]$ rise measured by Fura-2 appeared more strongly suppressed by Bcl-2 overexpression than the ER $[Ca^{2+}]$ drop measured by G-CEPIA1-ER. This may be due to differences in the dynamic range at which the measurements were performed or may be the result of the impact of Bcl-2 on non-ER Ca^{2+} -transport systems.

The IP₃R-inhibitory properties of purified Bcl-2 or of its BH4 domain decline with increasing [IP₃] or [agonist]. Next, we aimed to measure the direct effect of Bcl-2 on the activity of IP₃R1 channels in response to different concentrations of IP₃. We conducted IP₃R1 single-channel recordings by using the nuclear-membrane patch-clamp technique on isolated nuclei obtained from DT40-3KO cells ectopically expressing IP₃R1. When 1 μ M IP₃ was used to trigger IP₃R opening, application of 1 μ M purified Bcl-2 (6xHis-Bcl-2) decreased the open probability (P_o) of IP₃R1 by 4-fold, while when 10 μ M IP₃ was applied 6xHis-Bcl-2 did not affect the channel activity (**Fig. 2A-C**). This observation also strongly advocates a direct effect of Bcl-2 on the IP₃R in our results obtained with intact cells (**Fig. 1**).

The BH4 domain of Bcl-2 has been documented to be sufficient to inhibit IP₃R-mediated Ca^{2+} release in various experimental settings, including Ca^{2+} imaging in intact cells, unidirectional $^{45}Ca^{2+}$ fluxes in permeabilized cells and single channel recordings using nuclear patch [18, 23,

39]. In all these conditions, submaximal concentrations of IP₃ or agonist were applied to activate IP₃R channels. Here, we assessed the ability of Bcl-2's BH4 domain, produced as a synthetic peptide (BH4-Bcl-2), to inhibit IP₃R channels that were activated by varying concentrations of IP₃. The obtained results were in line with our data with recombinant Bcl-2. While as previously shown, 50 μM BH4-Bcl-2 strongly decreased the P_o triggered by 1 μM IP₃ [39], the peptide BH4-Bcl-2 failed to decrease the P_o of IP₃R1 triggered by 5 μM IP₃ (**Fig. 2D-F**).

Finally, we monitored the effect of the BH4-Bcl-2 on IP₃-induced Ca²⁺ release (IICR) by performing unidirectional ⁴⁵Ca²⁺ flux assays in saponin-permeabilized mouse embryonic fibroblasts (MEFs) (**Fig. 3**). These cells have low endogenous levels of Bcl-2 [16] and as such, they are a good system to study the activity of exogenous Bcl-2-protein functions. The non-mitochondrial Ca²⁺ stores were loaded with ⁴⁵Ca²⁺ and the unidirectional Ca²⁺ flux was measured in the presence of EGTA (1 mM) and thapsigargin (Tg) (4 μM). We quantified ⁴⁵Ca²⁺ release triggered by sub- and supramaximal concentrations of IP₃ in presence or absence of different concentrations of the BH4-Bcl-2 peptide. BH4-Bcl-2 was added from 2 min before until 2 min after IP₃ application. The results are plotted as the fractional loss (% / 2 min) over time. Consistent with previous reports, in these experiments BH4-Bcl-2 suppressed IICR triggered by a submaximal concentration of IP₃ (2 μM) with an IC₅₀ of about 20 μM (**Fig. 3A**). Surprisingly, the peptide appeared to be a less potent inhibitor of IICR when a higher concentration of IP₃ (4 μM) was applied (**Fig. 3B**). Increasing the IP₃ concentration to 10 μM (**Fig. 3C**) and 50 μM (**Fig. 3D**) led to the failure of the tested concentrations of BH4-Bcl-2 to inhibit IICR (**Fig. 3E**).

Taken together, our data clearly show that Ca²⁺ release evoked by high concentrations of IP₃ is neither subject to inhibition by Bcl-2 nor by its BH4 domain.

Bcl-2 remains capable of inhibiting IP₃R1 lacking a.a. 1389-1408. This amino acid stretch, previously recognized as responsible for the interaction of IP₃R1 with Bcl-2, is highly conserved among different IP₃R isoforms and species and even other intracellular Ca²⁺-release channels, such as ryanodine receptors [18, 41, 42]. Therefore, we anticipated that deleting this region in the full-length IP₃R1 channel would completely abrogate the ability of Bcl-2 to inhibit IP₃R1-mediated Ca²⁺ release. To test this hypothesis we transfected HEK-3KO cells in which all three IP₃R isoforms have been deleted by CRISPR/Cas9 [32] with wild-type IP₃R1 or IP₃R1^{Δ1389-1408} and 3xFLAG-Bcl-2-p2A-mCherry or empty plasmid (**Fig. 4A**). The expression levels of IP₃R1^{Δ1389-1408} appeared lower than those of the wild-type IP₃R1. A ratio IP₃R1:mCherry 3:1 was used, ensuring that all mCherry expressing cells contain IP₃R1 or IP₃R1^{Δ1389-1408}. We compared the effect of Bcl-2 overexpression on wild-type IP₃R1 *versus* IP₃R1^{Δ1389-1408}-mediated Ca²⁺ release triggered by 10 μM carbachol (CCH) by performing similar Fura-2 based measurements of [Ca²⁺] as described above. As expected, 3xFLAG-Bcl-2 decreased the number of responding cells expressing wild-type IP₃R1 as well as the magnitude of cytosolic [Ca²⁺] rises in response to 10 μM CCH (**Fig. 4B**). Importantly, cells expressing IP₃R1^{Δ1389-1408} were also responsive to CCH stimulation, although less cells responded and the response exhibited a lower amplitude compared to the cells expressing the wild-type IP₃R1 (**Fig. 4C**). This could be due to the lower activity of the IP₃R1^{Δ1389-1408} and/or its lower expression relative to the wild-type IP₃R1. In any case, these data indicate that the IP₃R1^{Δ1389-1408} is functional and thus can be used to assess whether or not its Ca²⁺-flux properties can be

1 inhibited by Bcl-2. Importantly, despite the fact that the IP₃R1^{Δ1389-1408} lacks the Bcl-2-binding
2 site in the modulatory region, Ca²⁺ release mediated by this mutated channel could still be
3 reduced by 3xFLAG-Bcl-2 overexpression (**Fig. 4D and E**). These findings suggest that Bcl-2
4 and its BH4 domain might target additional region(s) outside of the a.a. stretch 1389-1408 in
5 IP₃R1.

6 ***In silico* docking of 3D structures reveals a possible complex formation between the BH4**
7 **domain of Bcl-2 with the apo-, but not with the IP₃-bound form of the LBD of IP₃R1.**

8 Based on the functional data, suggesting that (i) IP₃ levels can modulate the extent of the action
9 of Bcl-2 on the IP₃R and (ii) Bcl-2, via its BH4 domain could interact with IP₃R outside of the
10 previously described binding site in the modulatory region, we hypothesized that this additional
11 target might be the LBD of IP₃R1. We first applied a modeling approach to assess complex
12 formation between the BH4 domain of Bcl-2 and the LBD of IP₃R1 and performed a targeted
13 docking experiment with ZDock [34]. Using the PDB-entries of Bcl-2 (4LXD, [36]) and of the
14 apo-form of the LBD of IP₃R1 (3UJ4, [35]) as templates, we obtained seven possible
15 complexes. Two of them were very alike, and in both models, Bcl-2 did not clash with the LBD
16 of IP₃R. We used FiberDock [37] to determine the most probable complex (the global energy
17 of each complex is given in Supplementary Material). This low-energy model is presented in
18 **Fig. 5A**. Next, we compared the conformational changes in the LBD after IP₃ binding (PDB-
19 entry 3T8S, [38]) with the apo-form of the LBD of IP₃R1. IP₃ binds in a cleft that is formed by
20 the two domains within the LBD, the suppressor domain (SD) and the IBC. This cleft closes
21 and becomes narrower when IP₃ is bound (**Fig. 5B**). Superposition of the docked complex
22 between Bcl-2 and the apo-form of IP₃R1 and the IP₃-bound form of IP₃R1 revealed that Bcl-2
23 might not be able to bind to the LBD in the presence of IP₃. This might be due to spatial
24 constraints, which result from conformational changes in IP₃R1 upon IP₃ binding; especially,
25 the cleft between the two domains of the LBD is rendered too narrow for Bcl-2 to bind (**Fig.**
26 **5C**).

27 **Bcl-2 targets the IBC of IP₃R1 via its BH4 domain.** We examined whether Bcl-2 is able to
28 directly bind to LBD and/or its subdomains, IBC and SD. Using purified GST-fused IP₃R1
29 fragments as bait, we performed GST-pull down experiments with lysates from COS-1 cells
30 overexpressing 3xFLAG-Bcl-2. The GST-Fragment 3 and GST-C-terminus served as positive
31 controls for Bcl-2 binding, while the parental GST formed the negative control. 3xFLAG-Bcl-
32 2 bound with similar efficiency to the GST-Fragment 3, GST-C-terminus, GST-LBD and GST-
33 BC, while a less prominent binding to the GST-SD was observed (**Fig. 6A and B**).

34 Second, we aimed to assess whether the BH4 domain of Bcl-2 directly binds to the LBD of
35 IP₃R1 using the surface plasmon resonance (SPR) technique. We used a biotinylated peptide
36 that covers the BH4 domain of Bcl-2 (biotin-BH4-Bcl-2), which was immobilized on a
37 streptavidin chip and we monitored its binding to purified GST-LBD (a.a. 1-604) (**Fig. 6C**),
38 GST-IBC (a.a. 226-604) (**Fig. 6D**) and GST-SD (a.a. 1-225) (**Fig. 6E**) of IP₃R1. As control
39 analytes, we used parental GST (negative control) and GST-Fragment 3 (a.a. 923-1581; positive
40 control), which was described as the main Bcl-2-binding region on IP₃R1. The signals were
41 corrected by subtracting the background binding to a control scrambled peptide, immobilized
42 on another channel on the same chip. The generated response curves display a concentration-
43 dependent increase in resonance units (RU) for the positive control GST-Fragment 3, GST-
44 LBD and GST-BC, indicating a specific binding of biotin-BH4-Bcl-2 to these fragments (**Fig.**

1 **6F).** With GST-SD the increase in RU was significantly less while parental GST did not show
2 any substantial binding to the biotin-BH4-Bcl-2.

3 **IP₃ and AdA disturb the interaction between BH4-Bcl-2 or full length Bcl-2 and the LBD**
4 **of IP₃R1.**

5 The results obtained above indicated that overexpressed Bcl-2 directly targets the
6 LBD of IP₃R1. Furthermore, we further narrowed down the interacting region to the BC,
7 containing the determinants of IP₃ binding. To test whether ligands binding to the LBD can
8 disturb the interaction between Bcl-2 and LBD, we performed GST-pull down experiments in
9 the presence of AdA, a more stable and potent IP₃R agonist [43]. Our rationale for utilizing
10 AdA over IP₃ in those experiments was that the presence of phosphatases and kinases in the
11 cell lysate, which can metabolize IP₃ might interfere with IP₃ stability [44]. Our results revealed
12 that the binding of full-length Bcl-2 to GST-LBD was severely impaired in the presence of
13 AdA, while the binding to GST-Fragment 3 was not affected (**Fig. 7A**). Next, we assessed
14 whether IP₃ and Bcl-2 bind to the same residues within the BC. We compared the ability of
15 3xFLAG-Bcl-2 to bind to GST-LBD and GST-LBD K508Q, a mutant version previously
16 shown to display severely reduced IP₃ binding capacity [45]. This analysis did not reveal any
17 significant differences in the binding affinity (**Fig. 7B**), which suggests that although Bcl-2
18 targets the IBC of IP₃R1, it does not interact with the same amino acids as IP₃.

19 In order to test whether IP₃R ligands could interfere with the interaction between the BH4
20 domain and LBD, we conducted SPR experiments in the presence of IP₃ and AdA. Here, we
21 used purified GST-fragments and peptides, thereby allowing the use of IP₃. The sensorgrams,
22 shown on **Fig. 7C**, reveal that the addition of IP₃ or AdA results in a decreased signal. The
23 compounds did not bind directly to the biotin-BH4-Bcl-2 and did not significantly affect the
24 binding of GST-Fragment 3 to the peptide (**Fig. 7D**). Based on these data, we concluded that
25 IP₃ and AdA disturb selectively the interaction between BH4-Bcl-2 and the LBD of IP₃R1. To
26 assess whether IP₃ and Bcl-2 bind to the same residues within the BC, we compared the ability
27 of biotin-BH4-Bcl-2 to bind to GST-LBD and GST-LBD K508Q. This analysis did not reveal
28 any significant differences in the binding affinity (**Fig. 7E**), confirming that like the full-length
29 Bcl-2, the BH4-Bcl-2 targets the IBC of IP₃R1, but not the same residues as IP₃.

29 **The BH4-Bcl-2 disrupts the interaction between LBD and a fluorescently labeled IP₃R**
30 **ligand.**

31 We showed that IP₃, as well as AdA, could interfere with BH4-Bcl-2 binding to the
32 LBD of IP₃R1. To address the opposite question, namely whether BH4-Bcl-2 might affect the
33 interaction of IP₃ with the LBD, we performed FRET-based measurements using a competitive
34 fluorescent ligand assay for IP₃ [46]. In this method, binding of a fluorescent low-affinity ligand
35 (FLL) and a fluorescent IP₃-binding protein (CFP-coupled LBD) generates FRET signals.
36 Ligands of the IP₃R, such as IP₃, reduce this FRET signal due to the decrease in the binding of
37 FLL to CFP-coupled LBD, and increase the fluorescence emission ratio of CFP/FLL (480
38 nm/535 nm) (**Fig. 8A**). We showed that application of BH4-Bcl-2, but not of a scrambled
39 version, caused an increase in the CFP/FLL signal, indicating that BH4-Bcl-2 decreased the
40 binding of FLL to the LBD (**Fig. 8A**). IP₃ (10 μ M) was applied at the end of the experiment to
41 determine the maximal response. BH4-Bcl-2 increased the fluorescence ratio in a
42 concentration-dependent manner, and the IC₅₀ value of BH4-Bcl-2 in the presence of 100 nM
43 FLL could be estimated to be approximatively about 20 μ M (**Fig. 8B**).

DISCUSSION

The main findings of this study are that (i) IP₃R1 inhibition by the anti-apoptotic Bcl-2 protein is modulated by the ligand, IP₃, and thus by the level of IP₃R activity; (ii) Bcl-2, via its BH4 domain, has a novel target, represented by the LBD of IP₃R1, a domain distinct from the modulatory region; (iii) Bcl-2 and IP₃ display a mutual antagonistic impact on their ability to bind the LBD of the IP₃R1.

Previous works suggested that IP₃R inhibition by Bcl-2 is not a static phenomenon, but can be dynamically modulated, perhaps partially by the level of IP₃ signaling in response to agonist stimulation [15, 27]. Yet, the molecular mechanisms that could account for the modulatory nature of Bcl-2-mediated IP₃R inhibition remained unresolved. After confirming in the present study that the level of IP₃ (or of agonist), indeed modulates the inhibitory effect of Bcl-2 and its BH4 domain on IP₃R activity, we demonstrated that Bcl-2 is able to target IP₃R1 outside of its modulatory domain. This unexpected finding sparked the identification of the LBD of IP₃R1 as a novel target of Bcl-2 and its BH4 domain.

The original approach for examining the IP₃R regions targeted by Bcl-2 was based on GST-pull down experiments [16]. This method makes use of GST-tagged proteins, representing the fragments obtained after limited proteolysis of the full-length IP₃R1 channel. However, this approach might miss certain functional regions, since limited proteolysis does not necessarily coincide with functional boundaries. For instance, the LBD covers a.a. 1-604, while limited proteolysis yield a Fragment 1 corresponding to a.a. 1-345 and a Fragment 2 corresponding to a.a. 346-922. As a consequence, an interaction, which would require the intact LBD, might be missed by using such an experimental paradigm. Instead, here, we made use of the functional domain (LBD) and its subdomains (IBC and SD) and revealed that Bcl-2 *via* its BH4 domain targets the same region of the LBD as IP₃, namely the BC. Nevertheless, our SPR and GST-pull down experiments, which show that Bcl-2 and its BH4 domain target the wild-type and the “loss of function” mutant LBD with similar affinity, indicate that Bcl-2 and IP₃ have different underlying binding determinants. We strongly believe that future identification of the IP₃R residues involved in the Bcl-2 binding is of a great importance. This might prompt the development of novel tools to disturb the Bcl-2 / IP₃R interaction, which has been shown to play crucial role in the survival of cancer cells [47, 48].

The fact that Bcl-2 and its ligand IP₃ target the same region but not necessarily the same residues, suggests that the Bcl-2 / IP₃R complex might be allosterically modulated by IP₃ levels. This is supported by (i) computational modelling showing that Bcl-2 binding to the LBD is sterically hindered by the conformational changes induced by IP₃ binding and (ii) experimental data demonstrating that IP₃ and AdA interfere with the Bcl-2 (BH4 domain) / LBD interaction. *Vice versa*, it seems that the BH4 domain of Bcl-2 decreased ligand binding to the LBD, in line with the previously reported ability of full-length Bcl-2 to decrease the affinity of IP₃ for binding to the full-length IP₃R [14]. Thus, Bcl-2 might serve as an efficient inhibitor of IP₃R channels, not only by targeting the modulatory region, thereby interfering with the conformational coupling of IP₃ binding to channel opening, but also by directly targeting the LBD and antagonizing the interaction with IP₃.

Previous work revealed that IP₃ binding to all 4 subunits in a tetrameric IP₃R channel is required for proper channel activation and IP₃R opening [32]. Thus, it is possible that the binding of the BH4 domain of Bcl-2 to just one of the monomers could be sufficient to inhibit IP₃R channel

opening. This may account for the very potent inhibition of IP₃Rs by Bcl-2 or its BH4 domain. Indeed, the BH4 domain of Bcl-2 is able to completely inhibit IP₃R-mediated Ca²⁺ release. As a consequence, higher concentrations of IP₃ would not only increase the probability that all 4 IP₃R subunits are occupied and thus trigger Ca²⁺ flux through the IP₃R, but also decrease the probability that Bcl-2 (or its BH4 domain) accesses the LBD and thus elicits IP₃R inhibition.

Our findings do not necessarily contradict the previously reported observations (replicated in this study) on Bcl-2 interaction with the modulatory region of IP₃R. In fact, the inhibition of IP₃R1^{Δ1389-1408}, lacking the Bcl-2-binding site in the modulatory region, appeared less prominent than the inhibition of the wild-type IP₃R1 by Bcl-2. This supports the concept that IP₃R modulation by Bcl-2 occurs via both Bcl-2-binding sites, one located in the LBD and the other one located between residues 1389-1408.

An inherent limitation of the current study is the link between the binding of Bcl-2 to the LBD in the full-length IP₃R and IP₃R inhibition by Bcl-2. The unequivocal proof of such link will require the identification of the amino acid stretch or specific residues within the LBD responsible for Bcl-2 binding, their deletion in the full-length IP₃R1 and IP₃R1^{Δ1389-1408} and assessment of Bcl-2 impact on them. Of course, an inherent prerequisite for such analysis is that deleting the Bcl-2-binding site in the LBD region in full-length IP₃R1 does not render the channel irresponsive to IP₃. However, we would like to stress that the complete IP₃R sequence was screened in previous studies for potential Bcl-2-binding sites. No other regions than the Fragment 3 and the C-terminus were identified. Fragment 3 is targeted by Bcl-2's BH4 domain [18], whereas the C-terminus serves to recruit Bcl-2, via its TMD, in the proximity of IP₃Rs [25]. Thus, it is very unlikely that other regions besides the LBD could account for the inhibition of IP₃R1^{Δ1389-1408} by Bcl-2.

From the IP₃R structure [21, 26, 49] (**Fig. 9**), it seems that both Bcl-2-binding regions are surface accessible. Interestingly, in this 3D structure, a.a. 1389-1408, representing the central Bcl-2-binding site, from one subunit appeared to be located in very close proximity of the LBD of the neighboring monomer. Therefore, the 3D structure supports the possibility of having two Bcl-2-binding sites in IP₃R1 in close proximity to each other. However, further work is needed to understand whether Bcl-2 can occupy both sites in neighboring monomers, whether binding of Bcl-2 to one site prevents the binding of Bcl-2 to the neighboring site or whether Bcl-2 can dynamically switch between both binding sites. Moreover, the loss of IP₃R inhibition by Bcl-2 in conditions of high [IP₃] suggest that in these conditions Bcl-2 binding to the central site may not be sufficient for IP₃R inhibition by Bcl-2. In any case, the Bcl-2 inhibitory effects on IP₃Rs present at low [IP₃] but not at high [IP₃] are not solely due to the interaction with the Fragment 3, since (i) Bcl-2 inhibits the channel lacking the binding site in Fragment 3 and (ii) the binding of Bcl-2 or its BH4 domain to Fragment 3 was not affected by the presence of IP₃R agonists. However, further work is needed to understand IP₃R/Bcl-2-complex formation at the molecular level.

It should be noted that the LBD has been previously identified as a binding site for other Bcl-2-family members. Indeed, Bcl-2 like protein (Bcl-2L10) [50] and its zebrafish orthologue Nr2 [51], have been shown to decrease IP₃ binding to the IP₃R, by targeting the LBD. Disruption of this interaction appeared as a potential strategy to target breast cancer [52]. Therefore, our study is revealing yet another human anti-apoptotic Bcl-2-protein that can target the LBD of IP₃Rs, interfering with IP₃ binding and suppressing IP₃R activity.

To conclude, IP₃ and Bcl-2 compete for the LBD, thereby antagonizing each other's effects on IP₃R function. On the one hand, Bcl-2 fails to inhibit IP₃R activity provoked by high [IP₃] or [agonist] in *in vitro* or *in cellulo* systems, respectively. Hence, this shows that IP₃R inhibition by Bcl-2 is modulated by the level of agonist stimulation. On the other hand, Bcl-2 or its BH4 domain counteract IP₃ binding to the IP₃R or LBD. This provides a novel molecular mechanism by which Bcl-2 proteins can inhibit IP₃R function, at least in conditions of moderate IP₃ signaling or agonist stimulation.

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REFERENCES

1. Tsujimoto, Y., et al., *Involvement of the bcl-2 gene in human follicular lymphoma*. Science, 1985. **228**(4706): p. 1440-3.
2. Vaux, D.L., S. Cory, and J.M. Adams, *Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells*. Nature, 1988. **335**(6189): p. 440-2.
3. Cuende, E., et al., *Programmed cell death by bcl-2-dependent and independent mechanisms in B lymphoma cells*. EMBO J, 1993. **12**(4): p. 1555-60.
4. Czabotar, P.E., et al., *Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy*. Nat Rev Mol Cell Biol, 2014. **15**(1): p. 49-63.
5. Brunelle, J.K. and A. Letai, *Control of mitochondrial apoptosis by the Bcl-2 family*. J Cell Sci, 2009. **122**(Pt 4): p. 437-41.
6. Cory, S. and J.M. Adams, *The Bcl2 family: regulators of the cellular life-or-death switch*. Nat Rev Cancer, 2002. **2**(9): p. 647-56.
7. Gross, A., J.M. McDonnell, and S.J. Korsmeyer, *BCL-2 family members and the mitochondria in apoptosis*. Genes Dev, 1999. **13**(15): p. 1899-911.
8. Antonsson, B., et al., *Inhibition of Bax channel-forming activity by Bcl-2*. Science, 1997. **277**(5324): p. 370-2.
9. Barclay, L.A., et al., *Inhibition of Pro-apoptotic BAX by a noncanonical interaction mechanism*. Mol Cell, 2015. **57**(5): p. 873-86.
10. Baffy, G., et al., *Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production*. J Biol Chem, 1993. **268**(9): p. 6511-9.
11. Vervliet, T., J.B. Parys, and G. Bultynck, *Bcl-2 proteins and calcium signaling: complexity beneath the surface*. Oncogene, 2016. **35**(39): p. 5079-92.

12. Pinton, P. and R. Rizzuto, *Bcl-2 and Ca^{2+} homeostasis in the endoplasmic reticulum*. Cell Death Differ, 2006. **13**(8): p. 1409-18.
13. Rong, Y. and C.W. Distelhorst, *Bcl-2 protein family members: versatile regulators of calcium signaling in cell survival and apoptosis*. Annu Rev Physiol, 2008. **70**: p. 73-91.
14. Chen, R., et al., *Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate*. J Cell Biol, 2004. **166**(2): p. 193-203.
15. Hanson, C.J., et al., *Bcl-2 suppresses Ca^{2+} release through inositol 1,4,5-trisphosphate receptors and inhibits Ca^{2+} uptake by mitochondria without affecting ER calcium store content*. Cell Calcium, 2008. **44**(3): p. 324-38.
16. Rong, Y.P., et al., *Targeting Bcl-2-IP₃ receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals*. Mol Cell, 2008. **31**(2): p. 255-65.
17. Monaco, G., et al., *Profiling of the Bcl-2/Bcl-X_L-binding sites on type 1 IP₃ receptor*. Biochem Biophys Res Commun, 2012. **428**(1): p. 31-5.
18. Monaco, G., et al., *Selective regulation of IP₃-receptor-mediated Ca^{2+} signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-X_L*. Cell Death Differ, 2012. **19**(2): p. 295-309.
19. Yoshikawa, F., et al., *Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor. Structural and functional coupling of cleaved ligand binding and channel domains*. J Biol Chem, 1999. **274**(1): p. 316-27.
20. Maes, K., et al., *Mapping of the ATP-binding sites on inositol 1,4,5-trisphosphate receptor type 1 and type 3 homotetramers by controlled proteolysis and photoaffinity labeling*. J Biol Chem, 2001. **276**(5): p. 3492-7.
21. Fan, G., et al., *Gating machinery of InsP₃R channels revealed by electron cryomicroscopy*. Nature, 2015. **527**(7578): p. 336-41.
22. Eckenrode, E.F., et al., *Apoptosis protection by Mcl-1 and Bcl-2 modulation of inositol 1,4,5-trisphosphate receptor-dependent Ca^{2+} signaling*. J Biol Chem, 2010. **285**(18): p. 13678-84.
23. Rong, Y.P., et al., *The BH4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding the regulatory and coupling domain of the IP₃ receptor*. Proc Natl Acad Sci U S A, 2009. **106**(34): p. 14397-402.
24. Rong, Y.P., et al., *Targeting Bcl-2 based on the interaction of its BH4 domain with the inositol 1,4,5-trisphosphate receptor*. Biochim Biophys Acta, 2009. **1793**(6): p. 971-8.
25. Ivanova, H., et al., *The trans-membrane domain of Bcl-2alpha, but not its hydrophobic cleft, is a critical determinant for efficient IP₃ receptor inhibition*. Oncotarget, 2016.
26. Fan, G., et al., *Cryo-EM reveals ligand induced allostery underlying InsP₃R channel gating*. Cell Res, 2018. **28**(12): p. 1158-1170.
27. Zhong, F., et al., *Bcl-2 differentially regulates Ca^{2+} signals according to the strength of T cell receptor activation*. J Cell Biol, 2006. **172**(1): p. 127-37.
28. Suzuki, J., et al., *Imaging intraorganellar Ca^{2+} at subcellular resolution using CEPIA*. Nat Commun, 2014. **5**: p. 4153.
29. Vervliet, T., et al., *Regulation of the ryanodine receptor by anti-apoptotic Bcl-2 is independent of its BH3-domain-binding properties*. Biochem Biophys Res Commun, 2015. **463**(3): p. 174-9.
30. Bultynck, G., et al., *Thimerosal stimulates Ca^{2+} flux through inositol 1,4,5-trisphosphate receptor type 1, but not type 3, via modulation of an isoform-specific Ca^{2+} -dependent intramolecular interaction*. Biochem J, 2004. **381**(Pt 1): p. 87-96.
31. Wagner, L.E., 2nd and D.I. Yule, *Differential regulation of the InsP₃ receptor type-1 and -2 single channel properties by InsP₃, Ca^{2+} and ATP*. J Physiol, 2012. **590**(Pt 14): p. 3245-59.
32. Alzayady, K.J., et al., *Defining the stoichiometry of inositol 1,4,5-trisphosphate binding required to initiate Ca^{2+} release*. Sci Signal, 2016. **9**(422): p. ra35.
33. Missiaen, L., et al., *Measurement of intracellular Ca^{2+} release in intact and permeabilized cells using $^{45}Ca^{2+}$* . Cold Spring Harb Protoc, 2014. **2014**(3): p. 263-70.
34. Pierce, B.G., et al., *ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers*. Bioinformatics, 2014. **30**(12): p. 1771-3.

35. Seo, M.D., et al., *Structural and functional conservation of key domains in InsP₃ and ryanodine receptors*. Nature, 2012. **483**(7387): p. 108-12.
36. Souers, A.J., et al., *ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets*. Nat Med, 2013. **19**(2): p. 202-8.
37. Mashiach, E., R. Nussinov, and H.J. Wolfson, *FiberDock: a web server for flexible induced-fit backbone refinement in molecular docking*. Nucleic Acids Res, 2010. **38**(Web Server issue): p. W457-61.
38. Lin, C.C., K. Baek, and Z. Lu, *Apo and InsP₃-bound crystal structures of the ligand-binding domain of an InsP₃ receptor*. Nat Struct Mol Biol, 2011. **18**(10): p. 1172-4.
39. Monaco, G., et al., *Alpha-helical destabilization of the Bcl-2-BH4-domain peptide abolishes its ability to inhibit the IP₃ receptor*. PLoS One, 2013. **8**(8): p. e73386.
40. Fouque, A., et al., *The apoptotic members CD95, Bcl-XL, and Bcl-2 cooperate to promote cell migration by inducing Ca²⁺ flux from the endoplasmic reticulum to mitochondria*. Cell Death Differ, 2016. **23**(10): p. 1702-16.
41. Ivanova, H., et al., *The BH4 domain of Bcl-2 orthologues from different classes of vertebrates can act as an evolutionary conserved inhibitor of IP₃ receptor channels*. Cell Calcium, 2017. **62**: p. 41-46.
42. Vervliet, T., et al., *Bcl-2 binds to and inhibits ryanodine receptors*. J Cell Sci, 2014. **127**(Pt 12): p. 2782-92.
43. Takahashi, S., T. Kinoshita, and M. Takahashi, *Adenophostins A and B: potent agonists of inositol-1,4,5-trisphosphate receptor produced by Penicillium brevicompactum. Structure elucidation*. J Antibiot (Tokyo), 1994. **47**(1): p. 95-100.
44. Sims, C.E. and N.L. Allbritton, *Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate by the oocytes of Xenopus laevis*. J Biol Chem, 1998. **273**(7): p. 4052-8.
45. Yoshikawa, F., et al., *Mutational analysis of the ligand binding site of the inositol 1,4,5-trisphosphate receptor*. J Biol Chem, 1996. **271**(30): p. 18277-84.
46. Oura, T., et al., *Highly Sensitive Measurement of Inositol 1,4,5-Trisphosphate by Using a New Fluorescent Ligand and Ligand Binding Domain Combination*. Chembiochem, 2016. **17**(16): p. 1509-12.
47. Greenberg, E.F., et al., *Synergistic killing of human small cell lung cancer cells by the Bcl-2-inositol 1,4,5-trisphosphate receptor disruptor BIRD-2 and the BH3-mimetic ABT-263*. Cell Death Dis, 2015. **6**: p. e2034.
48. Greenberg, E.F., A.R. Lavik, and C.W. Distelhorst, *Bcl-2 regulation of the inositol 1,4,5-trisphosphate receptor and calcium signaling in normal and malignant lymphocytes: potential new target for cancer treatment*. Biochim Biophys Acta, 2014. **1843**(10): p. 2205-10.
49. Hamada, K., et al., *IP₃-mediated gating mechanism of the IP₃ receptor revealed by mutagenesis and X-ray crystallography*. Proc Natl Acad Sci U S A, 2017. **114**(18): p. 4661-4666.
50. Bonneau, B., et al., *IRBIT controls apoptosis by interacting with the Bcl-2 homolog, Bcl2l10, and by promoting ER-mitochondria contact*. Elife, 2016. **5**.
51. Bonneau, B., et al., *The Bcl-2 homolog Nr2 inhibits binding of IP₃ to its receptor to control calcium signaling during zebrafish epiboly*. Sci Signal, 2014. **7**(312): p. ra14.
52. Nougarede, A., et al., *Breast cancer targeting through inhibition of the endoplasmic reticulum-based apoptosis regulator Nr2/BCL2L10*. Cancer Res, 2018. **78**(6): p. 1404-1417.

FIGURE LEGENDS

Fig. 1 Cytosolic $[Ca^{2+}]$ rises triggered by submaximal, but not supramaximal concentration of the agonist, are suppressed by Bcl-2. A-B: Cytosolic Ca^{2+} release in response to 0.5 (A) or 100 (B) μM ATP was measured in the mCherry-positive Fura-2-AM loaded COS-1 cells overexpressing 3xFLAG-empty vector or 3xFLAG-Bcl-2. The free extracellular Ca^{2+} was chelated by addition of 3 mM BAPTA. The obtained Fura-2 fluorescence signals (F340/F380) were calibrated and representative traces are plotted as $[Ca^{2+}]$. **C:** Quantitative analysis of the amplitude of the ATP-induced Ca^{2+} signals from at least 3 independent experiments, which were performed on different days after independent transfections (total number of cells for each $[ATP] > 90$) is plotted as mean $\pm$ S.E.M. **D:** Quantitative analysis of the number of cells responding to the agonist. **E-F:** Decrease in the ER Ca^{2+} content in response to 0.5 (E) or 100 (F) μM ATP was monitored using G-CEPIA1-ER in mCherry-positive COS-1 cells, overexpressing 3xFLAG-empty vector or 3xFLAG-Bcl-2. Extracellular Ca^{2+} was chelated by the addition of 3 mM BAPTA. Typical normalized (F/F_0) traces of the obtained fluorescence signals are presented. **G:** Quantitative analysis of the ER Ca^{2+} decrease in response to ATP. For each trace, the ATP-induced Ca^{2+} release was determined by subtracting the fluorescence after ATP addition (during plateau phase) from the fluorescence just before ATP addition after normalization. Values depict average $\pm$ S.E.M. from at least 3 independent experiments, which were performed on different days after independent transfections (total number of cells for each $[ATP] > 90$). **H:** Quantitative analysis of the number of cells responding to the agonist expressed as a % of the total number of measured cells. * stands for $p < 0.05$.

Fig. 2 Recombinant Bcl-2 and BH4-Bcl-2 fail to inhibit IP_3R activity induced by high concentrations of IP_3 in single-channel measurements. A-B: Representative IP_3R single-channel recordings from DT40-3KO cells ectopically expressing IP_3R . The channel opening was evoked by 1 (A) or 10 (B) μM IP_3 at 200 nM Ca^{2+} and 5 mM ATP, in the presence of PBS (A-B left) or in the presence of 1 μM 6xHis-Bcl-2 purified protein, which was dialysed against PBS (A-B right). **C:** Histogram depicting the open probability (P_o) $\pm$ S.E.M. for the IP_3R under the described conditions. The total number of recordings for each condition is indicated within every bar. **D-E:** Representative IP_3R single-channel recordings from DT40-3KO cells ectopically expressing IP_3R . The channel opening was evoked by 1 (D) or 5 (E) μM IP_3 at 200 nM Ca^{2+} and 5 mM ATP, in DMSO control condition (D-E left) or in presence of 50 μM BH4-Bcl-2 peptide (D-E right). **F:** Histogram depicting the P_o $\pm$ S.E.M. for the IP_3R under the described conditions. The total number of recordings for each condition is indicated within every bar. * stands for $p < 0.05$. *The single-channel recordings presented on panel D and the corresponding analyzed data on panel F for 1 μM IP_3 with and without 50 μM BH4-Bcl-2 were obtained from exactly the same data set, as previously published in [39]. All other results represent newly acquired data.*

Fig. 3 The BH4-Bcl-2 peptide fails to inhibit IICR triggered by high IP_3 concentrations A-D: Typical experiment of unidirectional $^{45}Ca^{2+}$ fluxes in permeabilized MEFs. Ca^{2+} release was induced by 2 (A), 4 (B), 10 (C) or 50 (D) μM IP_3 (the time of addition is indicated with a black bar) in control condition or in presence of different concentrations of BH4-Bcl-2 peptide (the time of addition is indicated with a grey bar). The results are plotted as fractional loss after 2 min of incubation with IP_3 minus the fractional loss before the addition of IP_3 (%/2 min) as a function of time. **E:** Concentration-response curve of the IICR as quantified from 4 independent experiments, performed on independently grown cell cultures. The values of IICR measured as fractional loss were calculated as percentage of the IICR in control condition (vehicle), which was set as 100%. * stands for $p < 0.05$.

Fig. 4 Bcl-2 inhibits IP_3R lacking the Bcl-2-binding site (a.a. 1389-1408) in the modulatory region. A-B: The cytosolic Ca^{2+} release in response to 10 μM CCH was measured in the mCherry-positive Fura-2-AM cells, co-transfected with 3xFLAG-empty vector or 3xFLAG-Bcl-2 and IP_3R (A) or $IP_3R^{\Delta 1389-1408}$ (B). The free extracellular Ca^{2+} was chelated by addition of 3 mM BAPTA. The obtained Fura-2 fluorescence signals (F340/F380) were calibrated and representative traces are plotted as $[Ca^{2+}]$. **C:** Quantitative analysis of the amplitude of the CCH-induced Ca^{2+} signals from at least 3 independent experiments performed after on independently transfected cell batches (total number of cells > 100 cells) is plotted as mean $\pm$ S.E.M. **D:** Quantitative analysis of the number of cells responding to the agonist. * stands for $p < 0.05$.

Fig. 5 Molecular docking of Bcl-2 and its BH4 domain on the LBD of human IP_3R . The color scheme: blue – LBD of apo IP_3R (PDB-entry 3UJ4, [35]); green – LBD of IP_3 -bound IP_3R (PDB-entry 3T8S, [38]); orange –

Bcl-2 (PDB-entry 4LXD, [36]); bright orange – BH4 of Bcl-2. The positively charged K508 residue, which was previously shown to be critical for IP₃ binding [45], is indicated. **A:** Proposed model of the interaction between Bcl-2 and the LBD of the apo-form of IP₃R1. The BH4 domain of Bcl-2 is colored in bright orange and its residues are represented as sticks. **B:** Superposition of the LBD of IP₃R in its apo- and IP₃-bound form. IP₃ binds the cleft between the two subdomains of the LBD. This cleft is narrower when IP₃ is bound. **C:** Binding of Bcl-2 to the IP₃-bound form of the LBD of IP₃R1 based on the proposed model of the interaction between Bcl-2 and the apo-form of IP₃R1. The narrower IP₃ binding cleft results in clashes between IP₃R and Bcl-2.

Fig. 6 The LBD of IP₃R is a novel target of full-length Bcl-2 and its BH4 domain. **A:** Representative GST-pull down experiments for assessing the binding of 3xFLAG-Bcl-2 from COS-1 cell lysate to GST-fused IP₃R1 fragments corresponding to the Fragment 3, C-term Dom, LBD, IBC and SD. The samples were analyzed via Western blot and stained with anti-FLAG antibody. Total COS-1 lysates (0.2 µg) were used as input. **B:** The immunoreactive bands from 3 independent experiments, using each time independently transfected cells and freshly prepared lysates, were quantified and normalized to the binding of 3xFLAG-Bcl-2 to GST-Fragment 3, which was set as 1. The data are plotted as mean ± S.E.M. * stands for p<0.05. **C-E:** Representative sensorgrams of the surface plasmon resonance experiments expressed in resonance units (RU) as a function of time. The biotin-BH4-Bcl-2 peptide and the scrambled peptide were immobilized on different channels of a streptavidin-coated sensor chip. The channels on the chip were exposed to the indicated concentrations of purified GST-LBD (**C**), GST-IBC (**D**) and GST-SD (**E**). Sensorgrams are obtained after background correction for binding to the respective scrambled versions of the biotinylated BH4-domain peptide. **F:** Quantitative analysis of the binding properties of biotin-BH4-Bcl-2 to the different GST-domains of IP₃R1. Values were obtained from three independent experiments (i.e. three independent immobilizations of the peptides) and are plotted as mean ± S.E.M.

Fig. 7 Bcl-2 and its BH4 domain compete with IP₃ and AdA for LBD, but they do not occupy the exact same site. **A:** Representative GST-pull down experiments for assessing the effect of AdA (5 µM) on the binding of 3xFLAG-Bcl-2 from COS-1 cell lysate to GST-fused IP₃R1 fragments corresponding to the Fragment 3 and LBD. The samples were analyzed via Western blot and stained with anti-FLAG antibody. 0.2 µg of total COS-1 lysates was used as input. The immunoreactive bands from 3 independent experiments, utilizing each time independently transfected cells and freshly prepared lysates, were quantified and normalized to the binding of 3xFLAG-Bcl-2 to GST-Fragment 3, which was set as 1. The data are plotted as mean ± S.E.M. * stands for p<0.05. **B:** Representative GST-pull down experiments for comparison of the binding of 3xFLAG-Bcl-2 from COS-1 cell lysate to GST-LBD and GST-LBD K508Q. The samples were analyzed via Western blot and stained with anti-FLAG antibody. Total COS-1 lysates (1 µg) were used as input. The immunoreactive bands from 3 independent experiments, utilizing each time independently transfected cells and freshly prepared lysates, were quantified and normalized to the binding of 3xFLAG-Bcl-2 to GST-LBD, which was set as 1. The data are plotted as mean ± S.E.M. * stands for p<0.05. **C-D:** Representative sensorgrams of the surface plasmon resonance experiments expressed in RU as a function of time. The biotin-BH4-Bcl-2 peptide and the scrambled peptide were immobilized on different channels of a streptavidin-coated sensor chip. The channels on the chip were exposed to 1,1 µM purified GST-LBD (**C**) or 0 and 1.1 µM GST-Fragment 3 (**D**) of IP₃R1 in presence of IP₃ (20 µM) or AdA (5 µM). **E:** The channels on the chip were exposed to different concentrations of GST-LBD or mutated GST-LBD K508Q, which fails to bind IP₃.

Fig. 8 BH4-Bcl-2 reduces the binding of FLL to LBD. **A:** Typical FRET-based measurements to assess the effect of the BH4-Bcl-2 or the scrambled peptide on the interaction between FLL and a fluorescent IP₃-binding protein (CFP-coupled LBD). The decrease in binding is shown by an increase in the emission ratio of CFP/FLL (480 nm/535 nm). IP₃ (10 µM) was added at the end of the experiment to obtain the maximal increase in CFP/FLL (maximal decrease of binding). **B:** Quantification of the increase in the FRET signal due to binding of 3, 10 or 30 µM BH4-Bcl-2 expressed as percentage of the maximal increase obtained after addition of 10 µM IP₃. The data from 3 or 4 independent experiments are plotted as mean ± S.E.M. * stands for p<0.05.

Fig. 9 Overview of the Bcl-2-binding sites on IP₃R1 structure. Structure of IP₃R1 as viewed from the cytosol. The C-terminal 6th TMDs, which are important for Bcl-2 binding to the C-terminus, are indicated in cyan; the previously identified Bcl-2-binding site (a.a. 1389-1408) in ARM2 is indicated in yellow. The IBC is indicated in green with the crucial IP₃-binding amino acids in red. Please note that the Bcl-2-binding site present in the ARM2

1 domain of one subunit is located in close proximity to the LBD of the neighboring subunit that contains the novel
2 N-terminal Bcl-2-binding site described in present study.

3 **SUPPLEMENTARY MATERIAL**

4 **Fig. S1 High [ATP] do not saturate Fura-2-AM signal:** The uncalibrated data from the measurement described
5 in **Fig. 1B** are shown. The addition of the agonist, ATP (100 μ M) and the ionomycin (5 μ M) for triggering the
6 maximal response, thereby the maximal Ca^{2+} release are indicated.

7 **Fig. S2** A representative Western blot analysis, showing the expression of the wild-type IP₃R1 and of the mutant
8 IP₃R1 Δ ¹³⁸⁹⁻¹⁴⁰⁸ (10 μ g lysate) using the isoform-specific antibody Rbt03. Cells transfected with 3xFLAG-Bcl-2
9 (analyzed with anti-FLAG antibody) served as a control to show absence of IP₃R expression in HEK-3KO cells.

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Fig. S1

16 Fig. S2