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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

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Introduction

Inositol 1,4,5-trisphosphate (InsP3) receptors (InsP3Rs) are channels responsible for calcium release from the endoplasmic reticulum (ER). We show that the anti-apoptotic protein Bcl-2 (either wild type or selectively localized to the ER) significantly inhibited InsP3-mediated calcium release and elevation of cytosolic calcium in WEHI7.2 T cells. This inhibition was due to an effect of Bcl-2 at the level of InsP3Rs because responses to both anti-CD3 antibody and a cell-permeant InsP3 ester were decreased. Bcl-2 inhibited the extent of calcium release from the ER of permeabilized WEHI7.2 cells, even at saturating concentrations of InsP3, without decreasing luminal calcium concentration. Furthermore, Bcl-2 reduced the open probability of purified InsP3Rs reconstituted into lipid bilayers. Bcl-2 and InsP3Rs were detected together in macromolecular complexes by coimmunoprecipitation and blue native gel electrophoresis. We suggest that this functional interaction of Bcl-2 with InsP3Rs inhibits InsP3R activation and thereby regulates InsP3-induced calcium release from the ER.

Abbreviations used in this paper: BN-PAGE, blue native–PAGE; ECB, extracellular buffer; D-myo InsP3, D-myo inositol 1,4,5-trisphosphate; ICB, intracellular buffer; IL-2, interleukin-2; InsP3, inositol 1,4,5-trisphosphate; InsP3R, InsP3 receptor; TCR, T cell receptor; TG, thapsigargin; TMRE, tetramethylrhodamine ethyl ester.
findings indicate that Bcl-2 localized specifically on the ER (Green and Reed, 1998; Gross et al., 1999). Recent reports have provided evidence that Bcl-2 preserves luminal calcium, whereas several recent papers suggest that Bcl-2 increases leakage of calcium from the ER and decreases luminal calcium (for review see Distelhorst and Shore, 2004).

The present study was undertaken to test the hypothesis that Bcl-2 on the ER regulates InsP$_3$-linked calcium signals that mediate cell cycle entry and apoptosis. We report that Bcl-2 inhibits InsP$_3$-induced calcium release from the ER by impeding calcium release through InsP$_3$Rs. This action of Bcl-2 was not due to an alteration in InsP$_3$R expression or luminal calcium concentration, but was mediated through a functional interaction of Bcl-2 with InsP$_3$Rs that inhibited their channel opening in response to InsP$_3$.

**Results**

**Bcl-2 inhibits anti-CD3–induced calcium elevation**

To investigate the effect of Bcl-2 on calcium homeostasis, the T cell receptor (TCR)–positive WEHI7.2 line was stably transfected with an expression vector encoding human Bcl-2 or empty control vector. Bcl-2 was not detected in nontransfected cells or in empty vector transfected control clones (Fig. 1 A; N1 and N2), but was readily detectable in Bcl-2–positive clones (Fig. 1 A; B1, B13, B17, and B27). Bcl-2 expression was monitored frequently by flow cytometry and only cultures with more than 85% of Bcl-2–positive cells were used in experiments. Bcl-2 expression conferred resistance to apoptosis induction by dexamethasone, staurosporine, and thapsigargin (TG; unpublished data). Substantial differences were not detected in the expression levels of InsP$_3$Rs (Fig. 1 B), SERCA pumps (Fig. 1 C), or luminal calcium binding proteins (not depicted).

Antibody to the CD3 component of the TCR induced a calcium elevation in control WEHI7.2 cells that was inhibited in Bcl-2–transfected cells (Fig. 2 A). Bcl-2 expression also reduced both the number of cells responding to anti-CD3 antibody (Fig. 2 B) and the amplitude of calcium elevation in responding cells (Fig. 2 C). In addition, Bcl-2 expression appeared to increase the latency up to 2 min between the time when anti-CD3 antibody was added and when the elevation of cytosolic calcium was first detected. The inhibitory effect of Bcl-2 on anti-CD3–induced calcium elevation was confirmed by directly comparing multiple Bcl-2–negative and –positive clones (Fig. 3, A and C). Also, Bcl-2 selectively targeted for expression on the ER inhibited anti-CD3–induced calcium elevation (Fig. 3 E).

**ER calcium levels are not affected by Bcl-2 expression**

We investigated several possible mechanisms by which Bcl-2 expression could decrease agonist-induced calcium signals. First, we tested whether or not Bcl-2 expression affected ER calcium levels. This test was performed by two complementary approaches: (1) quantitation of the TG releasable calcium pool and (2) direct measurement of free luminal calcium concentration. TG inhibits SERCA pumps and causes a passive leak of calcium from ER lumen into cytoplasm.
TG-induced calcium elevation is an indirect measure of ER calcium content. We found no difference in the magnitude of the TG releasable calcium pool (Fig. 3, B, D, and F). Therefore, absence of an effect of Bcl-2 on TG-induced calcium elevation suggests that the inhibitory effect of Bcl-2 on anti-CD3–induced calcium elevation is not secondary to a Bcl-2–imposed decrease in ER luminal calcium concentration.

Luminal calcium concentration was measured directly using the low affinity calcium-sensitive dye Fura-2FF AM. Optimal conditions for loading Fura-2FF AM into the ER were determined in preliminary experiments. The organelle distribution of Fura-2FF fluorescence was in a reticular pattern distinct from the punctate mitochondrial pattern detected with the potentiometric dye tetramethylrhodamine ethyl ester (TMRE; Fig. 4 A). To quantify the relative amount of Fura-2FF localized to the ER lumen, the plasma membrane was permeabilized with digitonin and cells were perfused with intracellular buffer (ICB) supplemented with an ATP-regenerating system, 10 μM InsP₃, and 100 μM MnCl₂ (Fig. 4 B). The initial decrease in the emission intensity at both 340 and 380 nm excitation (at ~80 s) signifies the point at which the plasma membrane is permeabilized. The subsequent decrease in 340 and 380 nm emission (at ~190 s) is due to InsP₃-induced opening of InsP₃Rs on the ER, allowing MnCl₂ to enter the ER lumen. Fura-2FF has high affinity for MnCl₂, which in turn quenches the dye. In multiple experiments, more than 90% of the fluorescence remaining after digitonin permeabilization was quenched by perfusing cells with 10 μM InsP₃ and 100 μM MnCl₂. Using this assay system, ER luminal calcium concentration was...
compared in Bcl-2–negative and –positive clones. A typical continuous single cell tracing is shown in Fig. 4 C. Cells were initially perfused with extracellular buffer (ECB) and then with ICB supplemented with an ATP regenerating system and digitonin. The 340:380 fluorescence emission ratio increased dramatically when cells were permeabilized by digitonin. The 340:380 ratio increases rapidly when cells are permeabilized with digitonin. The 340:380 ratio gradually reaches steady-state levels. (D) Summary of luminal ER calcium concentration based on steady-state 340:380 ratios after cell permeabilization with digitonin. Measurements were performed in six experiments constituting parallel comparisons of 59 Neo1 control cells versus 50 B27 cells, and in 13 experiments constituting parallel comparisons of 180 Neo2 control cells versus 85 B17 cells. Symbols represent mean ± SEM. Statistical analysis was performed with the Mann-Whitney U Test and confirmed with the Wilcoxon Rank Sum Test.

Figure 4. Bcl-2 does not decrease luminal free calcium concentration. Free luminal ER calcium concentration was measured using the low affinity calcium sensitive dye Fura-2FF AM. (A) Intracellular localization of Fura-2FF fluorescence (left) and fluorescence from the mitochondrial dye TMRE (right) was detected as nonconfocal Z-series stacks followed by deconvolution. Bars, 5 μm. (B) Typical single cell traces showing fluorescence emission at 340 and 380 nm excitation. Cells were loaded with Fura-2FF AM while suspended in ECB. Cells were then perfused with ICB supplemented with an ATP generating system and 10 μg/ml digitonin. Fluorescence at both 340 and 380 nm decreased dramatically when cells were permeabilized by digitonin. Cells were then perfused with ICB containing 10 μM InsP3 and 100 μM MnCl2. Mn2+ enters the ER and quenches luminal Fura-2FF after opening of InsP3Rs by InsP3. (C) Typical single cell traces monitoring the ratio of fluorescence at 340 and 380 nm excitation. The 340:380 ratio increases rapidly when cells are permeabilized with digitonin. The 340:380 ratio gradually reaches steady-state levels. (D) Summary of luminal ER calcium concentration based on steady-state 340:380 ratios after cell permeabilization with digitonin. Measurements were performed in six experiments constituting parallel comparisons of 59 Neo1 control cells versus 50 B27 cells, and in 13 experiments constituting parallel comparisons of 180 Neo2 control cells versus 85 B17 cells. Symbols represent mean ± SEM. Statistical analysis was performed with the Mann-Whitney U Test and confirmed with the Wilcoxon Rank Sum Test.

Bcl-2 targets the InsP3R to inhibit calcium release

To exclude the first possibility, the signal transduction pathway mediating the response to anti-CD3 antibody was bypassed by measuring cytoplasmic calcium elevation after addition of a cell-permeant InsP3 ester, D-myo InsP3 hexakisbutyryloxymethyl ester (D-myo InsP3BM), to intact Bcl-2–positive and –negative cells. After a brief delay required for de-esterification, D-myo InsP3BM induced an elevation in cytosolic calcium that had a shorter latency period, a more rapid rate of increase, and a higher peak amplitude in Bcl-2–negative cells compared with Bcl-2–positive cells (Fig. 5). These findings indicated that Bcl-2 acts at
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the level of the ER to inhibit anti-CD3–induced calcium elevation, rather than interfering with upstream components of the signal transduction pathway initiated by TCR activation.

To examine further if Bcl-2 was acting at the level of InsP$_3$Rs, the affinity of InsP$_3$Rs for InsP$_3$ was measured in microsomes isolated from Bcl-2–positive and –negative cells using a competitive binding assay. The $K_d$ for radiolabeled InsP$_3$ binding was consistently higher in Bcl-2–positive microsomes than in Bcl-2–negative microsomes (7.0 ± 1.3 nM vs. 4.8 ± 1.1 nM; $P < 0.001$), indicating that Bcl-2 decreases InsP$_3$R binding affinity. To determine if decreased InsP$_3$ binding affinity fully explains the inhibitory effect of Bcl-2 on InsP$_3$-induced calcium elevation, the relationship between InsP$_3$ concentration and calcium release from the ER was investigated. For this purpose, ER luminal calcium was continuously monitored with the low affinity calcium indicator Fura-2FF. After cell permeabilization with digitonin, increasing concentrations of InsP$_3$ were added sequentially while continuously monitoring the Fura-2FF 340:380 ratio. This finding was highly reproducible as shown in the InsP$_3$ dose response analysis and indicates that Bcl-2 inhibits the extent of InsP$_3$-induced calcium release from the ER even at saturating InsP$_3$ concentrations (Fig. 6 B). In contrast, the extent of calcium release induced by TG was similar in Bcl-2–positive and –negative cells (Fig. 6 C). These findings indicate that Bcl-2 inhibits InsP$_3$-induced calcium release, even at saturating InsP$_3$ concentrations well above the $K_d$ for InsP$_3$ binding. Thus, an alteration in InsP$_3$ binding affinity does not fully explain the inhibition of InsP$_3$-induced calcium release by Bcl-2. Nevertheless, these findings further

Figure 5. Bcl-2 inhibits cytoplasmic calcium elevation induced by cell-permeant InsP$_3$ ester. Cytosolic calcium concentration was measured by single cell calcium imaging using the calcium indicator Fura-2 AM in the presence of 1.3 mM extracellular calcium. (A) Representative calcium traces comparing Bcl-2–negative (N1) and –positive (B27) clones. (B) Summary of peak calcium concentration induced by 25 μM InsP$_3$ ester in three separate experiments on a total of 42 N1 cells and 31 B27 cells. Symbols represent mean ± SEM and statistical analysis was performed with the Mann-Whitney U Test and confirmed with the Wilcoxon Rank Sum Test.

Figure 6. Bcl-2 inhibits the extent of calcium release even at saturating concentrations of InsP$_3$. Cells were loaded with Fura-2FF AM to monitor ER luminal calcium as described in Fig. 4. After cell permeabilization with digitonin, increasing concentrations of InsP$_3$ were added sequentially while continuously monitoring the Fura-2FF 340:380 ratio. (A) Continuous recording of Fura-2FF ratio in neo control and Bcl-2 overexpressing WEHI7.2 cells. The stepwise decrease in the 340:380 ratio corresponds to the decline in luminal calcium secondary to InsP$_3$-induced release of calcium into the cytoplasm. (B) Linear least squares dose response analysis of multiple experiments like that shown in panel A (symbols represent mean ± SEM for nine separate experiments). (C) 1 μM TG was added to intact unpermeabilized Fura-2FF–loaded cells and the Fura-2FF 340:380 ratio was monitored continuously. The decrease in Fura-2FF ratio is due to a stepwise decline in luminal calcium induced by TG-mediated SERCA pump inhibition.
suggest that Bcl-2 regulates cellular calcium signaling at the level of the InsP3Rs.

Bcl-2 inhibits InsP3R channel opening in vitro

To determine if Bcl-2 regulates InsP3R channel activity, the effect of purified full-length Bcl-2 protein on single type I InsP3R channels was studied after addition of ~0.1 μM purified full-length Bcl-2 to the solution bathing the cytoplasmic face of the channel. (A) Representative single channel traces showing the level of activity of a channel under control conditions (top trace; cis/trans 220 mM CsCH3SO3/20 mM CsCH3SO3, pH 7.4) in the presence of 2 μM InsP3. The holding potential was 0 mV. Channel openings are seen as upward current deflections. The bottom trace shows the same InsP3R channel after addition of Bcl-2 to the cis compartment (cytoplasmic side of channel). Bcl-2 significantly reduces the open probability. The bar at the left indicates the zero current level (closed state). (B) All-points amplitude histogram of the experiment shown in A. The effect on open probability is demonstrated by a reduction of the peak representing the open level.

Figure 8. Bcl-2 is present in complexes with InsP3Rs. (A) Western blots showing levels of calnexin and cox IV in cell lysates and purified ER membrane from Bcl-2–expressing WEHI7.2 cells (clone 17). (B) Identification of protein complexes in purified ER membranes by BN-PAGE. MW, molecular size markers; ER, ER membrane. I–IV point to complexes that were cut from this gel and separated by SDS-PAGE. White line indicates that intervening lanes have been spliced out. (C) Complexes I–IV from B were resolved by SDS-PAGE and analyzed by Western blotting with antibody to Type III InsP3R (top) and antibody to human Bcl-2 (bottom).
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In this case, the total recording time considered in the analysis was much longer (120 s) for each condition. Addition of ~0.1 μM Bcl-2 to the cytoplasmic face of the InsP3R reduced the channel open probability by 2.92-fold. Potassium or cesium are commonly used instead of calcium because conductance of the channel for monovalent cations is much higher than that for divalent cations, improving the resolution (signal to noise ratio) of single channel currents without significantly changing gating properties. In summary, these results demonstrate an inhibitory interaction between Bcl-2 and InsP3Rs in vitro.

Bcl-2 and InsP3Rs form a macromolecular complex in WEHI7.2 cells

To determine if Bcl-2 associates with InsP3Rs in vivo, ER membranes were isolated from Bcl-2–expressing WEHI7.2 cells (clone 17) and subjected to blue native–PAGE (BN-PAGE), a technique in which protein complexes are separated in the first dimension on nondenaturing gels and protein complexes are then analyzed by SDS-PAGE/Western blotting in the second dimension (Schagger et al., 1994). Multiple high molecular mass complexes were resolved in first dimension gels (Fig. 8 B). The four largest complexes were cut from the gel and subjected to SDS-PAGE/Western blotting (Fig. 8 C). The findings indicate that at least a portion of Bcl-2 is located together with InsP3Rs in the largest complex. Bcl-2 was also detected in the smaller complexes analyzed, suggesting either that some of the Bcl-2 had dissociated from the InsP3R complexes during preparation or that not all of the Bcl-2 is associated with InsP3Rs.

The potential interaction of Bcl-2 and InsP3Rs was further analyzed by coimmunoprecipitation (Fig. 9). InsP3Rs were immunoprecipitated from Neo control cells (clone N1) and Bcl-2–expressing cells (clone B17), and immunoprecipitates were analyzed by Western blotting with an antibody specific for Bcl-2 (Fig. 9 A). The results indicate that Bcl-2 coimmunoprecipitates with InsP3Rs. Bcl-2 coimmunoprecipitation with InsP3Rs was detected using antibodies to each of the three InsP3R subtypes (Fig. 9 B). In the reciprocal experiment, Bcl-2 was immunoprecipitated and InsP3Rs were detected as coimmunoprecitating proteins by Western blotting, confirming the interaction between Bcl-2 and InsP3Rs (Fig. 9 C). To control for the specificity of interaction between Bcl-2 and InsP3Rs, immunoprecipitates were also analyzed by Western blotting using an antibody to SERCA3 (Fig. 9 D). This ER membrane protein did not coimmunoprecipitate with either Bcl-2 or InsP3Rs. Because the associations described in Fig. 9 (A–C) were shown in cells overexpressing Bcl-2, we next examined if similar interactions occurred in cells that endogenously expressed Bcl-2. For this purpose, we took advantage of another T cell line, S49.A2,
which has been demonstrated to express Bcl-2 (Wang et al., 2003). Coimmunoprecipitation of endogenous Bcl-2 with InsP₃Rs, using either anti–Bcl-2 or anti-InsP₃R antibody, was detected in these cells (Fig. 9 E). SERCA3 did not coimmunoprecipitate with either endogenous Bcl-2 or InsP₃Rs (Fig. 9 F). In summary, the findings of coimmunoprecipitation experiments suggest that Bcl-2 forms a complex with InsP₃Rs.

Discussion

Here we report that Bcl-2 inhibits InsP₃-induced calcium elevation in the WEHI7.2 T cell line. The focus has been on understanding the mechanism by which Bcl-2 inhibits anti-CD3–induced calcium elevation. The findings of a systematic series of experiments all point to a regulatory effect of Bcl-2 on InsP₃R function. First, the observation that Bcl-2 inhibits anti-CD3–induced calcium elevation was confirmed in multiple Bcl-2–expressing clones of the WEHI7.2 line by both fluorometric and digital imaging methods of calcium measurement. Second, an inhibitory effect of Bcl-2 on ER calcium release was detected when the signal transduction pathway mediating anti-CD3–induced InsP₃ synthesis was bypassed by adding a cell-permeant InsP₃ ester to cells or by adding InsP₃ to digitonin-permeabilized cells. Third, anti-CD3–induced calcium elevation was inhibited not only by wild-type Bcl-2, which localizes to both the ER and mitochondria, but also by Bcl-2 selectively targeted to the ER membrane. These findings indicate that the action of Bcl-2 resides at the level of the ER, rather than in the upstream signal transduction pathway that mediates InsP₃ synthesis. Fourth, a series of control experiments indicated that inhibition of InsP₃-induced calcium release by Bcl-2 was not due to decreased luminal calcium concentration, decreased InsP₃,R levels, or altered expression of luminal calcium binding proteins. Fifth, Bcl-2 inhibited the extent of InsP₃-induced calcium release even at saturating InsP₃ concentrations, indicating that the major action of Bcl-2 is to decrease the affinity of InsP₃,Rs for InsP₃, but to decrease InsP₃,R channel opening even under conditions of maximal stimulation. Although the complete mechanism is yet to be determined, based on coimmunoprecipitation and BN-PAGE experiments it appears that Bcl-2 coexists in a macromolecular complex with InsP₃,Rs, resulting in an inhibition of InsP₃-induced calcium release. This concept is further supported by in vitro evidence that purified Bcl-2 inhibited the frequency of InsP₃ channel opening when added to InsP₃,Rs integrated into planar lipid bilayers.

Although an inhibitory action of Bcl-2 on InsP₃-mediated calcium release has not been reported previously, an inhibitory effect of Bcl-2 on calcium-mediated signaling pathways has been reported, including the induction of the transcription factor c-fos (Qi et al., 1997). Significantly, Linette et al. (1996) demonstrated that Bcl-2 inhibits anti-CD3/TCR–mediated activation of NFATc and induction of interleukin-2 (IL-2) expression, thereby inhibiting cell cycle entry by delaying Go/G₁ transition into S phase and also inhibiting TCR activation-mediated apoptosis. Active NFATc is generated by calcineurin, which binds to and dephosphorylates NFATc in the cytoplasm, permitting NFATc to enter the nucleus. It has been suggested that Bcl-2 inhibits NFATc activation by sequestering calcineurin to intracellular membranes (Shibasaki et al., 1997). Our findings suggest that Bcl-2 may inhibit calcineurin activation by inhibiting InsP₃-mediated calcium release from the ER. In T cells, calcium/calcineurin-mediated activation of NFATc increases IL-2 expression, which in turn stimulates dual pathways, one leading to cell death and the other leading to cell survival. IL-2 induces cell death via Stat2-mediated induction of the death receptor ligand Fas, whereas IL-2 promotes cell survival via Akt-mediated induction of Bcl-2 expression (Parijs et al., 1999). The findings of the present paper raise the possibility that increased expression of Bcl-2 may form a feedback loop that dampens InsP₃-mediated calcium signals, thereby controlling T cell proliferation while maintaining cell survival.

InsP₃,Rs are known to associate with several factors that regulate InsP₃,R–gated channel activity (Mackrill, 1999; Roderick and Bootman, 2003). This paper is the first to suggest that Bcl-2 may interact with InsP₃,Rs and regulate their functional activity. Although the full functional significance of the inhibitory effect of Bcl-2 on InsP₃,R channel activity is as yet unknown, this action of Bcl-2 may contribute to the established inhibitory effects of Bcl-2 on cell cycle entry and/or apoptosis induction. In view of the wide range of cellular functions mediated by InsP₃,R–induced calcium signals (Berge et al., 2003), it will be interesting in future studies to determine if the function of Bcl-2 goes beyond that of regulating cell cycle entry and apoptosis.

Materials and methods

Reagents

TG, EGTA, digitonin, and other reagents were obtained from Sigma-Aldrich. D-myoinositol and L-myoinositol were obtained from Calbiochem. Fura-2 AM was obtained from Molecular Probes. Fura-2FF AM was obtained from Tef Labs. Hamster anti-mouse CD3ε epsilon chain mAb was obtained from BD Biosciences.

Cell culture and transfection procedures

WEHI7.2 and S49.A2 cells were cultured in DME supplemented with 10% serum, l-glutamine, and nonessential amino acids. Human Bcl-2 α cDNA from the p84 plasmid (American Type Culture Collection) was cloned into the pSFFV-Neo vector. Transfection and cloning were performed as described previously (Dieken and Miesfeld, 1992). Flag-tagged Bcl-2 was selectively localized to the ER by exchanging the COOH-terminal transmembrane sequence of Bcl-2 for the ER-targeting sequence of cytochrome b5, as described previously (Wang et al., 2001). Bcl-2 expression was monitored by flow cytometry of fixed cells using anti-Bcl-2 antibody (BD Biosciences; 15131A) at a 1:500 dilution and Alexa Fluor 488 goat anti–hamster IgG (H+L) conjugate (Molecular Probes; A-21120) as the secondary antibody at a dilution of 1:500.

Calcium fluorometry

Cells (10 ml volume, 1 million per milliliter) were incubated with 1 µM Fura-2 AM for 45 min at 25°C in ECB (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 25 mM Hepes, pH 7.5, 1 mg/ml BSA, and 5 mM glucose), after which they were pelleted and resuspended in ECB for an additional incubation at 25°C for 30 min to permit dye de-esterification. Fluorescence was continuously recorded at 37°C (alternating 340 and 380 nm excitation, 510 nm emission) in a fluorometer (Photon Technology Inc.). EGTA (final concentration 10 mM) was added to chelate extracellular calcium immediately before adding 100 nM TG, anti-CD3 antibody (BD Biosciences; 1:500 dilution), or 25 nM D-myoinositol. In experiments using D-myoinositol, the volume of cell suspensions was scaled down to 250 µl in a 300 µl cuvette. All measurements were performed in triplicate. Rₘₕ and Rₘₖ values were determined in each experiment by cell permeabilization with digitonin, followed by sequential addition of calcium and EGTA/
Tris. Calcium concentration was calculated, based on the published $K_d$ for Fura-2 of 220 nM, by the equation of Grynkiewicz et al. (1985) using Felix Software (Photon Technologies Inc.).

**Calcium imaging**

Cells adhered to poly-L-lysine-coated coverslips (15 mm) were loaded with 1 μM Fura-2-AM (Molecular Probes) as described in Calcium fluorometry. Coverslips were placed in a recording/perfusion chamber (model RC-25F; Warner Instruments) mounted on the stage of an inverted microscope (model Diaphot; Nikon) equipped with a 20× Fluor objective. Excitation light was alternated between 340 and 380 nm by a filter wheel (Sutter Instrument Co.) and emitted light was filtered at >510 nm and collected with an intensified charge-coupled device camera (model UltraView; PerkinElmer). The video signal was digitized using Ultraview software (PerkinElmer) and subsequently processed using Microsoft Excel.

Cells were perfused with ECB at 25°C and stock solutions of both anti-CD3 antibody (1:40 dilution) and 25 μM D-myo InsP3BM were diluted in ECB immediately before addition to the perfusion chamber. To determine $R_{\text{min}}$, Fura-2 of 220 nM, by the equation of Grynkiewicz et al. (1985) using Felix Origin 6.0 (Microcal).

ER calcium measurement

Luminal calcium measurement was modified after that of Hofer (1999). Cells, adhered to poly-L-lysine-coated coverslips were incubated with 1 μM Fura-2FF AM for 30 min at 37°C in ECB, after which the buffer was replaced with fresh ECB and the incubation continued for 45 min at 25°C to permit de-esterification. Cell imaging was performed as described in Calcium imaging. Cells were initially perfused with ECB at 25°C, and then with ECB (125 mM KCl, 25 mM NaCl, 10 mM Hepes, 0.5 mM Na2ATP, 200 μM CaCl2, and 500 μM EGTA, pH 7.25), supplemented with an ATP-regenerating system consisting of 10 μM creatine phosphokinese Type I and 10 mM phosphocreatine. As soon as cells were permeabilized, the perfusion buffer was switched to ICB plus ATP-regenerating system. After the fluorescence ratio reached steady-state, cells were perfused with the same buffer supplemented with D-myo-InsP3, at concentrations given in the text and accompanying figures. Stock solutions of D-myo-InsP3 and L-myo-InsP3, were prepared at a concentration of 3 mM in calcium-free water and stored at −20°C until use. After cell permeabilization with digitonin, the proportion of Fura-2FF located in the ER was determined by quenching luminal dye by perfusing with 10 μM D-myo-InsP3, in ECB supplemented with 100 μM MnCl2. To determine $R_{\text{max}}$, permeabilized cells were perfused with ECB deficient in calcium and supplemented with 4 mM EGTA and 10 μM iomycin. $R_{\text{max}}$ was obtained by perfusing permeabilized cells with ECB supplemented with 4 mM CaCl2 and 10 μM iomycin. Calcium concentration was calculated as described in Calcium fluorometry.

**Immunoprecipitation**

10°C cells were washed twice with PBS and incubated on ice 30 min with 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% CHAPS w/v, 50 mM NaF, 200 mM o-phenanthroline, 1 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin) and centrifuged at 100,000 g at 4°C. The supernatant was rotated with 0.25% 100 μl of 50% protein A or protein G agarose for 2 h at 4°C. After removing the beads, the supernatant was incubated with 1 μg of the following antibodies: anti–human Bcl-2 (BD Biosciences; 15131A, 1:2000), anti–mouse Bcl-2 (BD Biosciences; 610312, 1:2000), and anti-SERCA3 ATPase (Affinity Bioreagents, Inc.; PA1-910, 1:500).

**BN-PAGE**

ER membranes were isolated from WEHI7.2 cells as described previously (Krajewski et al., 1993). In brief, cells (1–10 x 10^6) were washed twice with ice-cold PBS (Invitrogen), pH 7.2, and resuspended in 1.5 ml of homogenization buffer (20 mM Hepes, pH 7.4, 2.5 mM EDTA, 250 mM sucrose, and protease inhibitor cocktail). After homogenization for 15–25 strokes with a Dounce homogenizer, samples were centrifuged at 3,000 g for 15 min at 4°C and 1 ml of supernatant was layered onto a 11-ml sucrose gradient (0.75 to 1.9 M sucrose in 20 mM Hepes, pH 7.4, and 2.5 mM MgCl2) and centrifuged at 110,000 g for 2 h at 4°C. Fractions of 0.8 ml were collected from the gradient and the distribution of ER and mitochondria was determined by Western blotting, Calnexin (anti-calnexin antibody, SPA-8600; StressGen Biotechnologies) and Cox IV (anti IV COX, A-6430; Molecular Probes) were used as markers of ER and mitochondria, respectively. Fractions containing purified ER membranes were pooled and mixed with three volumes of 20 mM Hepes, pH 7.4. The sample was centrifuged at 105,000 g for 1.5 h at 4°C and the resulting membrane pellet was used for BN-PAGE, performed as described previously (Schagger et al., 1994). The ER membrane was solubilized with 750 mM 6-aminoacaproic acid, 50 mM BisTris, pH 7.0, and 1% dodecyl maltoside (5 μg total protein per microliter), and centrifuged at 100,000 g for 15 min at 4°C. Before starting BN-PAGE, Coomassie blue was added to the resulting supernatant to 0.25%. 100 μg of protein was added to each well of a 4–10% linear gradient BN-PAGE. Single bands were excised from the blue native gel and applied to a 4–20% linear gradient SDS-PAGE with sample buffer, followed by Western blotting. Sizes of molecular complexes were estimated using a high molecular mass calibration kit for native electrophoresis (17-0445-01; Amersham Biosciences). After completion of electrophoresis, gels were subjected to Western blotting using antibodies to InsP3, and human Bcl-2 as described above.

**Organelle imaging**

Confocal Z-series stacks of TMRE-loaded (0.1 μM, 10 min) cells were acquired after permeabilization with digitonin in ECB at RT using an Ultra View LCI (PerkinElmer) with a microscope (model TE100; Nikon), ×100/1.3 objective (Nikon), and Orca ER camera (Hamamatsu) at excitation 568 nm, emission 600 ± 40 nm. The laser intensity was kept at a minimum to prevent irradiation-induced mitochondrial damage. Image acquisition was by UltraView software (PerkinElmer). Nonconfocal Z-series stacks of Fura-2FF loaded cells were obtained after permeabilization with digitonin in ECB at RT using an Ultra View LCI with a microscope, ×100/1.3 objective, epifluorescence arc-lamp as a light source, excitation 330–380 nm, dichroic 400/40LP, emissor 420/40LP, and Orca ER camera. Subsequent image restoration was achieved with the deconvolution software AutoDeblur (Aquantix).

**Western blotting**

Cell lysates containing 40–60 μg of protein were separated by SDS-PAGE (15% for Bcl-2 and 4–15% linear gradient for InsP3Rs) followed by electroblotting to nitrocellulose-Membran-Polyvinyl (PVDF) membranes. The following antibodies and their respective dilutions were used: anti–human Bcl-2 (BD Biosciences; 15131A, 1:2000), anti–mouse Bcl-2 (BD Biosciences; 554218, 1:1000), anti-actin (Santa Cruz Biotechnology, Inc.; sc-8432, 1:1000), anti-InsP3R Type I (Calbiochem; 407144, 1:2000), anti-InsP3R Type II (Chemicon; AB3000, 1:1000), anti-InsP3R Type III (BD Biosciences; 610312, 1:2000), and anti-SERCA3 ATPase (Affinity Bioreagents, Inc.; PA1-910, 1:500).

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Bcl-2 was purified from Escherichia coli M15 (pREP4) cells transformed with a pProex-1/hBcl-2 using methods described previously (Lam et al., 1998). Type I InsP3Rs were purified from microsomes isolated from COS-1 cells transfected with pInsP3R1/DTT-ALT plasmid as described previously (Mignery et al., 1989, 1990). Gradient fractions containing InsP3R protein were then identified by immunoblotting with Type 1 receptor antibody and reconstituted into proteoliposomes as previously described (Mignery et al., 1992; Perez et al., 1997). Planar lipid bilayers were formed across a 150-μm diameter aperture in the wall of a Delrin partition as described previously (Perez et al., 1997). Proteolipo-
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