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 (InsP$_3$) receptors (InsP$_3$Rs) 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 InsP$_3$-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 InsP$_3$Rs because responses to both anti-CD3 antibody and a cell-permeant InsP$_3$ ester were decreased. Bcl-2 inhibited the extent of calcium release from the ER of permeabilized WEHI7.2 cells, even at saturating concentrations of InsP$_3$, without decreasing luminal calcium concentration. Furthermore, Bcl-2 reduced the open probability of purified InsP$_3$Rs reconstituted into lipid bilayers. Bcl-2 and InsP$_3$Rs were detected together in macromolecular complexes by coimmunoprecipitation and blue native gel electrophoresis. We suggest that this functional interaction of Bcl-2 with InsP$_3$Rs inhibits InsP$_3$R activation and thereby regulates InsP$_3$-induced calcium release from the ER.

Abbreviations used in this paper: BN-PAGE, blue native–PAGE; ECB, extracellular buffer; D-myo InsP$_3$, D-myo inositol 1,4,5-trisphosphate; InsP$_3$R, inositol 1,4,5-trisphosphate receptor; TCR, T cell receptor; TG, thapsigargin; TMRE, tetramethylrhodamine ethyl ester.
mitochondria and the ER (Cory and Adams, 2002; Danial and Korsmeyer, 2004). Bcl-2 is an integral membrane protein that localizes to both the ER and mitochondria (Kaufmann et al., 2003). Although a significant proportion of Bcl-2 is on the ER (Kaufmann et al., 2003), its role in inhibiting cytochrome c release from mitochondria has been emphasized (Green and Reed, 1998; Gross et al., 1999). Recent findings indicate that Bcl-2 localized specifically on the ER also inhibits cytochrome c release and apoptosis in response to a variety of signals (Zhu et al., 1996; Hacki et al., 2000; Wang et al., 2001; Thomenius et al., 2003).

Previous studies have shown that changes in cellular calcium signaling can dramatically modulate the induction of apoptosis (for review see Hajnoczky et al., 2003; Orrenius et al., 2003). It has been proposed that the action of Bcl-2 is mediated in part due to its ability to regulate cytosolic and mitochondrial calcium changes. For example, it was demonstrated a decade ago that Bcl-2 dampens calcium oscillations and prevents redistribution of calcium from ER to mitochondria after growth factor withdrawal (Baffy et al., 1993; Magnelli et al., 1994). However, the absolute effect of Bcl-2 is unclear, with changes in cytosolic, ER, and mitochondrial calcium signaling being reported in different studies (Lam et al., 1994; Zornig et al., 1995; Marin et al., 1996; Ichimiya et al., 1998; Zhu et al., 1999; Williams et al., 2000; Pinton et al., 2001). Furthermore, the mechanism by which Bcl-2 interacts with calcium signaling systems is controversial. This is exemplified by the conflicting reports about Bcl-2’s effect on ER luminal calcium. We, and others, 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 InsP₃-linked calcium signals that mediate cell cycle entry and apoptosis. We report that Bcl-2 inhibits InsP₃-induced calcium release from the ER by impeding calcium release through InsP₃Rs. This action of Bcl-2 was not due to an alteration in InsP₃R expression or luminal calcium concentration, but was mediated through a functional interaction of Bcl-2 with InsP₃Rs that inhibited their channel opening in response to InsP₃.

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 InsP₃Rs (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. 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.

**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.

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 hexakisbutyryloxyethyl 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 InsP3Rs, the affinity of InsP3Rs for InsP3 was measured in microsomes isolated from Bcl-2–positive and –negative cells using a competitive binding assay. The $K_d$ for radiolabeled InsP3 binding was consistently higher in Bcl-2–positive microsomes than in Bcl-2–negative microsomes (7.0 ± 0.6 nM vs. 4.8 ± 1.1 nM; $P < 0.001$), indicating that Bcl-2 decreases InsP3R binding affinity. To determine if decreased InsP3 binding affinity fully explains the inhibitory effect of Bcl-2 on InsP3-induced calcium elevation, the relationship between InsP3 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, before and after adding InsP3 to cells in which the plasma membrane had been permeabilized by digitonin.

In typical calcium tracings, increasing concentrations of InsP3 induced a stepwise decrease in Fura-2FF ratio, reflecting a stepwise decline in luminal calcium due to InsP3-induced release of calcium into the cytoplasm (Fig. 6 A). This finding was highly reproducible as shown in the InsP3 dose response analysis and indicates that Bcl-2 inhibits the extent of InsP3-induced calcium release from the ER even at saturating InsP3 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 InsP3-induced calcium release, even at saturating InsP3 concentrations well above the $K_d$ for InsP3 binding. Thus, an alteration in InsP3 binding affinity does not fully explain the inhibition of InsP3-induced calcium release by Bcl-2. Nevertheless, these findings further

Figure 5. Bcl-2 inhibits cytoplasmic calcium elevation induced by cell-permeant InsP3 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 InsP3 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 InsP3. 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 InsP3 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 InsP3-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 measured under steady-state conditions. Single InsP₃R channels were incorporated into planar lipid bilayers. InsP₃R channel incorporation was visualized as a series of discrete positive (upward) current fluctuations in the presence of 2 μM InsP₃ and 250 nM calcium in the cis compartment (cytoplasmic side of the channel) as shown in control traces in Fig. 7 A. A conductance of 300 pS and reversal potential of −160 mV was measured from the current–voltage relationship (unpublished data), corresponding to those values expected for an InsP₃R channel under these experimental conditions (Ramos-Franco et al., 1998).

A reduction in single channel activity was observed upon addition of 0.1 μM Bcl-2 in the compartment bathing the cytoplasmic face of the channel (Fig. 7 A). Conductance and reversal potential (unpublished data) were not changed by adding Bcl-2. Thus, Bcl-2 only reduces open probability without important changes in conduction or gating properties. To quantify the change in open probability of the InsP₃R channel induced by Bcl-2, all-point histograms were constructed, permitting comparison of the relative areas for the control records and those obtained after the addition of Bcl-2 (Fig. 7 B). For clarity, only Gaussian distribution fits are shown. The total recording time considered in the analysis for each condition was 9 s. In the control situation (Fig. 7 B, dashed line), the closed state appears as an accumulation of points near 0 pA, whereas the open state is visualized as a peak at 8 pA. The probability in absence of added Bcl-2 was 0.27, calculated as the ratio between the area of the open state and the total area of the histogram. Addition of Bcl-2 reduced the probability by 4.5-fold to 0.058. This effect was seen as a reduction of the height of the peak at the open state (without change in the amplitude of the current) and a corresponding increase in the amount of points at the closed level (Fig. 7 B, solid line). Similar findings were obtained in experiments where the current carrier was potassium instead of cesium (cis/trans...
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Chen et al. 199250/50 mM KCl; unpublished data). 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, using antibodies specific for the three InsP3R subtypes (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 InsP₃,R 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 not 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,R-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.

Materials and methods

Reagents

TG, EGTA, digitonin, and other reagents were obtained from Sigma-Aldrich. D-myoinositol, 15,15,15,15-d₄-InsP₃, 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 DMEM supplemented with 10% fetal bovine serum, L-glutamine, and nonessential amino acids. Human Bcl-2 cDNA from the pB4 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-21110) 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; 1:50 dilution), or 25 μM 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. Rₘₐₓ and Rₘₚₐₜ values were determined in each experiment by cell permeabilization with digitonin, followed by sequential addition of calcium and EGTA/
immediately before addition to the perfusion chamber. To determine R min, Software (Photon Technologies Inc.).

Confocal Z-series stacks of TMRE-loaded (0.1 μM) cells were washed twice with ice-cold PBS (Invitrogen), pH 7.2, and resuspended in 150 mM NaCl. Images were captured in a confocal microscope with 1 μM Fura-2FF AM (Molecular Probes) as described in Calcium fluorescence. 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 (SutcliffeElmer) 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 after addition to the perfusion chamber. To determine R max, cells were perfused with ECB deficient in calcium and supplemented with 4 mM EGTA and 10 μM ionomycin. Calcium concentration was calculated as described in Calcium fluorescence.

ER calcium measurement
Luminal calcium measurement was modified after that of Hofer (1999). Cells adhered to poly-L-lysine–coated coverslips (15 mm) were loaded with 1 μM Fura-2-AM (Molecular Probes) as described in Calcium florescence. 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 (SutcliffeElmer) 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 after addition to the perfusion chamber. To determine R max, cells were perfused with ECB deficient in calcium and supplemented with 4 mM EGTA and 10 μM ionomycin. Calcium concentration was calculated as described in Calcium fluorescence.

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InsP3, binding
Specific binding of radiolabeled InsP3 to microsomes was measured as described previously (Riley et al., 2002). Microsomes (50 μg) were added in duplicate to 100 μl binding buffer (2 mM H-InsP3, [Dupont NET-911], 2 mM Tris, pH 9.0, 1 mM EDTA, and 1 mg/ml albumin, with or without a range of concentrations of unlabeled InsP3) and incubated on ice for 20 min. The mixture was centrifuged for 15 min at 20,000 g at 4°C. Pellets were solubilized in 100 μl water and added to 10 ml ACS scintillation cocktail (Amersham Biosciences) and their activity was determined by liquid scintillation counting. Nonspecific binding was determined using an excess of unlabeled InsP3. The Kd was calculated by Scatchard analysis.

Planar lipid bilayer analysis of InsP3R channel activity
Full-length human Bcl-2 was purified from Escherichia coli M-15 (pREP-4) cells transformed with a pProex-1/hBcl-2 using methods described previously (Lam et al., 1998). Type 1 InsP3Rs were purified from microsomes isolated from COS-1 cells transfected with pInsP3R, RI-DT1-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-
somes were added to the solution on one side of the bilayer (defined as the cis-chamber). The other side was defined as the trans-chamber. Standard solutions contained 220 mM CsCH₂SO₄ cis (20 mM trans), 20 mM Hepes, pH 7.4, and 1 mM EGTA ([Ca²⁺]free = 250 nM). A custom current/voltage conversion amplifier was used to optimize single-channel recording. Acquisition software (pClamp; Axon Instruments, Inc.), an IBM compatible 486 computer, and a 12-bit AD/DAC convertor (Axon Instruments, Inc.) were used. Single channel data were digitized at 5–10 KHz and filtered at 1 KHz. Channel sitedness was determined by InSP-sensitive sensitivity. The orientation of the channels studied was such that the InSP-sensitive side (i.e., cytoplasmic side) was in the cis compartment.

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