

# Bcl-2-dependent modulation of $\text{Ca}^{2+}$ homeostasis and store-operated channels in prostate cancer cells

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## Summary

**Antiapoptotic oncoprotein Bcl-2 has extramitochondrial actions due to its localization on the endoplasmic reticulum (ER); however, the specific mechanisms of such actions remain unclear. Here we show that Bcl-2 overexpression in LNCaP prostate cancer epithelial cells results in downregulation of store-operated  $\text{Ca}^{2+}$  current by decreasing the number of functional channels and inhibiting ER  $\text{Ca}^{2+}$  uptake through a reduction in the expression of calreticulin and SERCA2b, two key proteins controlling ER  $\text{Ca}^{2+}$  content. Furthermore, we demonstrate that  $\text{Ca}^{2+}$  store depletion by itself is not sufficient to induce apoptosis in Bcl-2 overexpressing cells, and that sustained  $\text{Ca}^{2+}$  entry via activated store-operated channels (SOCs) is required as well. Our data therefore suggest the pivotal role of SOCs in apoptosis and cancer progression.**

## Introduction

The oncoprotein Bcl-2 plays a prominent role in the regulation of apoptosis in a variety of cell models, and is by far the most studied antiapoptotic protein (Adams and Cory, 1998). However, while the antiapoptotic activity of Bcl-2 is now well established, its specific action mechanisms have not yet been fully elucidated. As mitochondria are known to play a central role in the induction of apoptosis in response to many apoptotic stimuli, the mitochondrial action of Bcl-2 has received major attention. However, there is an increasing body of evidence suggesting the role of extramitochondrial Bcl-2, especially localized on the ER, which is implicated in apoptosis as well. Indeed, it was recently reported that Bcl-2 targeted to ER was capable of blocking most, although not all, types of apoptosis (Zhu et al., 1996; Lee et al., 1999). Moreover, an elegant study by Häcki et al. (2000) demonstrated the existence of apoptotic crosstalk between the ER and mitochondria, controlled by the ER-localized Bcl-2. However, it is still unclear how Bcl-2 performs its survival function on the ER.

For years it was believed that  $\text{Ca}^{2+}$ -related apoptosis could be triggered by large, sustained increases in cytosolic  $\text{Ca}^{2+}$  (McConkey and Orrenius, 1997). However, recent studies indi-

cate that the  $\text{Ca}^{2+}$  status of ER lumen also affects cell sensitivity to apoptosis (Lam et al., 1994; Bian et al., 1997; Nakamura et al., 2000; Wertz and Dixit 2000; Pinton et al., 2001), and that Bcl-2 triggers a reduction in the ER  $\text{Ca}^{2+}$  content due to an increased leak (Pinton et al., 2000; Foyouzi-Youssefi et al., 2000).

The fact that Bcl-2 modulates the  $\text{Ca}^{2+}$ -filling status of the ER strongly suggests that this oncoprotein may interfere with the capacitative  $\text{Ca}^{2+}$  influx as well, which is activated by ER depletion (Putney, 1986). Capacitative  $\text{Ca}^{2+}$  entry is mediated by store-operated channels (SOCs) generating  $\text{Ca}^{2+}$  current,  $I_{\text{SOC}}$ . SOCs, located in the plasmalemma, may be activated by a variety of active or passive mechanisms, all of which share the property of depleting ER  $\text{Ca}^{2+}$  stores.  $\text{Ca}^{2+}$  entry via SOCs induces a sustained increase in cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) that helps to replenish ER. Therefore, when activated, SOCs regulate both cytoplasmic and ER intraluminal ( $[\text{Ca}^{2+}]_{\text{ER}}$ ) free  $\text{Ca}^{2+}$  concentrations. For this reason, SOCs are increasingly attracting attention as potential apoptosis regulators (Jayadev et al., 1999; Williams et al., 2000). However, the data concerning the role of SOCs in apoptosis of cancer cells in general, and SOCs regulation by Bcl-2 in particular, are rather contradictory,

## SIGNIFICANCE

**We describe a mechanism through which the commonly overexpressed Bcl-2 oncoprotein protects cancer cells against apoptosis. Cellular sensitivity to apoptotic induction is modulated by intracellular calcium concentrations, with increased cytoplasmic calcium levels promoting cell death. Here we show that Bcl-2 expression in LNCaP human prostate cancer epithelial cells causes a reduction in calcium entry from extracellular space that is associated with apoptotic resistance. Complementing its mitochondrial protective influence, reduced intracellular calcium levels in response to Bcl-2 overexpression could represent a key aspect of Bcl-2-mediated apoptotic protection. Since Bcl-2 overexpression induces transformation of androgen-sensitive LNCaP cells to an androgen-insensitive phenotype, our results also suggest an involvement of calcium flux in the progression to hormone-insensitive prostate cancer.**

and the molecular mechanisms involved have not yet been elucidated.

In our recent studies, we have identified  $\text{Ca}^{2+}$ -dependent mechanisms involved in the control of cell growth and apoptosis of human prostate cancer cells and characterized SOCs in these cells (Skryma et al., 2000; Legrand et al., 2001). We have also shown that in the androgen-sensitive human prostate cancer cell line LNCaP, intracellular  $\text{Ca}^{2+}$  store depletion is by itself (without any requirement for the  $[\text{Ca}^{2+}]_c$  elevation due to SOCs activation) sufficient to induce apoptosis (Skryma et al., 2000).

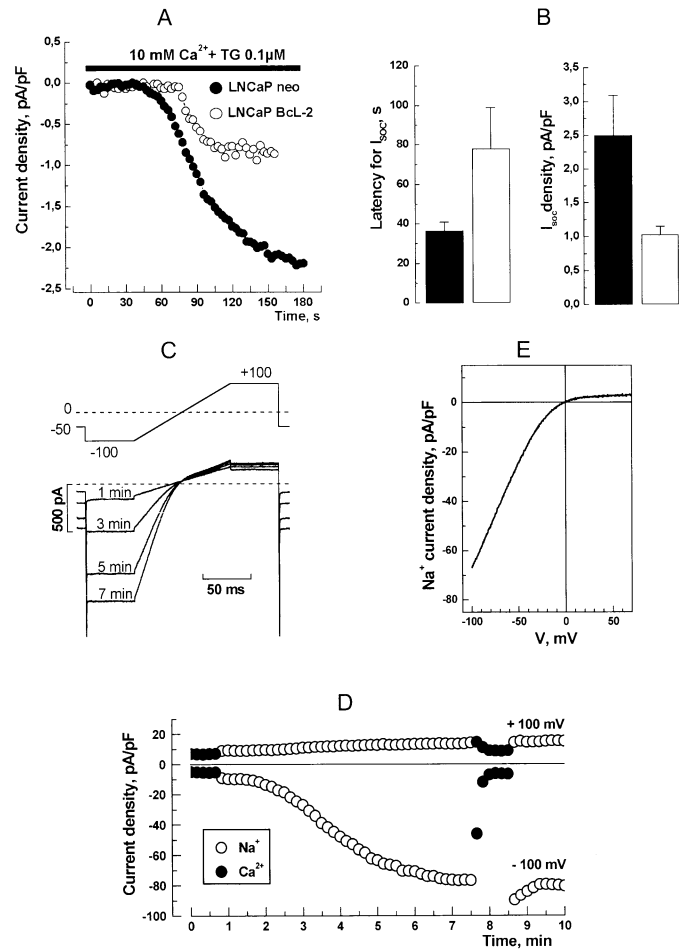
Here, we investigate the changes in  $\text{Ca}^{2+}$  homeostasis and  $I_{\text{SOC}}$  caused by Bcl-2 overexpression in LNCaP cells by using direct patch-clamp recordings combined with  $\text{Ca}^{2+}$  imaging and immunodetection techniques. With this complex approach we show that Bcl-2 downregulates  $I_{\text{SOC}}$ , most likely due to reduction of the number of functional channels, and decreases  $[\text{Ca}^{2+}]_{\text{ER}}$  due to diminished ER  $\text{Ca}^{2+}$  uptake associated with underexpression of two key  $\text{Ca}^{2+}$ -handling proteins, calreticulin and SERCA2b. We also show that  $\text{Ca}^{2+}$  store depletion by itself is not sufficient to induce apoptosis in Bcl-2-overexpressing cells, and that sustained  $\text{Ca}^{2+}$  entry via activated SOCs is required. Our data provide an insight into the role of Bcl-2 in regulating  $\text{Ca}^{2+}$  homeostasis in general and  $I_{\text{SOC}}$  in particular. Given that Bcl-2 overexpression confers an androgen-insensitive phenotype to prostate cells (Raffo et al., 1995), these data suggest a novel, potentially important SOC-dependent mechanism for the transition from hormone-sensitive to hormone-insensitive prostate cancer.

## Results

In this study we used human prostate cancer cells, LNCaP (Horszewicz et al., 1983), stably transfected with human Bcl-2 (LNCaP/Bcl-2) (Raffo et al., 1995) and compared the results with those obtained in LNCaP cells transfected with an empty vector (LNCaP/neo), which served as control. Both cell types were originally created and kindly provided to us by Dr. R. Buttyan (Columbia University, New York, NY) (Raffo et al., 1995). To exclude clonal variations in the results, two mixed populations of different individual LNCaP/Bcl-2 clones (designated as LNCaP/Bcl-2-1 and LNCaP/Bcl-2-2) and two mixed populations of different individual LNCaP/neo clones (designated as LNCaP/neo-1 and LNCaP/neo-2) were used in the experiments. Western blot analysis for the expression of various proteins (including Bcl-2) showed no difference between populations of the same cells (see below, Figure 4); therefore, in the functional studies ( $\text{Ca}^{2+}$  imaging and electrophysiology), we made no distinction between populations.

### Bcl-2 delays activation and reduces the density of $I_{\text{SOC}}$

In our previous work using perforated patch technique combined with Fura-2  $[\text{Ca}^{2+}]_c$  measurements (Skryma et al., 2000), we showed that the SERCA pump inhibitor, thapsigargin (TG), induces a sustained  $\text{Ca}^{2+}$  influx via activated SOCs. Figure 1A compares the time courses of the development of  $\text{Ca}^{2+}$ -carried  $I_{\text{SOC}}$  ( $I_{\text{SOC,Ca}}$ ) in response to 0.1  $\mu\text{M}$  TG application in representative LNCaP/neo and LNCaP/Bcl-2 cells. Bcl-2 overexpression resulted in a prolonged latency before  $I_{\text{SOC}}$  activation and a reduction in the maximal current density (by factors of 2.2 and 2.4, respectively; Figure 1B).



**Figure 1.** Bcl-2 overexpression is associated with downregulation of capacitative  $\text{Ca}^{2+}$  entry

**A:** Time courses of the development of  $I_{\text{SOC,Ca}}$  measured using perforated-patch technique at  $-80$  mV holding potential in response to 0.1  $\mu\text{M}$  TG in representative control LNCaP/neo and Bcl-2-overexpressing LNCaP/Bcl-2 cells. **B:** Cumulative data (mean  $\pm$  SEM) for the latency (left graph) and density (right graph) of  $I_{\text{SOC,Ca}}$  in LNCaP/neo and LNCaP/Bcl-2 cells. **C:** Traces of  $I_{\text{SOC,Na}}$  recorded in representative LNCaP/neo cells in response to the depicted pulse protocol at different times corresponding to the time course presented in **D** (B3 and P2 bath and pipette solutions, see Table 1). **D:** The time course of the development of  $I_{\text{SOC,Na}}$  in the same cell at  $+100$  mV (outward current) and  $-100$  mV (inward current), respectively; open and filled symbols correspond to the switches between DVC-free and normal bath solutions (B3 and B1, respectively; see Table 1). **E:** I-V relationship of fully developed  $I_{\text{SOC,Na}}$  derived from currents, presented in **C**.

### $I_{\text{SOC}}$ downregulation in Bcl-2 overexpressing cells is independent of charge carrier

Since in LNCaP/Bcl-2 cells,  $I_{\text{SOC,Ca}}$  was on average more than 2-fold smaller than in control cells (data not shown), and was usually too small to be analyzed reliably, we investigated whether removing divalent cations from the extracellular space would render SOCs in LNCaP cells permeable to monovalent cations, thus increasing  $I_{\text{SOC}}$  amplitude, as had been previously shown by Kerschbaum and Cahalan (1999).

In the conventional whole-cell configuration,  $\text{Na}^{+}$ -carried  $I_{\text{SOC}}$  ( $I_{\text{SOC,Na}}$ ) was activated by passive store depletion in response to dialyzing the cell with 10 mM EGTA while bathing the cell in

a DVC-free (divalent cation-free) extracellular solution supplemented with 10 mM EDTA to facilitate  $\text{Na}^+$  permeation. Figure 1C shows original traces of  $I_{\text{SOC,Na}}$  recorded in a representative LNCaP/neo cell in response to the depicted voltage-clamp protocol at various times after the whole-cell configuration was established. The inward  $\text{Na}^+$  current measured at  $-100$  mV started to develop after about 1.5 min and reached maximal amplitude at about 7 min (Figure 1D). The I-V relationship of the fully developed current showed strong inward rectification with a reversal potential near 0 mV (Figure 1E).

Under the same experimental conditions, activation of  $I_{\text{SOC,Na}}$  was also observed in LNCaP/Bcl-2 cells (Figure 2A). Figure 2B compares the average temporal parameters of  $I_{\text{SOC,Na}}$  development in LNCaP/neo ( $n = 27$ ) and LNCaP/Bcl-2 ( $n = 25$ ) cells. Bcl-2 overexpression resulted in a statistically significant increase in the latency period (from  $90.4 \pm 6.1$  s to  $202 \pm 27$  s) and decrease in maximal current density at  $-100$  mV (from  $57 \pm 4.2$  pA/pF to  $34 \pm 4.6$  pA/pF). The time to maximal  $I_{\text{SOC,Na}}$  activation (time to peak) in LNCaP/Bcl-2 cells was also significantly longer than in control ( $471 \pm 48$  s compared to  $380 \pm 22.5$  s), reflecting not only the longer latency period, but also the slower rate of current development ( $0.019 \pm 0.04$  pA/pF/s compared to  $0.066 \pm 0.024$  pA/pF/s).

We have also investigated whether Bcl-2 was capable of modulating the membrane resting potential. There was no statistically significant difference between the average values of resting potential in LNCaP/neo ( $-42 \pm 6$  mV,  $n = 11$ ) and LNCaP/Bcl-2 ( $-48 \pm 4$  mV,  $n = 6$ ) cells.

### Bcl-2 reduces the number of functional SOCs

To determine what kind of change, if any, in the properties of single SOCs might underlie the downregulation of the whole cell current by Bcl-2, we compared single channel activity in LNCaP/neo and LNCaP/Bcl-2 cells, by using  $\text{Na}^+$  as a charge carrier to permit its adequate resolution (Figures 2C and 2D). Experiments were carried out in cell-attached configuration on cells, continuously bathed in BAPTA-supplemented high- $\text{K}^+$  solution (B4, see Table 1) to provide for the zeroing of the cells' resting potential and passive store depletion, with DVC-free solution (B3, see Table 1) in the pipette. During recordings, the patch was held at 0 mV, and the currents were evoked by applying voltage pulses, consisting of hyperpolarizing to  $-100$  mV and depolarizing to  $+100$  mV portions connected with a voltage ramp (top panels of Figure 2C and 2D). Under these conditions we observed that in both cell types, single channel currents were directed inward and had an amplitude around 1.2 pA at  $-100$  mV (lower panels of Figure 2C and 2D). The following line of evidence suggested that these currents were associated with the activity of single SOCs: (1) the activity was never observed in the BAPTA-free high- $\text{K}^+$  bath that did not provide for store depletion (although the presence of TG in this solution was not critical for single channel activation); (2) inclusion of 1 mM  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  in the pipette instead of EDTA prevented the channel activity; and (3) the channel open probability decreased at less negative potentials, diminishing to nearly zero above 0 mV, consistent with the pattern of inward rectification of the whole-cell  $I_{\text{SOC,Na}}$ .

Linear fit of the unitary openings, generated in response to voltage ramps, yielded single channel conductance in LNCaP/Bcl-2 cells ( $11.8 \pm 2.4$  pS,  $n = 11$ ) almost identical to the control cells ( $12.1 \pm 3.2$  pS,  $n = 19$ ). At  $-100$  mV, the unitary amplitude

and open probability ( $P_o$ ) were quite similar as well ( $1.16 \pm 0.1$  versus  $1.19 \pm 0.12$  pA,  $n = 14$ , and  $0.31 \pm 0.12$  versus  $0.26 \pm 0.13$ ,  $n = 9$ , in LNCaP/Bcl-2 and LNCaP/neo cell, respectively), suggesting the decreased number of functional channels as the most probable reason for the observed downregulation of the capacitive  $\text{Ca}^{2+}$  entry and the whole-cell  $I_{\text{SOC}}$  in LNCaP/Bcl-2 cells.

### Bcl-2 reduces the amount of releasable $\text{Ca}^{2+}$

To investigate whether Bcl-2 was capable of modulating the amount of  $\text{Ca}^{2+}$  released from intracellular stores, we first examined the effects of TG in a  $\text{Ca}^{2+}$ -free medium. There was no difference between the resting level of  $[\text{Ca}^{2+}]_c$  in LNCaP/neo and LNCaP/Bcl-2 cells (Figure 3A and 3B). Adding 0.1  $\mu\text{M}$  TG in the absence of extracellular  $\text{Ca}^{2+}$  led to the transient increase in  $[\text{Ca}^{2+}]_c$  associated with the TG-induced release of  $\text{Ca}^{2+}$  from the ER in both cell types (Figure 3A). In LNCaP/Bcl-2 cells, however, the maximal increase in  $[\text{Ca}^{2+}]_c$  was substantially smaller than in the control (Figures 3A and 3B), suggesting that Bcl-2 overexpression reduces the amount of available  $\text{Ca}^{2+}$  that can be released. A similar result was obtained using the  $\text{Ca}^{2+}$  ionophore, ionomycin (IM) (Figure 3B).

We also studied the effects of TG and IM in the presence of two mitochondrial inhibitors, oligomycin (40  $\mu\text{M}$ ) and rotenone (20  $\mu\text{M}$ ). Under such conditions, the  $\text{Ca}^{2+}$  release induced by either TG or IM was still markedly smaller in LNCaP/Bcl-2 cells (data not shown), indicating that the decrease in the amount of  $\text{Ca}^{2+}$  releasable from intracellular stores in Bcl-2-overexpressing cells did not result from increased mitochondrial  $\text{Ca}^{2+}$  uptake.

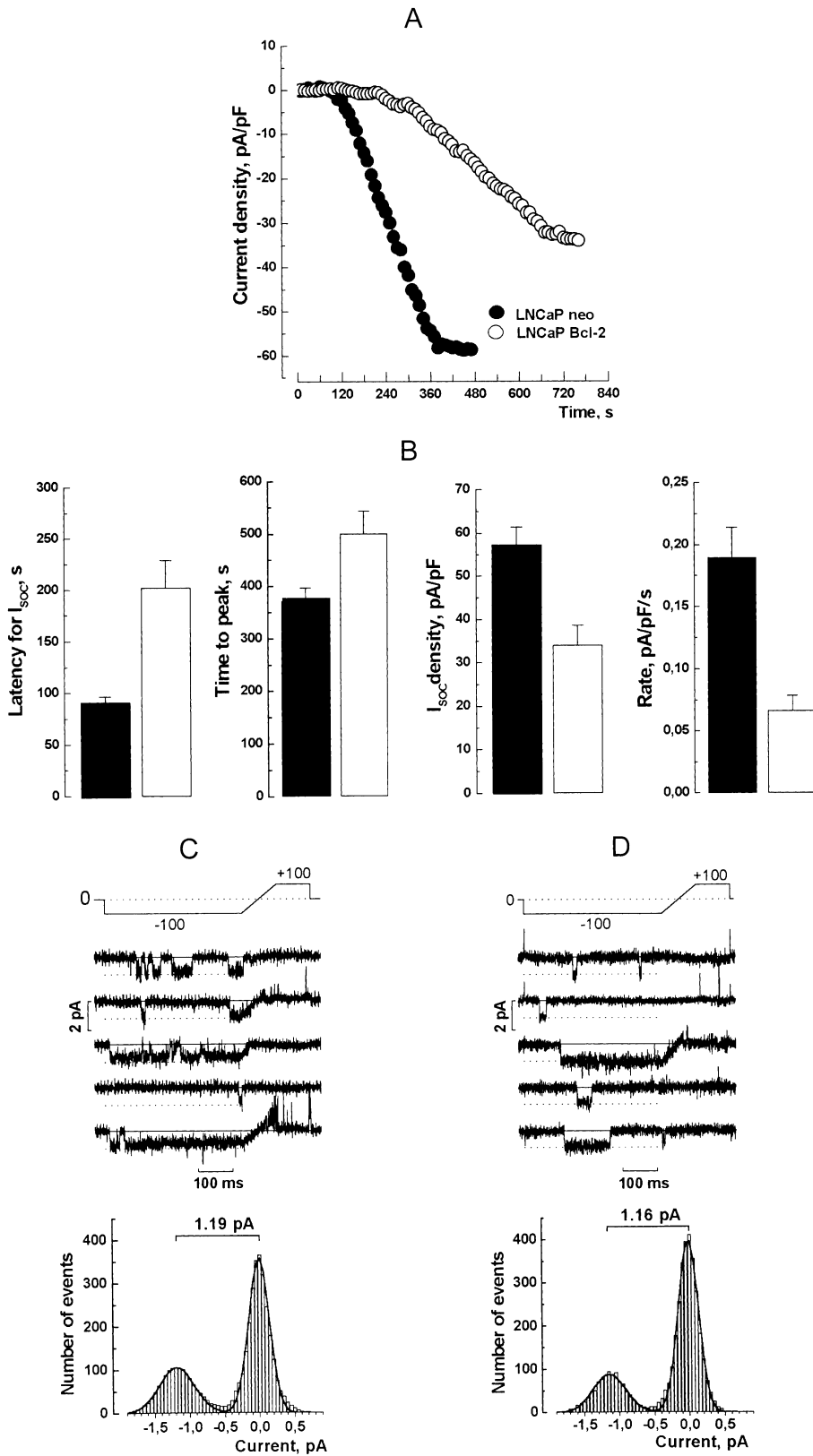
### Bcl-2 decreases the free $\text{Ca}^{2+}$ concentration in the ER

The ER  $\text{Ca}^{2+}$  content in LNCaP/neo and LNCaP/Bcl-2 cells was further investigated using the compartmentalized fluorescent  $\text{Ca}^{2+}$  indicator, Mag-fura 2-AM (Hofer and Schulz, 1996). Imaging experiments with Mag-fura 2-AM were conducted on cells permeabilized by mild digitonin treatment. Figure 3C shows a representative image of LNCaP/neo cells loaded with Mag-fura 2-AM following digitonin permeabilization, demonstrating  $\text{Ca}^{2+}$  localization in the subcellular compartments. Figure 3D shows an average of approximately 40% less  $[\text{Ca}^{2+}]_{\text{ER}}$  in LNCaP/Bcl-2 ( $215 \pm 4.3$   $\mu\text{M}$ ,  $n = 39$ ) compared to LNCaP/neo ( $352 \pm 6.8$   $\mu\text{M}$ ,  $n = 35$ ) cells.

The Bcl-2 dependence of  $[\text{Ca}^{2+}]_{\text{ER}}$  was also confirmed in an independent series of experiments on LNCaP/Bcl-2 cells subjected to antisense hybrid depletion of Bcl-2 mRNA. Figure 3F shows that treatment of LNCaP/Bcl-2 cells for 48 hr with Bcl-2 antisense oligonucleotides decreased Bcl-2 protein expression by about 40% compared to the sense oligonucleotides treatment. Reduction of Bcl-2 protein in antisense-treated cells was accompanied by a nearly 2-fold increase of their  $[\text{Ca}^{2+}]_{\text{ER}}$  (Figure 3E), suggesting strict correlation between Bcl-2 expression and  $\text{Ca}^{2+}$  feeling status of the ER.

### Bcl-2 decreases SERCA2b and calreticulin expression

One of the mechanisms by which Bcl-2 may decrease the  $\text{Ca}^{2+}$  filling status of the ER is by affecting the expression of ER-specific  $\text{Ca}^{2+}$ -handling proteins, such as endolemmal SERCA pump and/or calreticulin. We therefore compared the expression of SERCA isoforms (SERCA2a, SERCA2b, and SERCA3) and calreticulin in LNCaP/neo and LNCaP/Bcl-2 cells. Only the



**Figure 2.** Effect of Bcl-2 on the whole-cell and single-channel  $I_{SOC,Na}$ .

**A:** Two representative time courses of the whole-cell  $I_{SOC,Na}$  development in LNCaP/neo and LNCaP/Bcl-2 cells. **B:** Cumulative data (mean  $\pm$  SEM) for the latency, time to peak, maximal current density, and rate of development of  $I_{SOC,Na}$  in LNCaP/neo and LNCaP/Bcl-2 cells. **C and D:** Representative recordings of the activity of single store-operated channels in cell-attach patches in LNCaP/neo (**C**) and LNCaP/Bcl-2 (**D**) cells. Lower graphs represent amplitude histograms for single store-operated channels at  $-100$  mV in the respective cells.

**Table 1.** Compositions of bath and pipette solutions used for electrophysiological recordings

Salt	Bath				Pipette			
	Normal (B1)	Na <sup>+</sup> -free (B2)	DVC-free (B3)	High-K <sup>+</sup> (B4)	Basic (P1)	DVC-free 1 (P2)	DVC-free 2 (P3)	Perfor. Patch (P4)
NaCl	120	–	120	–	–	–	–	–
KCl	5	–	–	20	–	–	–	–
K(OH)	–	–	–	120	–	–	–	–
CsCl	–	–	–	–	120	120	120	40
Cs <sub>2</sub> SO <sub>4</sub>	–	–	–	–	–	–	–	50
CaCl <sub>2</sub>	2	10	–	–	1	–	–	1
MgCl <sub>2</sub>	2	–	–	–	1	–	–	7
Glucose	5	5	5	5	–	–	–	5
HEPES	10	10	10	10	10	10	10	10
EDTA	–	–	10	–	–	–	–	–
EGTA	–	–	–	–	10	10	–	–
BAPTA	–	–	–	10	–	–	10	–
TEA-Cl	–	120	20	–	–	–	–	–
pH-adj.	Na(OH)	TEA(OH)	MSF	Glut.	Cs(OH)	Cs(OH)	Cs(OH)	Cs(OH)

Concentrations are given in mM, and pH of all solutions was adjusted to 7.3. Osmolarity of the bath and pipette solutions was approximately 300 and 280 mosm/l, respectively. Abbreviations: MSF, methansulfonic acid; Glut., L-glutamic acid; DVC, divalen cations.

SERCA2b isoform was detected. Figure 4 shows semiquantitative Western blot analysis of the protein lysates derived from two populations of LNCaP/neo and two populations of LNCaP/Bcl-2 cells (see Experimental Procedures and the first paragraph of Results) on the presence of five proteins: Bcl-2 (to confirm overexpression), SERCA2b, calreticulin, calnexin (an integral ER membrane protein not involved in drug-induced apoptosis [Nakamura et al., 2000] to check for constancy of total ER), and actin (for control purposes, to confirm equal loading of the protein samples). Both SERCA2b and calreticulin expression were markedly lower in LNCaP/Bcl-2 compared to LNCaP/neo cells, while calnexin and actin expression was unchanged.

#### Bcl-2 enhances the rate of passive Ca<sup>2+</sup> leak from the ER and decreases Ca<sup>2+</sup> reuptake

The Ca<sup>2+</sup> permeability of the ER and Ca<sup>2+</sup> uptake were investigated on digitonin-permeabilized cells using the fluorescent Ca<sup>2+</sup> indicator, Mag-fura 2-AM. Figure 5A shows the time course of a typical experiment, involving ratiometric measurements of [Ca<sup>2+</sup>]<sub>ER</sub> in representative LNCaP/neo cells, in response to IP<sub>3</sub> (100 μM) and TG (0.1 μM). Application of IP<sub>3</sub> triggered a rapid drop in [Ca<sup>2+</sup>]<sub>ER</sub> due to IP<sub>3</sub>-induced release, followed by [Ca<sup>2+</sup>]<sub>ER</sub> recovery after IP<sub>3</sub> withdrawal due to Ca<sup>2+</sup> reuptake into intracellular stores. Subsequent exposure of the cell to TG produced a gradual reduction of [Ca<sup>2+</sup>]<sub>ER</sub>, due to passive leakage under inhibited uptake conditions. The rate of [Ca<sup>2+</sup>]<sub>ER</sub> recovery in response to IP<sub>3</sub> withdrawal is determined by SERCA-mediated Ca<sup>2+</sup> reuptake into the ER, while the rate of TG-induced leakage is indicative of the degree of ER permeability.

Figures 5B and 5C present quantification of the Ca<sup>2+</sup> leakage and reuptake rates, derived from Mag-fura 2-AM measurements in LNCaP/neo and LNCaP/Bcl-2 cells. Bcl-2 overexpression resulted in a substantially increased rate of Ca<sup>2+</sup> leakage from the ER (Figure 5B). At the same time, the Ca<sup>2+</sup> uptake rate in LNCaP/Bcl-2 cells (Figure 5C) was 32% slower than in control, consistent with the lower levels of SERCA protein expression in these cells.

The increased rate of Ca<sup>2+</sup> leakage from the ER in LNCaP/Bcl-2 cells was also confirmed using the TG and ionomycin

coapplication approach, as was previously reported by Foyouzi-Youssefi et al. (2000) (data not shown).

#### SOCs activation is required for TG-induced apoptosis in Bcl-2 overexpressing cells

We have also examined whether Bcl-2 overexpression interferes with the ability of TG to induce apoptosis. In our previous study, we have shown that chronic treatment of LNCaP cells with 0.1 μM TG was able to induce apoptosis irrespective of capacitative Ca<sup>2+</sup> influx (Skryma et al., 2000).

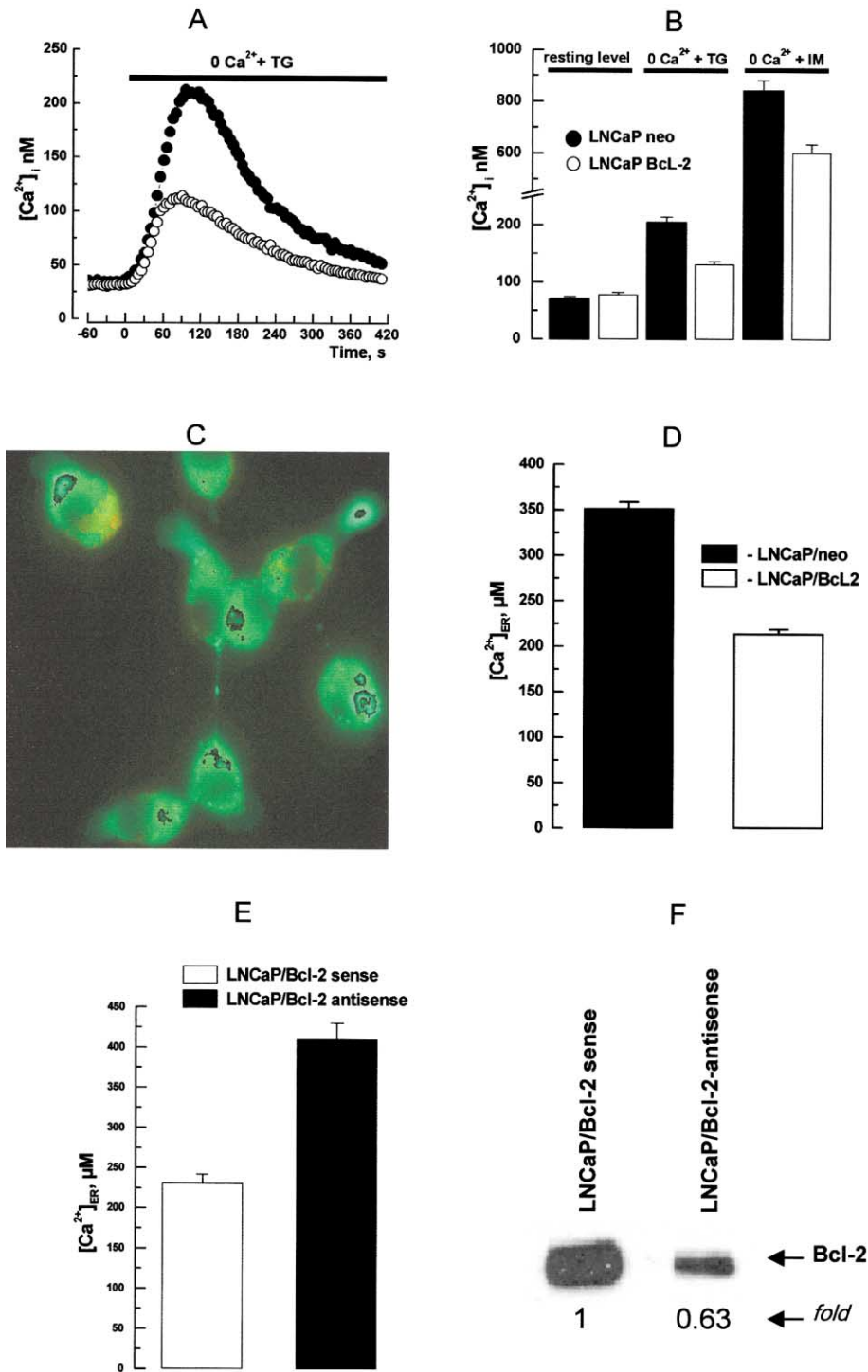
Assessment of TG-induced apoptosis in LNCaP/Bcl-2 cells at different extracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>out</sub>) has shown much stronger [Ca<sup>2+</sup>]<sub>out</sub> dependence compared to LNCaP/neo cells (Figure 6). Indeed, at low [Ca<sup>2+</sup>]<sub>out</sub> (0.4 mM), TG, even after 48 hr of exposure, was able to induce apoptosis in only about 2.6% of LNCaP/Bcl-2 cells, compared to around 39% of LNCaP/neo cells (Figure 6A). Following transition to high [Ca<sup>2+</sup>]<sub>out</sub> (2 mM), the percentage of TG-induced apoptotic LNCaP/Bcl-2 cells increased 14-fold (to 37%), compared to only about 2-fold for LNCaP/neo cells (to 75%) (Figure 6B). These results indicate that under conditions of weak capacitative Ca<sup>2+</sup> entry (i.e., low [Ca<sup>2+</sup>]<sub>out</sub>), emptying of Ca<sup>2+</sup> stores by TG is not sufficient to induce apoptosis in Bcl-2-overexpressing cells, and that sustained Ca<sup>2+</sup> entry via activated SOC is required for the apoptosis to occur.

#### Discussion

##### Bcl-2 overexpression and capacitative calcium entry

Bcl-2 overexpression was reported to induce the downregulation (Pinton et al., 2000) as well as upregulation (Foyouzi-Youssefi et al., 2000; Williams et al., 2000) of the capacitative Ca<sup>2+</sup> entry. These contradictions may be at least partly explained by the fact that in all quoted studies, the effects of Bcl-2 on capacitative Ca<sup>2+</sup> influx were assessed indirectly on the basis of [Ca<sup>2+</sup>]<sub>c</sub> measurements, while only direct electrophysiological recordings of I<sub>SOC</sub> combined with [Ca<sup>2+</sup>]<sub>c</sub> and [Ca<sup>2+</sup>]<sub>ER</sub> measurements can provide reliable results.

In our recent work, we identified the TG-induced I<sub>SOC</sub> in



**Figure 3.** Bcl-2 reduces the free Ca<sup>2+</sup> concentration within ER lumen

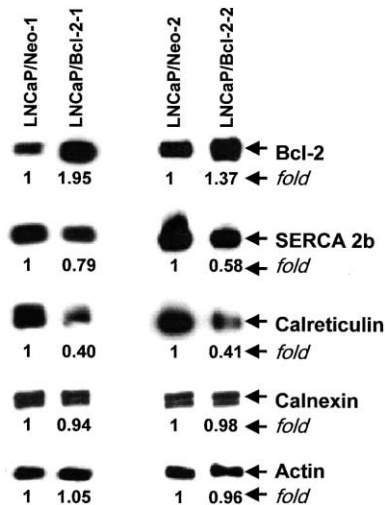
**A:** Typical [Ca<sup>2+</sup>]<sub>i</sub> traces in the control LNCaP/neo and Bcl-2-overexpressing LNCaP/Bcl-2 cells in response to TG (0.1 μM) in Ca<sup>2+</sup>-free medium. **B:** Cumulative data (mean ± SEM) for the resting [Ca<sup>2+</sup>]<sub>i</sub> and peak [Ca<sup>2+</sup>]<sub>i</sub> increases evoked by TG (0.1 μM) and ionomycin (IM, 2 μM) in LNCaP/neo and LNCaP/Bcl-2 cells. **C:** Representative image of the control LNCaP cells loaded with Mag-fura-2-AM demonstrating Ca<sup>2+</sup> localization in the ER. **D:** Resting [Ca<sup>2+</sup>]<sub>ER</sub> values (mean ± SEM) in LNCaP/neo and LNCaP/Bcl-2 cells derived from Mag-fura-2-AM measurements. **E:** Resting [Ca<sup>2+</sup>]<sub>ER</sub> values (mean ± SEM) derived from Mag-fura-2-AM measurements in LNCaP/Bcl-2 cells treated with sense and antisense oligonucleotides. **F:** Semiquantitative Western blot analysis of Bcl-2 expression in LNCaP/Bcl-2 treated for 48 hr with Bcl-2 mRNA sense and antisense oligonucleotides.

LNCaP cells (Skryma et al., 2000), while here we extend our study by showing that Bcl-2 overexpression quite dramatically downregulates I<sub>SOC</sub> by prolonging the latency for current activation and significantly reducing its maximal amplitude.

In spite of considerable recent progress in understanding SOC regulation (Yao et al., 1999; Ma et al., 2000), the molecular identity of these channels and the type of signal that activates them is still open to debate. It was suggested that the depletion of Ca<sup>2+</sup> stores was the *sine qua non* for capacitative Ca<sup>2+</sup> entry

through SOCs (Broad et al., 1999). An alternative hypothesis, first proposed by Barritt (1998) and then experimentally verified by Krause et al. (1999), does not link SOC activation directly to the depletion of the ER, but rather postulates that channels open in response to decreasing [Ca<sup>2+</sup>]<sub>c</sub> levels in subplasmalemmal spaces in close proximity to SOCs.

We therefore investigated whether Bcl-2 modulated the cytoplasmic Ca<sup>2+</sup> concentration and/or ER Ca<sup>2+</sup> content in LNCaP cells. Similar to those of Pinton et al. (2000), our experiments



**Figure 4.** Bcl-2 decreases SERCA2b and calreticulin expression

Semiquantitative Western blot analysis of Bcl-2, SERCA2b, calreticulin, calnexin, and actin expression in two populations of control (LNCaP/neo-1 and LNCaP/neo-2) and in two populations of Bcl-2 overexpressing (LNCaP/Bcl-2-1 and LNCaP/Bcl-2-2) LNCaP cells. See text for details.

showed no difference in the resting  $[Ca^{2+}]_c$  levels between Bcl-2-transfected and control cells. However, high basal  $[Ca^{2+}]_c$  was observed in Bcl-2-expressing A20 cells by Foyouzi-Youssefi et al. (2000). The authors suggested that this increase resulted from an enhanced unstimulated  $Ca^{2+}$  influx in Bcl-2-overexpressing cells, but no evidence was presented that this basal  $Ca^{2+}$  influx was indeed mediated by SOCs.

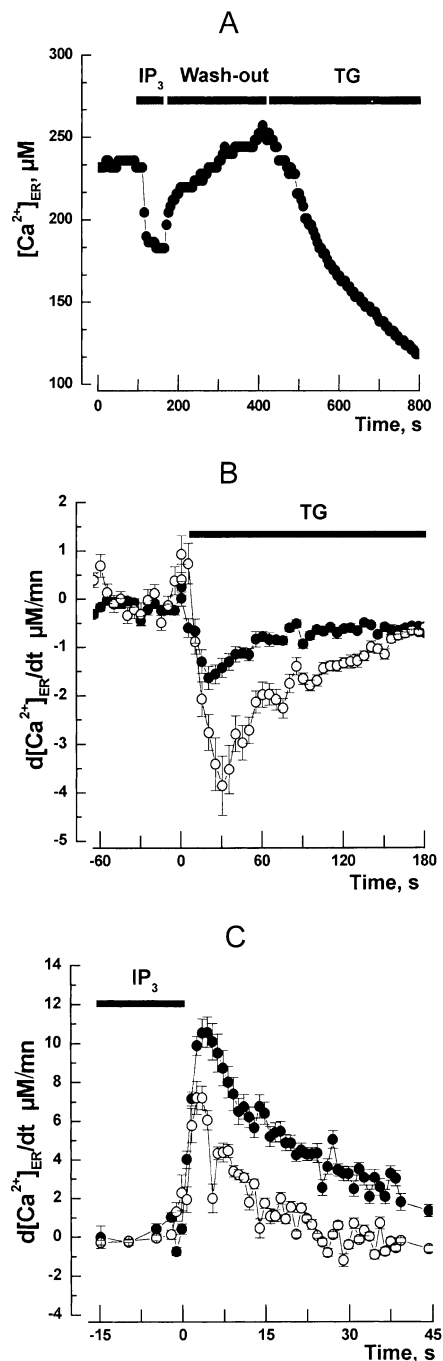
In contrast to  $[Ca^{2+}]_c$ , the status of intracellular  $Ca^{2+}$  stores was significantly modified in LNCaP/Bcl-2 cells: direct  $[Ca^{2+}]_{ER}$  measurements using Mag-fura 2-AM showed about 40% less ER  $Ca^{2+}$  content in LNCaP/Bcl-2 cells compared to the control.

Capacitative  $Ca^{2+}$  current in LNCaP cells is increased following membrane hyperpolarization (Skryma et al., 2000), suggesting that changes in  $I_{SOC}$  could be secondary to the possible Bcl-2-mediated modulation of the resting potential. Indeed, there is data that indicates that Bcl-2 overexpression may affect voltage-gated  $K^+$  channels (Ekhterae et al., 2001) and that hyperpolarization associated with  $K^+$  channel activation could underlie protection from the cell death conferred by members of the Bcl-2 family of proteins, such as *mcl-1* (Wang et al., 1999b). We have also investigated whether Bcl-2 was capable of modulating the LNCaP cells' resting potential and found no difference between LNCaP/neo and LNCaP/Bcl-2 cells, indicating that the effects of Bcl-2 on  $I_{SOC}$  could not be attributed to the modulation of membrane potential.

#### Possible mechanisms for the Bcl-2-induced decrease in $[Ca^{2+}]_{ER}$

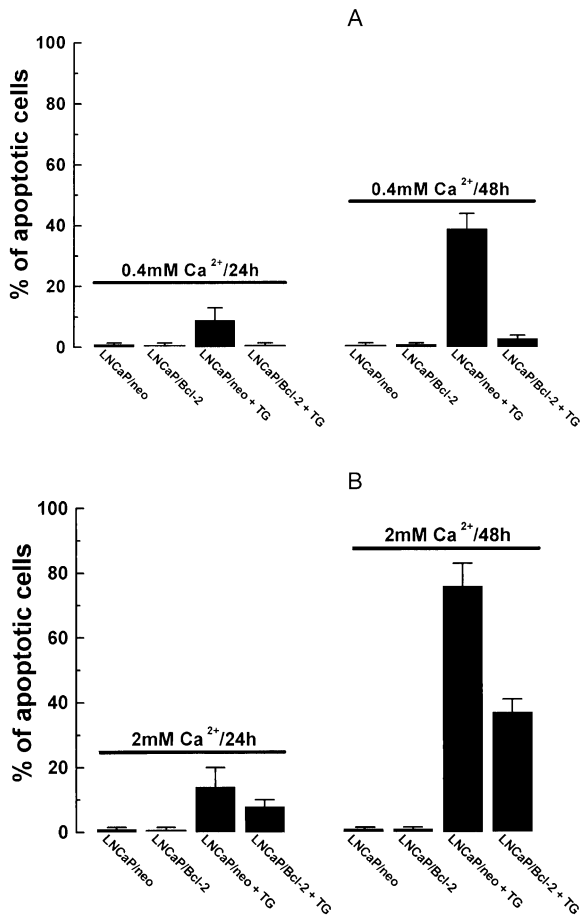
The basal filling status of intracellular  $Ca^{2+}$  stores depends on the balance between  $Ca^{2+}$  efflux via leak channels and immediate reuptake by SERCA pumps. Our results show that both of these mechanisms are affected by Bcl-2.

The molecular origin and functioning of ER leak channels are not clearly understood. In light of the evidence that Bcl-2 may be an ion channel (Antonsson et al., 1997; Minn et al.,



**Figure 5.** Bcl-2 enhances the rate of passive  $Ca^{2+}$  leak from ER and decreases  $Ca^{2+}$  reuptake

**A:** Time course of a typical experiment on the estimation of the passive  $Ca^{2+}$  leak and  $Ca^{2+}$  uptake in digitonin-permeabilized cells with the use of  $IP_3$  and TG. **B:** The apparent  $Ca^{2+}$  leak rate plotted as a function of time after TG application configuration in LNCaP/neo and LNCaP/Bcl-2 cells. **C:** The apparent  $Ca^{2+}$  uptake rate plotted as a function of time following  $IP_3$  removal in LNCaP/neo and LNCaP/Bcl-2 cells. The data in **B** and **C** are means  $\pm$  SEM for 8 cells of each type.



**Figure 6.** Bcl-2 overexpression increases cell resistance to TG-induced apoptosis

**A:** Temporal changes in the apoptosis of LNCaP/neo cells (filled bars). Cells were treated with 0.1  $\mu$ M TG in a medium containing 0.4 mM  $\text{Ca}^{2+}$ . The level of apoptosis was estimated from the number of apoptotic bodies visualized by the Hoechst staining. **B:** Effect of extracellular  $\text{Ca}^{2+}$  increase (from 0.4 mM to 2 mM) on the 0.1  $\mu$ M TG-induced apoptosis in LNCaP/Bcl-2 cells.

1997; Schendel et al., 1997), the simplest explanation for the increased passive leakage observed by us in Bcl-2-overexpressing cells would be a higher density of endolemmal Bcl-2 channels. However—although this is an attractive hypothesis—it is still not known whether Bcl-2 is able to conduct  $\text{Ca}^{2+}$ . Identification of the precise mechanisms by which Bcl-2 may activate an endogenous leak remains an intriguing subject of future research.

Besides increasing passive leakage, Bcl-2 also reduces  $\text{Ca}^{2+}$  reuptake, and this effect is most probably associated with downregulation of SERCA2b protein expression. An additional factor that may contribute to the reduced ER filling status in Bcl-2 overexpressing cells is altered expression of ER  $\text{Ca}^{2+}$  binding proteins, in particular calreticulin, the most important ER protein in apoptosis regulation. It was recently shown that its overexpression increased cell sensitivity to TG- and staurosporine-induced apoptosis and, conversely, that cells lacking calreticulin showed considerable resistance to drug-induced apoptosis (Nakamura et al., 2000). It was concluded that the

ER luminal environment in general, and calreticulin in particular, play a significant role in determining cell sensitivity to apoptosis (Michalak et al., 1999). Moreover, calreticulin may interact with SERCA2b, and calreticulin binding to SERCA2b may be regulated by changes in  $[\text{Ca}^{2+}]_{\text{ER}}$  (John et al., 1998; Vassilakos et al., 1998). It has also been hypothesized that calreticulin may act as a  $\text{Ca}^{2+}$  sensor for SERCA in the ER lumen (Corbett et al., 2000) and play a role in  $\text{Ca}^{2+}$  storage (Nakamura et al., 2001). Our results demonstrate that Bcl-2 decreases the expression of calreticulin protein, which is normally abundant in LNCaP cells. The low levels of calreticulin and  $[\text{Ca}^{2+}]_{\text{ER}}$  may in turn be responsible for the reduction in SERCA2b expression.

#### Why does Bcl-2 downregulate $I_{\text{SOC}}$ ?

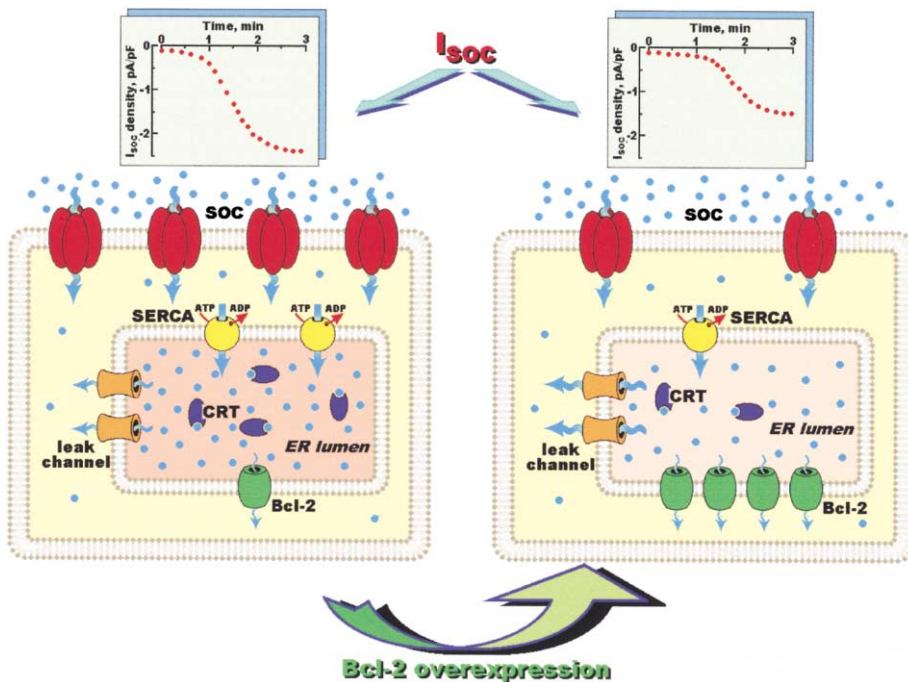
The reduced  $\text{Ca}^{2+}$  status of intracellular stores in LNCaP/Bcl-2 cells had thus been demonstrated, but it was not yet clear whether this change in ER status is responsible for downregulating  $I_{\text{SOC}}$ . In fact,  $I_{\text{SOC}}$  downregulation could be a direct consequence of Bcl-2 overexpression or an adaptive mechanism to the long-term reduction in ER  $\text{Ca}^{2+}$  content. However, even if one accepts the adaptation hypothesis, the mechanism by which the Bcl-2-induced reduction in ER  $\text{Ca}^{2+}$  content results in increased latency and lower amplitude of  $I_{\text{SOC}}$  still needs to be elucidated. The delay in  $I_{\text{SOC}}$  activation may be due either to the time needed to deplete intracellular stores below a certain threshold level that triggers the activation process, or the time required to synthesize and/or release endoplasmic messenger(s) (Huang and Putney, 1998). Several studies have demonstrated that  $I_{\text{SOC}}$  could be fully activated with only partial depletion of intracellular  $\text{Ca}^{2+}$  stores (Huang and Putney, 1998; Krause et al., 1999). Moreover, the  $\text{Ca}^{2+}$  pool involved in controlling  $I_{\text{SOC}}$  is very small compared to the total TG-sensitive  $\text{Ca}^{2+}$  pools (Huang and Putney, 1998), suggesting that only specialized subcompartments of the ER directly govern  $I_{\text{SOC}}$  activation. We favor the hypothesis that autowdownregulation by reduced store content of synthesis and/or release of endoplasmic messenger(s) is one of the primary reasons for the increased latency and decreased amplitude of  $I_{\text{SOC}}$ .

We also observed downregulation of  $I_{\text{SOC,Na}}$  in Bcl-2-overexpressing LNCaP cells. It was recently demonstrated that  $I_{\text{SOC,Na}}$  can be activated by cytosolic medium with significantly reduced concentration of divalent cations by means of chelating agents, without any store depletion (Braun et al., 2001). This indicates that Bcl-2 also plays a role in modulating the properties and/or total number of store-operated channels. Given that the open probability and unitary conductance of SOCs in LNCaP/Bcl-2 cells hardly change compared to the control, our results suggest that Bcl-2 reduces the number of available SOCs. Therefore, Bcl-2-induced downregulation of the whole-cell  $I_{\text{SOC}}$  amplitude may be, at least partially, attributed to the decreased number of channels. A schematic diagram summarizing our findings is presented in Figure 7.

#### Downregulation of SOCs by Bcl-2 and apoptosis: What is the significance?

While the implication of  $\text{Ca}^{2+}$  ions in the induction of apoptosis is now generally accepted, the data concerning the role of SOCs in this process are rather contradictory. Two hypotheses have been proposed. The first one assumes that apoptosis may be triggered by ER depletion without any requirement for the cytosolic  $\text{Ca}^{2+}$  elevation due to store-operated  $\text{Ca}^{2+}$  entry, while the





**Figure 7.** Schematic diagram showing in simplified form the major effects of Bcl-2 overexpression in prostate cancer cells evident in this study. The left panel presents the control conditions characterized by the low levels of expression of the ER-localized Bcl-2 (depicted as bearing presumed  $\text{Ca}^{2+}$  channel-like function) and the basal expression of the ER leak channels, SERCA pump, intraluminal calreticulin (CRT), and plasma membrane SOCs, the combined activity of which results in the background ER  $\text{Ca}^{2+}$  concentration and store-operated  $\text{Ca}^{2+}$  entry ( $I_{\text{SOC}}$ , see upper left graph) typical of control cells. Overexpression of Bcl-2 (right) results in the lowered ER intraluminal  $\text{Ca}^{2+}$  concentration due to downregulated expression of SERCA pump and CRT, combined with the enhanced  $\text{Ca}^{2+}$  leak via ER leak channels (probably aided by Bcl-2's own channel-like function) and decreased store-operated  $\text{Ca}^{2+}$  entry most probably associated with the diminished density of SOCs.

second one ignores ER and associates the initiation of apoptosis with sustained elevation in cytosolic  $\text{Ca}^{2+}$  to a certain critical level. This last hypothesis mostly relies on the experiments on TG-induced apoptosis in androgen-insensitive human prostate cancer cell lines TSU-Pr1, DU-145, and PC-3 (Furuya et al., 1994; Wang et al., 1999a). However, recent studies, including our own, have shown that in the androgen-sensitive human prostate cancer cell line, LNCaP, intracellular  $\text{Ca}^{2+}$  store depletion is by itself sufficient to induce apoptosis (Wertz and Dixit, 2000; Skryma et al., 2000). In the present study we find that, in Bcl-2 overexpressing LNCaP cells,  $\text{Ca}^{2+}$  store depletion per se is no longer sufficient to induce apoptosis, and that additional sustained  $\text{Ca}^{2+}$  entry via activated SOCs is required for the apoptosis to occur.

It is important to note that LNCaP cells have been shown to acquire the phenotype of androgen-insensitive cells after Bcl-2 overexpression (Raffo et al., 1995). Our results, therefore, raise an interesting question about the modulation of  $\text{Ca}^{2+}$ -dependent mechanisms of apoptosis during transition from hormone-sensitive to hormone-insensitive prostate cancer. The reasons for the differences in  $\text{Ca}^{2+}$  store-dependent mechanisms involved in apoptosis between androgen-sensitive and androgen-insensitive prostate cancer cells are intriguing. One of them may be differential expression of apoptosis-regulating proteins, such as Bcl-2 and calreticulin, as well as of SOCs proteins. Our results show that Bcl-2 downregulates calreticulin expression and decreases  $[\text{Ca}^{2+}]_{\text{ER}}$ , and that under such conditions, ER store depletion by itself is not sufficient to induce apoptosis. Since induction of apoptosis in Bcl-2-overexpressing cells also requires sustained  $\text{Ca}^{2+}$  influx via activated SOCs, the downregulation of these channels could be a key component in the protective action of Bcl-2 against apoptosis in hormone-insensitive cancer cells. Thus, our results present the evidence for a potentially important SOC-dependent mechanism govern-

ing the transition from hormone-sensitive to hormone-insensitive prostate cancer.

In conclusion, our study demonstrates events in intracellular  $\text{Ca}^{2+}$  homeostasis induced by antiapoptotic protein Bcl-2 and thereby may offer novel therapeutic strategies interfering with the mechanisms controlling  $[\text{Ca}^{2+}]_{\text{ER}}$  and  $\text{Ca}^{2+}$  entry via store-operated channels.

#### Experimental procedures

##### Cell lines

The human prostate cancer cell line LNCaP was originally derived from a patient with metastatic prostate cancer to the pelvic lymph nodes (Horoszewicz et al., 1983). LNCaP/neo and LNCaP/Bcl-2 cells, were a generous gift from Dr. R. Buttyan (Department of Urology, College of Physicians and Surgeons of Columbia University, New York, NY). The procedure of derivation of Bcl-2-overexpressing LNCaP cells is detailed in the original article (Raffo et al., 1995). Briefly, LNCaP cells were transfected with either Bcl-2 cDNA-containing neomycin-selectable pSFFV/Bcl-2 plasmid (LNCaP/Bcl-2 cells) or control neomycin-selectable pBK-CMV plasmid (LNCaP/neo cells), and several positive LNCaP/Bcl-2 and LNCaP/neo clones were selected and expanded (Raffo et al., 1995). We were provided with two batches of mixed populations of LNCaP/Bcl-2 (designated as LNCaP/Bcl-2-1 and LNCaP/Bcl-2-2) cells and two batches of mixed populations of LNCaP/neo (designated as LNCaP/neo-1 and LNCaP/neo-2). Cells were routinely maintained in a neomycin selection culture medium and prepared for electrophysiological and  $\text{Ca}^{2+}$  imaging experiments as described elsewhere (Skryma et al., 2000).

##### Electrophysiology and solutions

Regular and perforated whole-cell as well as single-channel patch-clamp techniques were used for current recording as detailed elsewhere (Skryma et al., 2000). The compositions of all bath (extracellular) and patch pipette solutions used for electrophysiological recordings are presented in Table 1. The free concentrations of divalent cations in the solutions containing chelating agents (EDTA, EGTA, BAPTA) were estimated using WinMaxc 1.7 software (Brooks and Storey, 1992).

### Fluorescence measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$

$[Ca^{2+}]_c$  was measured using fura-2 (the detailed procedure has been described previously [Skryma et al., 2000]). The composition of normal extracellular solution is presented in Table 1 (B1). For imaging of  $Ca^{2+}$  within the ER, LNCaP cells were loaded with 2  $\mu$ M of the AM-ester derivative of Mag-fura 2 for 45 min at 37°C. After incubation with the dye, the cells were rinsed briefly in a high  $K^+$  solution (in mM): 125 KCl, 25 NaCl, 10 HEPES, 0.1  $MgCl_2$  [pH 7.2], and then exposed for 2 min to an "intracellular buffer" at 37°C and 5  $\mu$ g/ml digitonin. Digitonin-permeabilized cells were continuously superfused with digitonin-free "intracellular buffer" supplemented with 0.2 mM Mg-ATP and free  $[Ca^{2+}]$  clamped to 170 nM using  $Ca^{2+}$ /EGTA buffer. The Mag-fura 2-AM fluorescence ratio was calibrated using exposure to 10  $\mu$ M ionomycin and 15 mM  $Ca^{2+}$  or 10 mM EGTA, assuming a dissociation constant for  $Ca^{2+}$ -Mag-fura 2 at room temperature of 53  $\mu$ M (Hofer and Schulz, 1996). Ratio imaging measurements of Mag-fura 2-AM fluorescence were made using a commercial imaging system (Quanticell 900, Applied Imaging, UK).

### Determination of apoptosis

The level of apoptosis was estimated from a number of apoptotic bodies visualized by the Hoechst staining (the detailed procedure has been described previously [Skryma et al., 2000]). Apoptosis was also detected by flow cytometry using AnnexinV-fluorescein isothiocyanate (FITC) to detect phosphatidylserine exposure on the cell surface, and propidium iodide to assess loss of plasma membrane integrity (Boehringer Mannheim, IN).

### Western analysis and antisense hybrid depletion

Western analysis of protein expression was as described previously (Legrand et al., 2001). Antibody anti-Bcl-2 was from Santa Cruz Biotechnology Inc., CA; antibodies anti-calreticulin and anti-calnexin were from StressGen Biotechnologies Corp., CA. Anti-SERCA2a and anti-SERCA2b were generously provided by Dr. F. Wuytack and anti-SERCA3 by Dr. B. Papp. The procedure of Bcl-2 mRNA antisense hybrid depletion, including selection of mRNA regions targeted by sense and antisense oligonucleotides, was the same as described elsewhere (Meilhac et al., 1999).

### Chemicals

All chemicals were from Sigma except for fura-2AM and thapsigargin, which were purchased from Calbiochem, and Mag-fura 2-AM, which was from Molecular Probe.

### Data analysis and statistics

Each experiment was repeated several times. The data were analyzed using PulseFit (HEKA Electronics, Germany) and Origin 5.0 (Microcal, Northampton, MA) software. Results were expressed as mean  $\pm$  standard deviation, where appropriate. Student's *t* test was used for statistical comparison of the differences, and  $P < 0.05$  was considered significant. Characteristic times of membrane current or  $[Ca^{2+}]_c$  response to any intervention were determined as follows: the time intervals from the onset of the intervention until the current or  $[Ca^{2+}]_c$  signal reached 0.05 ( $A_{max} - A_{base}$ ) and 0.95 ( $A_{max} - A_{base}$ ) were considered as latency (or delay) and time-to-peak response periods, respectively. The time interval between 0.05( $A_{max} - A_{base}$ ) and 0.95( $A_{max} - A_{base}$ ) was considered to be the response development time ( $A_{base}$  is the baseline signal before the intervention, and  $A_{max}$  is the maximal signal amplitude).

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