

Effect of Bcl-2 Overexpression on Mitochondrial Structure and Function*

Received for publication, July 31, 2002

Published, JBC Papers in Press, August 30, 2002, DOI 10.1074/jbc.M207765200

Alicia J. Kowaltowski^{‡§}, Ricardo G. Cosso^{¶||}, Cláudia B. Campos^{¶**}, and Gary Fiskum^{‡‡}

From the [‡]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, 05508-900, Brazil, the [¶]Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, 13083-970, Brazil, and the ^{‡‡}Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

Overexpression of the antiapoptotic Bcl-2 protein enhances the uptake of fluorimetric dyes sensitive to mitochondrial membrane potential, suggesting that Bcl-2 changes the mitochondrial proton gradient. In this study, we performed calibrated measurements of mitochondrial respiration, membrane potential, Δ pH, and intramitochondrial $[K^+]$ in digitonin-permeabilized PC12 and GT1-7 neural cells that either do not express human Bcl-2 (control transfectants) or that were transfected with and overexpressed the human *bcl-2* gene to evaluate whether Bcl-2 alters mitochondrial inner membrane ion transport. We found that although Bcl-2-overexpressing cells exhibit higher fluorescence responses to membrane potential, pH, and K^+ -sensitive dyes, this increased response is due to an enhanced accumulation of these dyes and not an increased mitochondrial membrane potential, Δ pH, or $[K^+]$. This result is supported by the presence of equal respiratory rates in Bcl-2+ and Bcl-2- cells. Possible structural alterations in Bcl-2+ mitochondria that could account for increases in fluorescent dye uptake were evaluated using flow cytometry particle sizing and light scattering determinations. These experiments established that Bcl-2-overexpressing mitochondria present both increased volume and structural complexity. We suggest that increased mitochondrial volume and structural complexity in Bcl-2+ cells may be related to many of the effects of this protein involved in the prevention of cell death.

ten associated with mitochondrial cytochrome *c* release and subsequent cell death (8, 11), and Bcl-2 is also capable of inhibiting cytochrome *c* release pathways independent of mitochondrial permeability transition, such as Bid- and Bax-mediated cytochrome *c* release (12, 13).

Bcl-2 effects on mitochondria determined to date involve almost exclusively studies conducted under conditions leading to cell death. Although these studies are essential to understand the antiapoptotic effects of this protein, it is important to determine whether Bcl-2 can affect mitochondrial function under basal conditions. Understanding the changes promoted by Bcl-2 on mitochondrial function in healthy cells may determine how these cells respond to potentially deadly stimuli and uncover the common roots of the distinct antiapoptotic effects of this protein. Furthermore, Bcl-2 may have an unknown role in the regulation of basal mitochondrial energy metabolism. In fact, some previous data from both our group and others (9, 10) suggest that Bcl-2 may regulate mitochondrial proton transport across the inner membrane resulting in higher mitochondrial membrane potentials since Bcl-2-overexpressing mitochondria take up larger quantities of membrane potential-sensitive dyes. This increased mitochondrial membrane potential could explain other Bcl-2 effects such as increased H_2O_2 generation (14–16) since mitochondrial reactive oxygen species generation is strongly inhibited at lower membrane potentials (17). Unfortunately, the cause of these mitochondrial changes promoted by Bcl-2 has never been carefully studied, and these results are often obtained from a single transfected cell line, raising the possibility that these are not universal Bcl-2 effects. In this study, we investigated the effects of Bcl-2 on mitochondrial function and structure using two separate cell lines and found that overexpression of this protein does not affect the membrane potential, respiration, Δ pH, or intramitochondrial $[K^+]$ but does increase mitochondrial volume and structural complexity. This increase in volume and structural complexity explains changes in fluorimetric membrane potential determinations conducted previously. Based on our results, we propose a model in which enhanced mitochondrial volume and structural complexity mediate many of the Bcl-2 effects related to the prevention of cell death.

The Bcl-2 protein, originally described in lymphoma cells (1) and then found to be widely distributed in a variety of cancerous tissues (2, 3), is a potent inhibitor of cell death, both programmed and accidental (4, 5). Bcl-2 is located in biological membranes, including mitochondria (6, 7), and acts to inhibit mitochondrially controlled steps leading to cell death. The effects of Bcl-2 on mitochondrial control of cell death are variable according to the experimental conditions studied, indicating a multifunctional role for this protein. For example, Bcl-2 inhibits mitochondrial permeability transition (8–10), a process of-

* This project was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grant 00/09642-5 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Produtividade em Pesquisa Grant 300843/00-3. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Fax: 55-11-3815-5579; E-mail: alicia@iq.usp.br.

|| Supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) scholarship.

** Supported by a FAPESP scholarship.

EXPERIMENTAL PROCEDURES

Cell Cultures—PC12 pheochromocytoma and immortalized hypothalamic GT1-7 neuronal cell lines transfected with the human *bcl-2* gene (Bcl-2+) or with a control retroviral construct (Bcl-2-) were maintained as described previously (4). Prior to the experiments the cells were trypsinized and suspended in growth medium supplemented with 10 mM Hepes, pH 7.0. Suspended cells were kept at room temperature for up to 5 h. Cell viability, as assessed by a cell count in trypan blue, was above 95% even after 5 h at room temperature. The suspended cells were centrifuged and resuspended in the medium used in the experiment just prior to each determination. Cell protein content was deter-

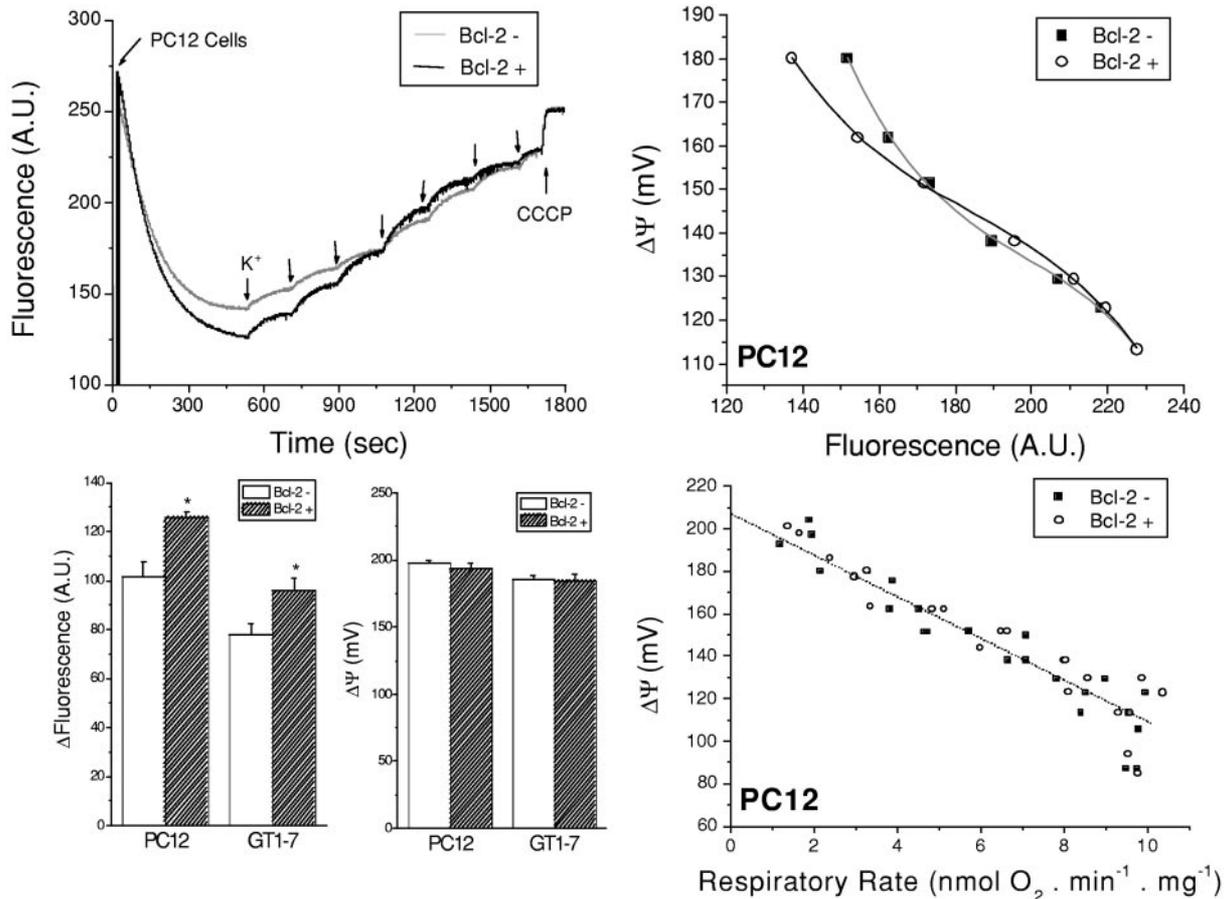


FIG. 1. **Bcl-2 overexpression increases safranin quenching but not mitochondrial $\Delta\Psi$.** In the *top panels*, 10 mg/ml PC12 cells were incubated in 250 mM sucrose, 5 mM pyruvate, 5 mM malate, 5 mM glutamate, 100 μ M EGTA, 1 mg/ml bovine serum albumin, 0.004% digitonin, 5 μ M safranin O, 1 μ g/ml valinomycin, and 10 mM Hepes, pH 7.2 (NaOH), and safranin fluorescence was measured as described under "Experimental Procedures" in the presence of increasing concentrations of K^+ (0.15, 0.3, 0.45, 0.75, 1.05, 1.35, and 1.95 mM) and 5 μ M CCCP, added where indicated. The respective $\Delta\Psi$ for each K^+ concentration was calculated using the Nernst equation (see "Experimental Procedures"), and the best fitting for the fluorescence *versus* $\Delta\Psi$ plot (*upper right*) was used to estimate $\Delta\Psi$ in the absence of added K^+ for both PC12 and GT1-7 cells incubated under similar conditions (*lower left*). Δ Fluorescence represents fluorescence readings in the presence of CCCP minus readings without added K^+ . In the *lower right panel*, PC12 respiratory rates and $\Delta\Psi$ (estimated through calibrated safranin fluorescence changes) were measured in parallel and plotted against each other under conditions similar to those described above in which K^+ (0.15–1.95 mM) and CCCP (0.1–5 μ M) concentrations were varied. *, $p < 0.05$ when compared with Bcl-2⁻ cells. A.U., arbitrary units.

mined using the Biuret method. All experiments were conducted at 37 °C.

Mitochondrial Membrane Potential ($\Delta\Psi$)—Mitochondrial $\Delta\Psi^1$ was estimated through fluorescence changes of safranin O (5 mM) at excitation and emission wavelengths of 485 and 586 nm, respectively (10, 18). Data were calibrated using a K^+ gradient as described by Akerman and Wikstrom (19), and the membrane potential obtained for each K^+ concentration was determined using the Nernst equation assuming intramitochondrial $[K^+]$ to be 150 mM, a value quite close to the experimentally determined $[K^+]$ in GT1-7 cells (see Fig. 3). A calibration curve was constructed and fitted using Origin[®] software, and all subsequent fluorescence traces were transformed into $\Delta\Psi$ using the same fitting equation.

Intramitochondrial pH—Cells (10 mg/ml) were suspended in medium containing 10 μ M BCECF-AM, 250 mM sucrose, 5 mM pyruvate, 5 mM malate, 5 mM glutamate, 100 μ M EGTA, 1 mg/ml bovine serum albumin, 0.001% or 0.004% digitonin (GT1-7 and PC12 cells, respectively), and 10 mM Hepes, pH 7.2 (KOH) and incubated at 25 °C for 20 min. The permeabilized cells with BCECF-loaded mitochondria were then diluted to 2 mg/ml in 4 °C buffer devoid of BCECF, centrifuged, and resuspended in the same medium. BCECF fluorescence emission was measured at 550 nm with variable excitation wavelengths. Intramitochondrial pH was calculated from the ratio between fluorescence

levels at 509 and 450 nm as described by Molecular Probes and Jung *et al.* (20, 21). Cells were treated with CCCP and nigericin (1 μ M), and the extracellular medium pH (measured using a standard pH meter) was manipulated between 7 and 8 by adding HCl and KOH. A plot relating the measured pH to the 509/450 nm fluorescence ratio was used to determine intramitochondrial pH in the absence of ionophores. All experiments were conducted within 30 min of mitochondrial loading with BCECF.

Intramitochondrial $[K^+]$ —Cells (10 mg/ml) were suspended in medium containing 20 μ M PBFI-AM (a K^+ indicator marketed by Molecular Probes) and treated in the same manner as those loaded with BCECF. PBFI fluorescence emission was measured at 500 nm with variable excitation wavelengths. Intramitochondrial $[K^+]$ was calculated from the ratio between fluorescence levels at 320 and 360 nm as described by Molecular Probes and Jung *et al.* (20, 21). Cells were treated with CCCP and nigericin (1 μ M), and spectra were collected in the presence of varying medium $[K^+]$ (50–200 mM). A plot relating $[K^+]$ to the fluorescence ratio was used to determine intramitochondrial $[K^+]$ in the absence of ionophores.

Mitochondrial Isolation—Mitochondria were isolated from digitonin-permeabilized GT1-7 and PC12 cells exactly as described by Moreadith and Fiskum (22) in isolation buffer containing 210 mM mannitol, 75 mM sucrose, 1 mg/ml bovine serum albumin, 5 mM Hepes, and 1 mM EGTA, pH 7.2 (KOH).

Mitochondrial Particle Sizing and Light Scattering—Isolated mitochondria (~0.2 mg/ml) were incubated in 250 mM sucrose, 10 mM Hepes, 100 μ M EGTA, pH 7.2 (KOH), containing 1 μ M rotenone and 5 mM K^+ succinate. The suspension was analyzed by a Becton Dickinson FACS-

¹ The abbreviations used are: $\Delta\Psi$, membrane potential; AM, acetoxyethyl ester; BCECF, 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FSC, forward scattering; SSC, side scattering.

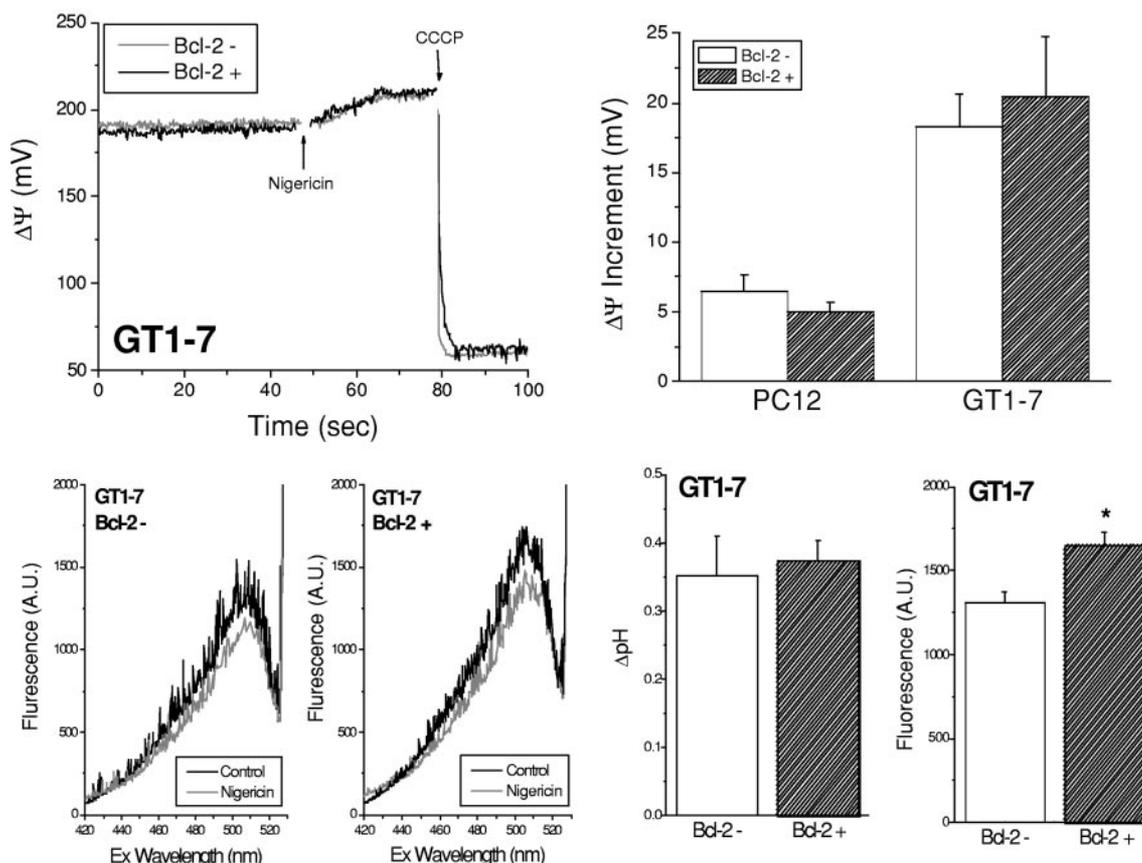


FIG. 2. **Bcl-2 overexpression does not alter mitochondrial ΔpH .** In the *top panels*, digitonin-permeabilized cells were incubated under the same conditions as in Fig. 1 except no valinomycin was added, and the pH of the reaction medium was adjusted using KOH. $\Delta\Psi$ was measured before and after the addition of 50 nM nigericin and 5 μ M CCCP as shown. The average increment in $\Delta\Psi$ observed after the addition of nigericin in three different experiments is shown at the *top right*. In the *bottom panels*, GT1-7 cells with BCECF-loaded mitochondria (see "Experimental Procedures") were used. Fluorescence spectra of these cells are shown at the *bottom left* in the presence and absence of 50 nM nigericin. ΔpH values and fluorescence at excitation (E_x) = 500 nm were calculated for three similar determinations with each cell type (*lower right*) as described under "Experimental Procedures." *, $p < 0.05$ when compared with Bcl-2⁻ cells. A.U., arbitrary units.

Calibur flow cytometer, and detected with a 488 nm laser. Particle size (forward scattering (FSC)) and light scattering (side scattering (SSC)) characteristics were analyzed using CellQuest software.

Reagents—BCECF-AM and PBF1-AM were purchased from Molecular Probes. Safranin O, EGTA, digitonin, malate, glutamate, pyruvate, bovine serum albumin, CCCP, nigericin, and valinomycin were from Sigma.

Data Analysis—Data presented as traces are representative of at least three similar repetitions. Averages represented in bar graphs were calculated from data collected in at least three repetitions using different cell preparations. Error bars indicate standard errors (S.E.), and significant differences were calculated using pairwise Tukey tests conducted by SigmaStat®.

RESULTS

Previous studies have shown that Bcl-2 overexpression causes an increase in the uptake of fluorescent dyes sensitive to the mitochondrial $\Delta\Psi$, a result interpreted as an increase in $\Delta\Psi$ induced by this protein (9, 10). This $\Delta\Psi$ effect could explain many changes observed in Bcl-2-overexpressing mitochondria, including increased reactive oxygen species generation (14–16), enhanced Ca^{2+} uptake capacity, and larger quantities of reduced pyridine nucleotides (10, 14, 23). To understand the mechanism through which Bcl-2 apparently enhances $\Delta\Psi$, we performed measurements of mitochondrial uptake of the $\Delta\Psi$ -sensitive probe safranin O and calibrated the data by using K^+ gradients and applying the Nernst equation (see "Experimental Procedures" and Ref. 19). These experiments were conducted using cultured cells in which low digitonin concentrations were added to selectively permeabilize the plasma

membrane, promoting a large dilution of cytosolic components while maintaining cell architecture and mitochondrial function unaltered (18). This is the preferred method to study the effects of Bcl-2 in mitochondria from transfected cell lines since mitochondrial isolation may promote damage to the organelle in a Bcl-2-inhibited manner (18). As noted previously (9, 10), PC12 pheochromocytoma cells overexpressing human Bcl-2 (Bcl-2+ cells) decrease safranin fluorescence more intensely than control transfectant cells (Bcl-2-) and present an enhanced difference in fluorescence in the presence and absence of the proton ionophore CCCP (Δ Fluorescence) when respiring on NADH-linked substrates (Fig. 1, *upper* and *lower left*), an effect compatible with a higher $\Delta\Psi$. A similar increase in safranin Δ Fluorescence was observed in a second transfected cell line (GT1-7 hypothalamic tumor cells; Fig. 1, *lower left*). However, when we calibrated $\Delta\Psi$ using a K^+ distribution curve (Fig. 1, *upper panels*, see "Experimental Procedures" and Ref. 19), we found that Bcl-2+ mitochondria presented larger safranin responses to equal $\Delta\Psi$ changes (more change in fluorescence with equal K^+ additions). By using the best fittings for the fluorescence *versus* $\Delta\Psi$ plots (Fig. 1, *upper right*), we were able to estimate Bcl-2- and Bcl-2+ $\Delta\Psi$ in the absence of added K^+ and found these to be equal in both cell lines studied (Fig. 1, *lower left*). Thus, Bcl-2 increases safranin fluorescence changes dependent on $\Delta\Psi$, but this effect seems to be related to an altered calibration curve and not enhanced $\Delta\Psi$.

We also determined mitochondrial respiratory rates in Bcl-2+ and Bcl-2- cells (Fig. 1, *lower right*) and plotted them

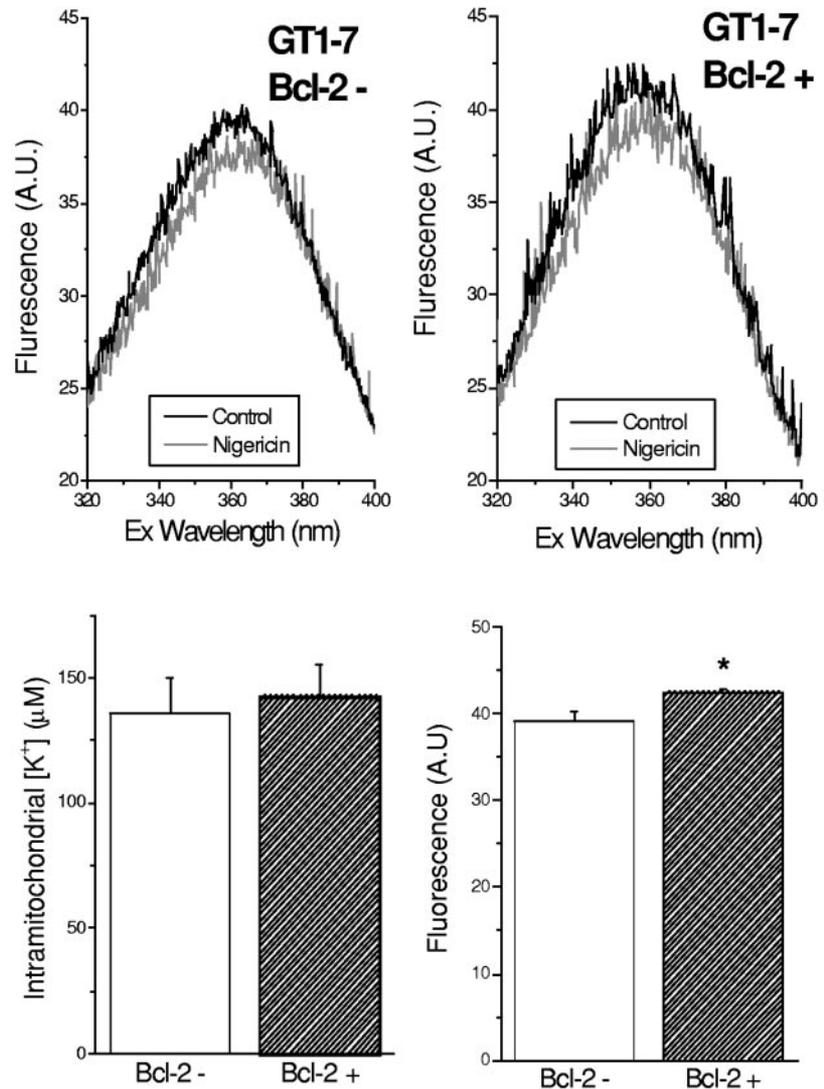


FIG. 3. **Bcl-2 overexpression does not alter intramitochondrial $[K^+]$.** GT1-7 cells with PBFI-loaded mitochondria (see “Experimental Procedures”) were used. Fluorescence spectra of these cells are shown in the *top panels* in the presence and absence of 50 nM nigericin. Intramitochondrial $[K^+]$ values and fluorescence at excitation (Ex) = 368 nm were calculated for three similar determinations with each cell type as described under “Experimental Procedures” (*lower panels*). *, $p < 0.05$ when compared with Bcl-2 $-$ cells. A.U., absorbance units.

against the measured membrane potential in the presence of increasing K^+ and CCCP concentrations. A change in the linear $\Delta\Psi$ /respiration plot would be indicative of altered proton pumping/oxygen consumption ratios at the mitochondrial respiratory chain as proposed previously to explain the apparent higher $\Delta\Psi$ in Bcl-2+ mitochondria (9). We found that Bcl-2 does not change the correlation between mitochondrial oxygen consumption and H^+ pumping, a result which supports the finding that mitochondrial $\Delta\Psi$ is equal in Bcl-2+ and Bcl-2 $-$ cells.

Safranin is a lipophilic cation that accumulates within or in near proximity to the mitochondrial inner membrane, reducing the fluorescence of the suspension in a manner proportional to the negative charge of the mitochondrial matrix (19). Thus, safranin fluorescence traces measure only changes in charge across the inner membrane and are insensitive to a second component of the mitochondrial H^+ gradient, ΔpH . In addition, the estimated $\Delta\Psi$ calculated using the Nernst equation in Fig. 1 assumes intramitochondrial K^+ concentrations to be ~ 150 mM and equal in Bcl-2+ and Bcl-2 $-$ cells. To ascertain that Bcl-2 affects safranin distribution and not the mitochondrial proton gradient, we measured both ΔpH and K^+ concentrations in Bcl-2+ and Bcl-2 $-$ mitochondria.

The experiments shown in Fig. 2 compare ΔpH levels in Bcl-2 $-$ and Bcl-2+ mitochondria. We found that the addition of

nigericin, a K^+/H^+ exchanger that reduces ΔpH and increases $\Delta\Psi$, promotes very similar effects on $\Delta\Psi$ measured by calibrated safranin fluorescence in Bcl-2 $-$ and Bcl-2+ mitochondria (Fig. 2, *upper panels*). To confirm that Bcl-2 did not affect ΔpH , we loaded GT1-7 mitochondria with the esterified form of the pH-sensitive dye BCECF. PC12 mitochondria were not used in this experiment since they loaded very poorly with this dye, and the final fluorescence levels were insufficient to accurately estimate ΔpH . In GT1-7 Bcl-2+ cells, BCECF fluorescence was more intense and responded more significantly to the addition of nigericin than that in Bcl-2 $-$ cells (Fig. 2, *bottom left*). However, by calibrating the fluorescence traces (see “Experimental Procedures”), we found no difference in intramitochondrial pH levels in Bcl-2 $-$ and Bcl-2+ mitochondria despite a consistently higher BCECF load (Fig. 2, *bottom right*). These results indicated that, although GT1-7 mitochondria are more intensely loaded with BCECF, there is no difference in ΔpH between Bcl-2 $-$ and Bcl-2+ mitochondria.

Intramitochondrial K^+ levels were determined in Fig. 3 by loading mitochondria with the K^+ probe PBFI-AM. Again PC12 cells loaded very poorly with the dye, so only GT1-7 cells were used. We found that, although Bcl-2+ mitochondria loaded more dye and presented more intense fluorescence (Fig. 3, *upper panels* and *lower right*), no difference in intramitochondrial K^+ concentrations could be detected when the data were

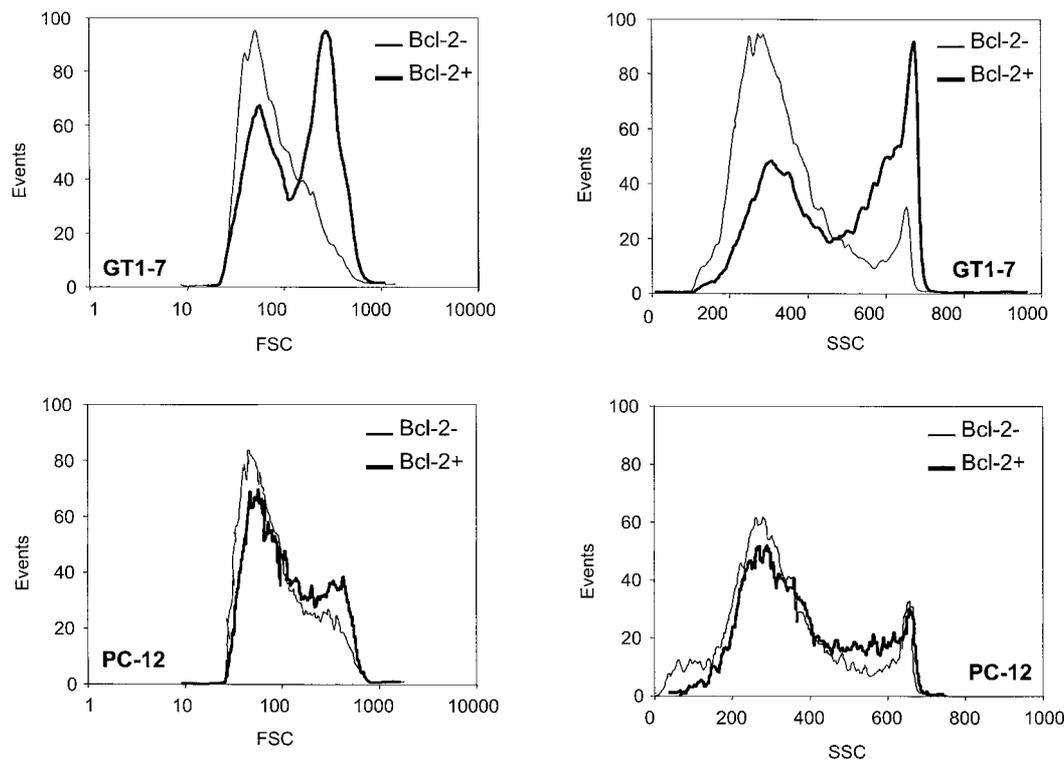


FIG. 4. **Bcl-2 increases mitochondrial volume and structural complexity.** Particle size, measured by FSC, and particle complexity, measured by SSC, of mitochondria isolated from Bcl-2⁻ and Bcl-2⁺ cells were determined using flow cytometry as described under "Experimental Procedures."

calibrated (*lower left*). Indeed, intramitochondrial K⁺ concentrations determined using PBF1 were quite close to the estimated K⁺ concentrations used to calibrate $\Delta\Psi$ determinations in Figs. 1 and 2, ensuring the accuracy of our $\Delta\Psi$ estimation.

In the absence of any difference in $\Delta\Psi$, ΔpH , or intramitochondrial [K⁺], the increased fluorescence response to three different dyes observed in the Bcl-2⁺ mitochondria suggests the presence of a larger mitochondrial membrane surface (to increase safranin distribution since safranin accumulates in close contact to or within the inner membrane, Ref. 19) and matrix volume (to increase intramitochondrial BCECF and PBF1 accumulation). To investigate this surprising possibility, we isolated mitochondria from GT1-7 and PC12 Bcl-2⁻ and Bcl-2⁺ cells and evaluated their size and structural complexity using flow cytometry (Fig. 4).

FSC measurements using a flow cytometer can be used to estimate particle size since the intensity of light scattered at small angles from an incident laser beam is proportional to particle volume as demonstrated by Mullaney *et al.* (24). In the *top panels* of Fig. 4, we compared FSC in Bcl-2⁻ and Bcl-2⁺ mitochondria isolated from GT1-7 cells. We found that Bcl-2⁺ mitochondria present two populations of distinct sizes and that the average particle size of these mitochondria is larger than Bcl-2⁻ mitochondria (Fig. 4, *top left*). In addition, flow cytometry can determine mitochondrial structural complexity as measured by particle light scattering (SSC), which is dependent on the refractive index of each particle. Side scattering measurements show that GT1-7 Bcl-2⁺ mitochondria present increased structural complexity in relation to Bcl-2⁻ mitochondria (Fig. 4, *top right*). In PC12 cells, mitochondrial volume and complexity increases in Bcl-2⁺ cells were less pronounced but still evident (Fig. 4, *lower panels*). The increase in both mitochondrial size and complexity, as determined by increases in forward and side scattering, excludes the possibility that the difference between Bcl-2⁺ and Bcl-2⁻ mitochondria is due to

membrane damage promoted by mitochondrial isolation since mitochondria with more permeable membranes present increased size and decreased light scattering (25). Thus, in two distinct cell lines, Bcl-2 overexpression enhanced the mean size and complexity of mitochondria. The enhanced size and structural complexity of Bcl-2⁺ mitochondria explain why these organelles present larger responses to fluorescent dyes without changes in the mitochondrial function these dyes measure.

DISCUSSION

In this study, we examined the effects of Bcl-2 overexpression on mitochondrial energetics and structure using two unrelated *bcl-2*-transfected cell lines. We found that Bcl-2⁺ mitochondria, previously thought to present higher $\Delta\Psi$ due to increased uptake of membrane potential probes such as safranin and rhodamine 123 (9, 10), do not show any difference in $\Delta\Psi$ when this measurement is calibrated using K⁺ gradients (Fig. 1). Instead Bcl-2⁺ mitochondria present a larger ability to promote fluorescence changes not only of the membrane potential probe safranin but also of pH- and K⁺-sensitive probes BCECF and PBF1 (Figs. 2 and 3) without apparent changes in $\Delta\Psi$, ΔpH , or intramitochondrial [K⁺]. This finding indicates that studies comparing non-calibrated fluorescence responses in cell lines with different Bcl-2 expression levels may, in fact, misinterpret fluorescence signals and should be carefully reevaluated.

Previous studies involving isolated mitochondria suggest that the increase in response promoted by Bcl-2 in fluorescence $\Delta\Psi$ measurements is not due to a larger number of mitochondria in Bcl-2-overexpressing cells (26), a result supported by the fact that no difference in respiratory activity can be measured between Bcl-2⁺ and Bcl-2⁻ cells (Fig. 1, *lower left*). This observation suggests that Bcl-2⁺ mitochondria present both a more extensive membrane surface, interacting more intensely with membrane-accumulated probes such as safranin, and

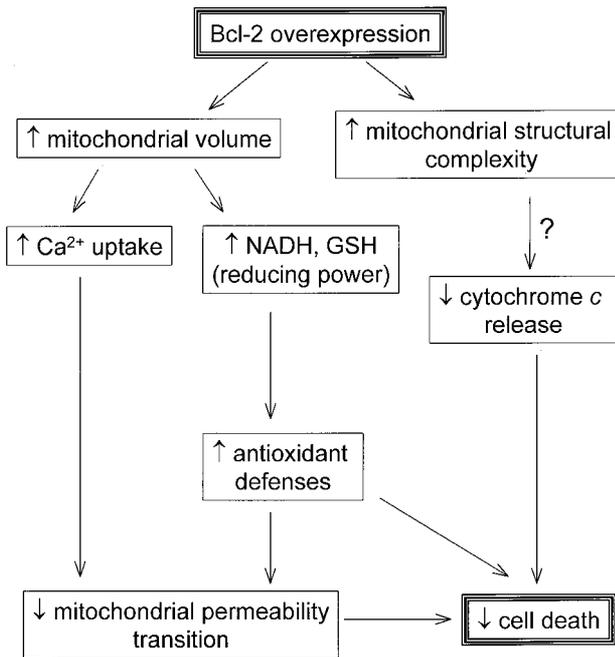


FIG. 5. Proposed model of Bcl-2-promoted mitochondrial alterations leading to the prevention of cell death. Bcl-2 increases mitochondrial volume, leading to greater Ca^{2+} accumulation capacity and enhanced levels of NADPH and GSH, which increase mitochondrial antioxidant capacity. These effects can prevent mitochondrial permeability transition-dependent and -independent cell death. In addition, more structurally complex mitochondria in Bcl-2-overexpressing cells may present more inner membrane folds and release less cytochrome *c*, avoiding cytochrome *c*-triggered apoptosis or cell death resulting from energetic depletion.

larger matrix volumes to enhance the accumulation of intramitochondrial probes such as BCECF and PBFI. These differences in mitochondrial volume and membrane content were confirmed by using a flow cytometer to measure particle size (forward scattering) and structural complexity (side scattering) of isolated Bcl-2+ and Bcl-2- mitochondria (Fig. 4). The exact nature of the structural changes present in Bcl-2+ mitochondria is still not clear and will have to be investigated using three-dimensional imaging techniques since conventional electron microscopy does not show any striking differences between Bcl-2- and Bcl-2+ mitochondrial morphology (10).

Independently of the exact nature of the structural alterations promoted by Bcl-2, our data using both fluorescent dyes and light scattering of individual mitochondria through flow cytometry clearly indicate that Bcl-2+ mitochondria are larger. The presence of larger mitochondria and, most probably, larger matrix volumes may explain why Bcl-2-overexpressing mitochondria have been previously found to present a larger capacity to accumulate Ca^{2+} ions (Ref. 26, and see the scheme in Fig. 5) independently of their increased resistance to undergo non-selective inner membrane permeabilization following excessive Ca^{2+} uptake (mitochondrial permeability transition, Refs. 10 and 11). It is also possible that the increased structural complexity of Bcl-2+ mitochondria is related to changes in membrane structure such as increases in cristal folds, resulting in resistance to cytochrome *c* loss under conditions in which the outer membrane is permeabilized (27). Cytochrome *c* normally

interacts closely with the inner membrane and must be displaced to the intermembrane space to be released into the cytosol (28). Finally, larger matrix volumes may explain why Bcl-2+ cells present higher quantities of matrix-soluble components such as NADPH, NADH, and glutathione (10, 14, 23). The antioxidant effects of NADPH and GSH are related to the increased resistance Bcl-2+ cells present to oxidative damage (10, 14, 16, 23).

In summary, we found that Bcl-2 does not affect mitochondrial $\Delta\Psi$, ΔpH , or intramitochondrial K^{+} concentrations but alters mitochondrial structure, resulting in increased size and complexity. These changes are accompanied by an enhancement in the response to fluorescent dyes, including those that measure mitochondrial $\Delta\Psi$. Enhanced volume and structural complexity may affect the response presented by Bcl-2+ cells to normally deadly stimuli, inhibiting apoptosis and necrosis by preventing cytochrome *c* release, increasing Ca^{2+} uptake capacity, and enhancing antioxidant defenses (see Fig. 5 for a proposed model).

Acknowledgments—We acknowledge Edson Alves Gomes for excellent technical assistance and Prof. A. E. Vercesi for stimulating discussions and for allowing ready access to the flow cytometer. Dr. A. Starkov and Prof. E. J. Bechara are thanked for critical reading of the manuscript.

REFERENCES

1. Tsujimoto, Y., Ikegaki, N., and Croce, C. M. (1987) *Oncogene* **2**, 3–7
2. Reed, J. C. (1995) *Curr. Opin. Oncol.* **7**, 541–546
3. Adams, J. M., and Cory, S. (2001) *Trends Biochem. Sci.* **26**, 61–66
4. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) *Science* **262**, 1274–1277
5. Kroemer, G., Dallaporta, B., and Resche-Rigon, M. (1998) *Annu. Rev. Physiol.* **60**, 619–642
6. Monaghan, P., Robertson, D., Amos, T. A., Dyer, M. J., Mason, D. Y., and Greaves, M. F. (1992) *J. Histochem. Cytochem.* **40**, 1819–1825
7. Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., and Reed, J. C. (1993) *Cancer Res.* **53**, 4701–4714
8. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. (1996) *J. Exp. Med.* **183**, 1533–1544
9. Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H., and Tsujimoto, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 1455–1459
10. Kowaltowski, A. J., Vercesi, A. E., and Fiskum, G. (2000) *Cell Death Differ.* **7**, 903–910
11. Kowaltowski, A. J., Castilho, R. F., and Vercesi, A. E. (2001) *FEBS Lett.* **495**, 12–15
12. Polster, B. M., Kinnally, K. W., and Fiskum, G. (2001) *J. Biol. Chem.* **276**, 37887–37894
13. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4997–5002
14. Esposti, M. D., Hatzinisiriou, I., McLennan, H., and Ralph, S. (1999) *J. Biol. Chem.* **274**, 29831–29837
15. Steinman, H. M. (1995) *J. Biol. Chem.* **270**, 3487–3490
16. Armstrong, J. S., and Jones, D. P. (2002) *FASEB J.* **16**, 1263–1265
17. Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997) *FEBS Lett.* **416**, 15–18
18. Fiskum, G., Kowaltowski, A. J., Andreyev, A. Y., Kushnareva, Y. E., and Starkov, A. A. (2000) *Methods Enzymol.* **322**, 222–234
19. Akerman, K. E., and Wikstrom, M. K. (1976) *FEBS Lett.* **68**, 191–197
20. Jung, D. W., Apel, L., and Brierley, G. P. (1990) *Biochemistry* **29**, 4121–4128
21. Jung, D. W., Baysal, K., and Brierley, G. P. (1995) *J. Biol. Chem.* **270**, 672–678
22. Moreadith, R. W., and Fiskum, G. (1984) *Anal. Biochem.* **137**, 360–367
23. Ellerby, L. M., Ellerby, H. M., Park, S. M., Holleran, A. L., Murphy, A. N., Fiskum, G., Kane, D. J., Testa, M. P., Kayalar, C., and Bredesen, D. E. (1996) *J. Neurochem.* **67**, 1259–1267
24. Mullaney, P. F., Van Dilla, M. A., Coulter, J. R., and Dean, P. N., (1969) *Rev. Sci. Instrum.* **40**, 1029–1032
25. Garlid, K. D., and Beavis, A. D. (1985) *J. Biol. Chem.* **260**, 13434–13441
26. Murphy, A. N., Bredesen, D. E., Cortopassi, G., Wang, E., and Fiskum, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 9893–9898
27. Green, D. R., and Reed, J. C. (1998) *Science* **281**, 1309–1312
28. Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 1259–1263