Modulation of Mitochondrial Ca\textsuperscript{2+} Homeostasis by Bcl-2\textsuperscript{*}

Liping Zhu\textsuperscript{+,} Song Ling\textsuperscript{+,} Xiao-Dan Yu\textsuperscript{+,} L. K. Venkatesh\textsuperscript{§}, T. Subramanias\textsuperscript{§}, G. Chinnadurai\textsuperscript{§}, and Tuan H. Kuo\textsuperscript{++,}\textsuperscript{†}

From the \textsuperscript{§}Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201 and the \textsuperscript{++}Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110

We have investigated the role of mitochondrial Ca\textsuperscript{2+} (Ca\textsubscript{m}) homeostasis in cell survival. Disruption of Ca\textsubscript{m} homeostasis via depletion of the mitochondrial Ca\textsuperscript{2+} store was the earliest event that occurred during staurosporine-induced apoptosis in neuroblastoma cells (SH-SY5Y). The decrease of Ca\textsubscript{m} preceded activation of the caspase cascade and DNA fragmentation. Overexpression of the anti-apoptosis protein Bel-2 led to increased Ca\textsubscript{m} load, increased mitochondrial membrane potential (\(\Delta \Psi \textsubscript{m}\)), and inhibition of staurosporine-induced apoptosis. On the other hand, ectopic expression of the pro-apoptotic protein Bik led to decreased Ca\textsubscript{m} load and decreased \(\Delta \Psi \textsubscript{m}\). Inhibition of calcium uptake into mitochondria by ruthenium red induced a dose-dependent apoptosis as determined by nuclear staining and DNA ladder assay. Similarly, reducing the Ca\textsubscript{m} load by lowering the extracellular calcium concentration also led to apoptosis. We suggest that the anti-apoptotic effect of Bel-2 is related to its ability to maintain a threshold level of Ca\textsubscript{m} and \(\Delta \Psi \textsubscript{m}\) while the pro-apoptotic protein Bik has the opposite effect. Furthermore, both ER and mitochondrial Ca\textsuperscript{2+} stores are important, and the depletion of either one will result in apoptosis. Thus, our results, for the first time, provide evidence that the maintenance of Ca\textsubscript{m} homeostasis is essential for cell survival.

* This work was supported by National Institutes of Health Grants HL-39481 (to T. H. K.) and CA33616 (to G. C.) and by a grant-in-aid from the American Heart Association of Michigan (to T. H. K.). 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: Dept. of Pathology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201. Tel.: 313-577-1131; Fax: 313-577-0057; E-mail: tkuo@med.wayne.edu.

The abbreviations used are: ER, endoplasmic reticulum; Ca\textsubscript{m}, mitochondrial Ca\textsuperscript{2+}; Ca\textsubscript{c}, cytoplasmic Ca\textsuperscript{2+}; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Tg, thapsigargin; SR, sarcoplasmic reticulum; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; STS, staurosporine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; \(\Delta \Psi \textsubscript{m}\), mitochondrial membrane potential; MPT, mitochondrial permeability transition; TMRM, tetramethylrhodamine methyl ester.

This paper is available on line at http://www.jbc.org

MATERIALS AND METHODS

Cells and Viruses—SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 25 \(\mu\)g/ml gentamycin at 37 °C. Recombinant adenovirus type 5 expressing human Bcl-2 and Escherichia coli LacZ have been described (20). Construction of recombinant adenovirus expressing human Bik tagged with HA
33268

Mitochondrial Ca\(^{2+}\) Homeostasis and Bcl-2

epitope will be described elsewhere. To overexpress Bcl-2, LacZ, or Bik, cells were infected with the appropriate virus at 100 plaque-forming units/cell in DMEM with 5% serum. After 6 h of infection, the medium was replaced with DMEM growth medium containing 10% serum. Cells were used 24 h later after virus infection. The expression of the Bcl-2 or HA-Bcl-2 was verified by Western blot analysis.

Western Analysis—For detecting Bcl-2 or Bik protein expression, 70–80% confluent cells were lysed and protein samples were separated on 12% polyacrylamide gel for Western blotting as described previously (18). The Bcl-2 antibody was obtained from Dako. A monoclonal antibody 12CA5 that recognizes HA epitope was used for Bik detection.

Measurement of Mitochondrial Membrane Potential—Mitochondrial membrane potential (DCM) was measured using fura-2. SH-SY5Y cells grown on coverslip (approximately 5 × 10\(^4\) cells/coverslip) were loaded with Fura2 (4 μM) in loading buffer A containing (in mM) 5.4 KCl, 137 NaCl, 0.44 KH\(_2\)PO\(_4\), 4.2 NaHCO\(_3\), 0.34 Na\(_2\)HPO\(_4\), 1 MgCl\(_2\), 5 Hepes (pH 7.4), 11.1 μg/mL bovine serum albumin. Fura 2 fluorescence was recorded at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. To begin the fluorescence measurement, cells were rinsed after the dye loading in loading buffer A without Ca\(^{2+}\). After establishing the baseline, cells were then treated with either ionomycin (10 μM) plus 0.1 mM EGTA or thapsigargin (1 μM) plus 0.1 mM EGTA. A standard curve was used to convert the fluorescence ratio to Ca\(^{2+}\) concentration.

Measurement of Mitochondrial Ca\(^{2+}\) Concentration—Rhod2-AM was used to measure mitochondrial Ca\(^{2+}\) according to the procedure of Hajnoczky et al. (14). Rhod2-AM has a net positive charge, which facilitates its sequestration into mitochondria due to membrane potential-driven uptake. The use of dihydro-rhod-2-AM enhances the selectivity for mitochondrial loading because this dye exhibits Ca\(^{2+}\)-dependent fluorescence only after it is oxidized and this occurs preferentially within mitochondria. Cells were loaded with 4 μM dihydro-rhod2-AM for 60 min. The residual cytosolic fraction of the dye was eliminated when the cells were kept in culture for an additional 18 h after loading, whereas the mitochondrial dye fluorescence was maintained. Cellular fluorescence was measured by the Photon Technology International system with excitation at 550 nm and emission at 580 nm or by image acquisition using the Meridian laser confocal microscope. The fluorescence units (counts/s) or the average pixel intensity/cell was determined. Rhod2 fluorescence was not calibrated in terms of [Ca\(^{2+}\)]\(_i\), since it is not a radiometric dye and is localized in only a small compartment within the cell (14).

Measurement of Mitochondrial Membrane Potential—The dye tetra-methylrhodamine methyl ester (TMRRM) was used to measure mitochondrial membrane potential (ΔΨ\(_m\)) according to Ichas et al. (22). Cells infected with recombinant adenovirus (for 24 h) were loaded with 0.2 μM TMRRM for 20 min in culture medium. Cells were then rinsed and transferred to a chamber on the microscope stage in the same loading buffer A (containing glucose and no Ca\(^{2+}\)) as the Fura-2 experiments. Images were collected at every 10 s using the Meridian ACS laser confocal microscope with excitation at 514 nm and emission at 575 nm, and the average intensity/cell was determined.

Apoptotic Nuclei—Cells were fixed in 4% paraformaldehyde and stained with the DNA-binding dye Hoechst 33258 according to Jacobson and Raff (23). Apoptotic nuclei were visualized and counted under epifluorescence illumination (340 nm excitation and 510 nm barrier filter) using a 40× oil immersion objective (more than 200 cells/culture determined by Student’s t test. A value of p < 0.05 was considered to be significant.

RESULTS

Increased Capacity of Internal Ca\(^{2+}\) Store by Bcl-2—We have examined the effect of Bcl-2 overexpression on the Ca\(^{2+}\) load of the internal store in the neuroblastoma cells (SH-SY5Y). For this purpose, cells infected with recombinant adenovirus expressing Bcl-2 (Ad-Bcl2) were compared with cells infected with a recombinant adenovirus expressing E. coli LacZ (Ad-LacZ). Fig. 1A shows the Western analysis of Bcl-2 protein in the cell lysate prepared 24 h after infection. Results from three experiments indicated that there was a low level of endogenous Bcl-2 present in control cells infected with Ad-LacZ, while the level of Bcl-2 was enhanced by more than 2-fold in cells infected with Ad-Bcl2. To estimate the loading state of the internal stores, Bcl-2 overexpressing cells and control cells were treated with ionomycin, a calcium ionophore that releases Ca\(^{2+}\) from the ER and the mitochondrial stores. After reaching a peak within 60 s, the Ca\(^{2+}\) started to decline to near basal level. Fig. 1B shows that the resting level of Ca\(^{2+}\) was similar for Bcl-2 expressing cells and control cells (ranging from 66 ± 2.4 nM for control, and 70 ± 6.5 nM for Bcl-2 cells, n = 5). However, the ionomycin-induced release of Ca\(^{2+}\) was significantly higher in Bcl-2 expressing cells (151 ± 22 nM) as compared with controls (87 ± 9.7, p < 0.001, n = 5). The results suggest increased loading of the internal store in the Bcl-2 overexpressing cells. Since the ionomycin-sensitive stores encompass both...
the ER and the mitochondria compartments, we then examined the effect of Bcl-2 on the Ca\(^{2+}\) load of the ER store, which is sensitive to thapsigargin (Tg, 1 \(\mu\)M), a specific inhibitor of the ER calcium ATPase. Fig. 1C shows that Tg induced a larger increase of \(C_{\text{a}}\) in Bcl-2 overexpressing cells than controls. Results from five independent experiments indicated that the increase of Ca\(^{2+}\) was 90 \(\pm\) 7.8 nm for Bcl-2 and 73 \(\pm\) 4.6 nm for controls (\(p < 0.01\)). These results are consistent with our previous report of enhanced ER Ca\(^{2+}\) load in the breast epithelial cells by Bcl-2 overexpression (18).

**Increased Loading of the Mitochondrial Ca\(^{2+}\) Store by Bcl-2**—To further test if the mitochondria Ca\(^{2+}\) store is also increased by Bcl-2, we evaluated the mitochondrial store in SH-SY5Y cells infected with Ad-Bcl2 or Ad-LacZ by using rhod2 as the specific probe for mitochondria Ca\(^{2+}\) (see “Materials and Methods”).}

**FIG. 2.** Increased loading of the mitochondrial Ca\(^{2+}\) store by Bcl-2. SH-SY5Y cells (5 \(\times\) 10\(^4\) cells) were infected with Bcl-2 adenovirus (Bcl-2) or LacZ virus (Control). A, mitochondrial Ca\(^{2+}\) (\(C_{\text{m}}\)) response induced by FCCP (4 \(\mu\)M) in Bcl-2 versus control cells. Rhod2 was used for the measurement of mitochondrial Ca\(^{2+}\) concentration as described under “Materials and Methods.” B, cytoplasmic Ca\(^{2+}\) (\(C_{\text{c}}\)) response induced by FCCP (4 \(\mu\)M) in Bcl-2 versus control cells. Results are typical of two or more independent experiments.

**FIG. 3.** The dependence of \(C_{\text{a}}\) release on FCCP concentration. SH-SY5Y cells (5 \(\times\) 10\(^4\) cells) were infected with Bcl-2 adenovirus (Bcl-2) or LacZ virus (Control). A and B, \(C_{\text{a}}\) response induced by increasing concentrations of FCCP (4-20 \(\mu\)M) in control and Bcl-2 cells, respectively. C, \(C_{\text{a}}\) response versus FCCP concentration. Results are typical of three or more experiments.

**Mitochondrial Ca\(^{2+}\) Homeostasis and Bcl-2**

The dependence of \(C_{\text{a}}\) release on FCCP concentration—We have determined the optimal concentration of FCCP required for the maximal release of \(C_{\text{a}}\) for Bcl-2 cells and control cells. Fig. 3 (A and B) shows the dose-response experiments of the control and Bcl-2 groups, respectively. Initially, at 4 \(\mu\)M FCCP, the release of \(C_{\text{a}}\) was minimal. Replacement of 4 \(\mu\)M FCCP with 6 \(\mu\)M FCCP led to further release of \(C_{\text{a}}\). Successive additions of higher concentration of FCCP were accompanied by incremental release of \(C_{\text{a}}\) until maximal response was reached. Fig. 3C shows the dependence of \(C_{\text{a}}\) release on FCCP concentration for both Bcl-2 cells and control cells. There was a linear relationship between the FCCP concentration and \(C_{\text{a}}\) release until maximal release was obtained. Results from several independent experiments indicated that the release of Ca\(^{2+}\) required an FCCP concentration of 6 \(\mu\)M, confirming the dependence of \(C_{\text{a}}\) release on FCCP concentration.

In agreement with Fig. 2, there was a larger release of \(C_{\text{a}}\) for Bcl-2 cells than controls at 4 \(\mu\)M of FCCP, confirming the enhanced Ca\(^{2+}\) load in the Bcl-2 cells. The present study of the living cell is consistent with a study of isolated mitochondria showing enhanced calcium uptake by Bcl-2 (26). Because uptake of \(C_{\text{a}}\) by the uniporter is directly proportional to mitochondrial membrane potential (27, 28), the increased \(C_{\text{a}}\) ac-
Mitochondrial Ca\(^{2+}\) Homeostasis and Bcl-2

**Fig. 4. Decreased loading of the mitochondrial Ca\(^{2+}\) store by Bik.** SH-SY5Y cells (5 \(\times\) 10\(^5\) cells) were separately infected with Bcl-2 adenovirus (Bcl-2), LacZ adenovirus (Control), or Bik adenovirus (Bik). A, Western blot showing expression of HA-Bik protein after infection with Bik virus as compared with controls. B, C\(_{am}\) response to 20 \(\mu\)M FCCP in Bcl-2, control, and Bik groups at the single cell level.

Cumulation in the Bcl-2 cells suggests an increased \(\Delta\Psi_m\) in the Bcl-2 mitochondria (to be shown later).

**Decreased Loading of the Mitochondrial Ca\(^{2+}\) Store by Pro-apoptotic Protein Bik**—In order to understand the functional significance of mitochondrial Ca\(^{2+}\) loading, we proceeded to examine the status of the C\(_{am}\) in cells that overexpress Bik, a pro-apoptotic protein that has antagonistic function to Bcl-2 (29). Neuroblastoma cells were infected with the adenovirus vector expressing human Bik insert (see “Materials and Methods”). After 24 h, the cells were lysed and analyzed for Bik expression by Western blot. The results indicated that there was no HA-Bik expression in the control cells (infected with Ad-LacZ) while HA-Bik expression was prominent in cells infected with the Ad-Bik (Fig. 4A). The status of the C\(_{am}\) load was examined in a laser cytometer (Meridian Instrument), where changes of fluorescence image were followed at the single cell level for Bcl-2, control and Bik groups (Fig. 4B). Comparison of basal fluorescence indicated that the average pixel intensity per cell was 822 ± 151 \((n = 13)\) for Bcl-2, 580 ± 110 \((n = 5)\) for control, and 413 ± 100 \((n = 10)\) for Bik-expressing cells. More quantitative evaluation of the C\(_{am}\) load by FCCP-sensitive release (Fig. 4C) also indicated that the addition of 20 \(\mu\)M FCCP caused 14.8 ± 4.5\% \((n = 8)\) of C\(_{am}\) release in Bcl-2, 9.0 ± 1.7\% \((n = 6)\) for control, and 5.1 ± 4\% \((n = 9)\) for Bik cells. Thus the C\(_{am}\) load was highest in the Bcl-2 group, intermediate in the control group, and lowest in the Bik group. Interestingly, this study at the single cell level also revealed that Bcl-2 cells were more resistant to 20 \(\mu\)M FCCP treatment than Bik-expressing cells (compare Bcl-2 panel with the Bik panel in Fig. 4B). While the release of C\(_{am}\) by FCCP was completed in 30 s for the Bik-expressing cells, it required almost 300 s to deplete the C\(_{am}\) in the Bcl-2 cells.

**Association of Mitochondrial Calcium Load with Membrane Potential**—We also measured mitochondrial membrane potential (\(\Delta\Psi_m\)) in cells that overexpress Bcl-2 or Bik. A cationic fluorophore, TMRM, was used as an indicator of mitochondrial membrane potential \(\Delta\Psi_m\) (30, 31). After loading (20 min) and removal of excess TMRM, the fluorescence in the cells were compared at \(t = 0\). There was stronger TMRM fluorescence (warmer pseudocolored image) in a typical Bcl-2-expressing cell than a typical Bik-expressing cell (data not shown), suggesting higher membrane potential in the former than in the latter. Furthermore, the fluorescence was stable in the Bcl-2-expressing cell during the measurement when image scan was continuously monitored at every 10-s interval for 3 min. In contrast, the fluorescence of Bik-expressing cells was continuously declining indicating the loss of \(\Delta\Psi_m\). In fact, it required the addition of oligomycin, a mitochondrial ATPase inhibitor, to prevent the hydrolysis of cellular ATP and thereby stabilize the \(\Delta\Psi_m\) temporarily. Hence, the measurement of TMRM fluorescence in the presence of oligomycin represented an overestimate of \(\Delta\Psi_m\) for the Bik cells. Results from three independent experiments indicated that the Bcl-2 cells had TMRM fluorescence of 719 ± 159 units versus 283 ± 12 units \((n = 3, p < 0.02)\) for the Bik cells. Therefore the \(\Delta\Psi_m\) of the Bcl-2 cells was at least 2.6 ± 0.4-fold higher than Bik cells. Taken together, these experiments indicate that higher \(\Delta\Psi_m\) is associated with increased C\(_{am}\) load in Bcl-2 cells and lower \(\Delta\Psi_m\) is associated with decreased C\(_{am}\) load in Bik cells.

**Mitochondrial Calcium Load Is Decreased by Apoptosis-inducing Agent Staurosporine**—To further demonstrate that C\(_{am}\) load is important for normal mitochondrial function and cell survival, we tested the effect of staurosporine (STS), a commonly used apoptosis inducer. It has been shown in SH-SY5Y cells that treatment with 0.5 \(\mu\)M STS leads to translocation of endogenous Bax from cytosol to mitochondria at 15 min, release of cytochrome \(c\) at 1 h, activation of caspase at 2–4 h, and initiation of apoptosis at 4 h (32). We have also confirmed these observations. In addition, we demonstrate here that STS triggers an immediate release of C\(_{am}\) in these cells (which precedes Bax translocation and cytochrome \(c\) release). Fig. 5A shows that STS caused an immediate release of C\(_{am}\) similar to the effect of FCCP shown in Fig. 2A. The STS-induced C\(_{am}\) release was also concomitant with an increase of C\(_{am}\) (data not shown). Comparing control cells (Fig. 5A) with Bcl-2-overexpressing cells (Fig. 5B) indicated that Bcl-2 cells were more resistant to STS treatment. While it was sufficient to use 0.5 \(\mu\)M STS to completely deplete the C\(_{am}\) store in control cells, the same STS concentration caused only partial depletion of the store in Bcl-2 cells. Presumably this remaining C\(_{am}\) load may be sufficient for cell survival. We have also determined caspase activity using cell extracts that was prepared after 4 h of treatment with 0.5 \(\mu\)M STS (see “Materials and Methods”). Fig. 5C shows that STS treatment for 4 h led to prominent activation of caspase in control cells but no activation in the Bcl-2 cells (data were taken continuously at 30-s intervals during the 30-min assay). The caspase 3-like activity was 1.8 OD/min/mg of cell extract for control cells, while Bcl-2 cells had zero activity. Since at 0.5 \(\mu\)M, STS caused complete depletion of C\(_{am}\) in control cells and only partial depletion in Bcl-2 cells, the results suggest that maintenance of C\(_{am}\) load at a threshold level by Bcl-2 relates to its anti-apoptotic activity.

**The Pro-apoptotic Effect of Ruthenium Red**—To demonstrate a direct relationship between mitochondrial Ca\(^{2+}\) uptake and

---

2 L. Zhu, S. Ling, X.-D. Yu, and T. H. Kuo, unpublished results.
Mitochondrial Ca$^{2+}$ Homeostasis and Bcl-2

FIG. 5. Decreased loading of the mitochondrial Ca$^{2+}$ store by staurosporin. SH-SYSY cells (5 x 10$^6$ cells) were infected with Bcl-2 adenovirus (Bcl-2) or LacZ virus (Control). A, Ca$_{mit}$ response induced by increasing concentrations of STS (0.1–0.8 μM) in control cells. B, Ca$_{mit}$ response induced by increasing concentrations of STS (0.1–1.5 μM) in Bcl-2 cells. The results show that STS induces a complete depletion of Ca$_{mit}$ at 0.5 μM and Bcl-2 group is able to maintain a threshold level of Ca$_{mit}$ at this dose. C, control or Bcl-2-expressing cells were treated with 0.5 μM STS for 4 h. Cell lysates were prepared and assayed for caspase activity. Data were taken at 30-s intervals, and the assay was carried out for 30 min. The results show the inhibition of caspase activity in the Bcl-2 group as compared with controls.

Cell survival, we then treated normal SH-SYSY cells with ruthenium red, an inhibitor of the mitochondrial uniporter. Prior control study has indicated that ruthenium red (25 μM) was effective to block the mitochondrial Ca$^{2+}$ uptake (data not shown). The ability of ruthenium red to promote apoptosis was judged by the Hoechst dye staining of the apoptotic nuclei (see “Materials and Methods”). It should be mentioned that this assay gave a conservative estimate of apoptosis because only cells attached to the coverslip were counted, while the dead cells that became detached from the coverslip were not counted. Results from four independent experiments (Fig. 6A), indicated that while the untreated control group had negligible number of apoptotic nuclei per 200 counted cells (0.4 ± 0.2, n = 10), treatment with ruthenium red (25 μM, 24 h) led to significant increase in the number of apoptotic nuclei (3.3 ± 1.8, p < 0.001, n = 8). This result was confirmed by the DNA ladder assay (Fig. 6B), where increasing ruthenium red concentration from 25 μM to 1 mM produced a dose-dependent increase in the ladder formation as compared with untreated control. These data demonstrate that inhibition of mitochondrial Ca$^{2+}$ uptake by ruthenium red results in efficient apoptosis.

The Effect of Extracellular Calcium on Cell Viability—We reasoned that if depletion of Ca$_{mit}$ leads to cell death, then strategies to increase or maintain the mitochondrial Ca$^{2+}$ store should promote cell survival. Therefore we examined the effect of extracellular calcium (Ca$_{o}$) on cell viability. Cells were grown on coverslips (1 x 10$^5$) in culture medium. For this experiment, the regular growth medium was replaced with serum-free DMEM that contained variable concentration of Ca$_{o}$ from zero to 3 mM. After 6 h or 16 h of incubation, cells were fixed and stained for apoptotic nuclei with the Hoechst 33258 dye. Comparison between two groups was made using 1.8 mM Ca$_{o}$ as the reference because it approximates the normal growth condition. Fig. 7A shows a time-dependent induction of apoptosis when cells were incubated in zero Ca$_{o}$ for 6 h and 16 h. There was a significant number of apoptotic nuclei per 200 counted cells (4.5 ± 1.3 at 6 h, and 9.3 ± 2.9 at 16 h, n = 10, p < 0.001) as compared with that in 1.8 mM Ca$_{o}$ (0.1 ± 0.3 at 6 h, and 0.5 ± 1.0 at 16 h, n = 10). Increase [Ca$_{o}$] to 0.4 mM led to reduced number in apoptotic nuclei (0.9 ± 0.9 at 6 h, and 4.0 ± 1.9 at 16 h, n = 10) which was still significantly higher than the 1.8 mM group (p < 0.001). There was also complete protection of cells grown in medium containing 3 mM Ca$_{o}$ (0.3 ± 0.4 at 6 h, and 0.5 ± 0.8 at 16 h). This result was confirmed by the quantitative analysis of apoptotic cells using a flow cytometric method (data not shown). The results suggest that external calcium (1.8 mM) is important for cell survival. This supposition is consistent with recent reports showing that reduced capacitative calcium entry correlates with apoptosis (33–35). In a separate experiment, the level of mitochondrial calcium (Ca$_{mit}$) in cells treated with various [Ca$_{o}$] was determined using the rhod2 dye. After 16 h of incubation, the Ca$_{mit}$ load was measured as the amount of Ca$^{2+}$ releasable by 20 μM FCCP. The percent of release was calculated to estimate the Ca$_{mit}$ load in these cells. Fig. 7B shows a linear relationship between Ca$_{mit}$ and Ca$_{o}$. The results (Fig. 7, A and B) indicated that both zero and 0.4 mM Ca$_{o}$ led to decreased Ca$_{mit}$ and more reduction of Ca$_{mit}$ is associated with more apoptosis. It is worth mentioning that this experiment does not exclude the possibility that external Ca$^{2+}$ may affect the levels of other internal stores such as ER.
Mitochondrial Ca\(^{2+}\) Homeostasis and Bcl-2

It is now recognized that there are dynamic interactions among various intracellular Ca\(^{2+}\) stores such as mitochondria and ER (36–38). The close physical interaction between the mitochondria and ER network has been demonstrated by using high speed imaging system that allows a three-dimensional fluorescence image of high resolution (37). The consequence of such interaction is the modulation of mitochondrial Ca\(^{2+}\) by ER (38) and the influence of ER Ca\(^{2+}\) release by mitochondria Ca\(^{2+}\) uptake (8, 22). It has been demonstrated in isolated cardiomyocytes that focal SR calcium release results in calcium microdomains sufficient to promote local mitochondrial calcium uptake (38). This has suggested a tight coupling of calcium signaling between SR release sites and nearby mitochondria. Previously, another laboratory and ours have reported the modulation of ER Ca\(^{2+}\) load by Bcl-2 (17, 18). The tight coupling between ER and mitochondria compartments has suggested the coordinated regulation of both the ER Ca\(^{2+}\) and mitochondrial Ca\(^{2+}\) by Bcl-2.

Indeed, we found that ectopic expression of Bcl-2 results in elevated loading of Ca\(^{2+}\) in the mitochondria in addition to enhanced loading of the ER Ca\(^{2+}\) (Figs. 1 and 2). This phenomenon is not restricted to the neuroblastoma cells (the present study) but also true for breast epithelial cells (18) and cardiomyocytes (data not shown). Therefore, the enhanced loading of both mitochondrial and ER Ca\(^{2+}\) stores appears to be a general phenomenon. While we have shown that the increased ER Ca\(^{2+}\) is due to the increased SERCA gene expression by Bcl-2 (18), the reason for the enhanced mitochondrial Ca\(^{2+}\) load is not clear. One possibility is that the Bcl-2-expressing cells have more mitochondria and therefore more Ca\(^{2+}\) uptake. This possibility has been ruled out by the Western blot experiment showing no increase in the cytochrome c protein in the Bcl-2-expressing cells (data not shown). Another possibility is that higher mitochondrial membrane potential in the Bcl-2-expressing cells allows the \(\Delta \Psi_{m}\)-dependent uptake of Ca\(^{2+}\) by the uniporter. Alternatively, the expression of the uniporter gene could be up-regulated by Bcl-2 similar to the up-regulation of SERCA gene expression by Bcl-2 in epithelial cell (18). While this possibility cannot be ruled out, the data in Fig. 3 and 4 are consistent with the interpretation that increased Ca\(_{\text{m}}\) load by Bcl-2 is related to an increase of \(\Delta \Psi_{m}\). Still an even more intriguing possibility is that increased Ca\(_{\text{m}}\) load may be a consequence of increased ER Ca\(^{2+}\) load in the Bcl-2-expressing cells. The tight coupling of Ca\(^{2+}\) signaling between the ER and mitochondria compartment (38) suggest that local Ca\(^{2+}\) release from ER could lead to local Ca\(^{2+}\) uptake into mitochondria. Interestingly, in parallel studies of Bik-expressing cells, we found a decreased ER Ca\(^{2+}\) load as compared with controls (data not shown). This decrease in ER Ca\(^{2+}\) could contribute to the decreased Ca\(_{\text{m}}\) load in Bik cells.

The present study suggests that maintenance of both mitochondrial and ER Ca\(^{2+}\) stores is important for cell survival. It has been well documented that depletion of ER Ca\(^{2+}\) by thapsigargin treatment leads to apoptosis in all cell types tested (33, 34, 39–41). Here we show for the first time that depletion of mitochondrial Ca\(^{2+}\) is the earliest event that occurs during staurosporine-induced apoptosis. The STS-induced efflux of Ca\(_{\text{m}}\) is followed by cytochrome c release at 1 h, caspase activation at 2 h, and initiation of apoptosis at 4 h (Fig. 5 and data not shown). Furthermore, this STS-induced caspase activation and apoptosis in SH-SY5Y cells are inhibited by Bcl-2 (Fig. 5C). A direct relationship between Ca\(_{\text{m}}\) load and apoptosis is demonstrated by the use of ruthenium red, an inhibitor of the uniporter. Blocking Ca\(^{2+}\) uptake into mitochondria by treatment with ruthenium red leads to apoptosis in 24 h (Fig. 6). Moreover, incubation of SH-SY5Y cells in medium containing no calcium also leads to apoptosis in 24 h, and cell death can be blocked by the addition of 1.8 mM calcium to the medium (Fig. 7). Thus, for cell survival, it is crucial to maintain a threshold level of Ca\(_{\text{m}}\) below that cells will die. The results suggest that Bcl-2 by maintaining Ca\(_{\text{m}}\) at the threshold level can protect neuroblastoma cells from STS-induced caspase activation and apoptosis (Fig. 5C). On the other hand, Bik is antagonistic to Bcl-2; it promotes apoptosis by lowering the Ca\(_{\text{m}}\) load.

Our study showing the ability of ruthenium red to promote apoptosis in SH-SY5Y cells is in direct contrast to a recent report indicating the prevention of STS-induced apoptosis by ruthenium red in PC12 cells (42). The discrepancy between these two studies on the ruthenium red effect is not clear. In the PC12 cell study, the authors have observed a mitochondrial Ca\(^{2+}\) overload during STS-induced apoptosis. However, this increase in Ca\(_{\text{m}}\) is a delayed event, occurring at 8 h, well after the activation of caspase (42). According to the emergent view on the role of mitochondria in apoptosis, once the cell releases cytochrome c and activates caspases, it is committed to die by either a rapid apoptotic mechanism or a slower necrotic process (43). A recent confocal study on STS-induced apoptosis in PC-6 cells also indicates that mitochondrial depolarization (and permeability transition) accompanies cytochrome c release (30). These observations suggest that the increase in Ca\(_{\text{m}}\) in PC-12 cells (42) is a consequence but not the cause of mitochondrial permeability transition (MPT) and apoptosis.

How depletion of mitochondria Ca\(^{2+}\) triggers apoptosis is not clear. One possibility is that depletion of Ca\(_{\text{m}}\) directly accompanies MPT, thereby leading to cell death. Our results (Fig. 4) of a correlation of mitochondrial potential (\(\Delta \Psi_{m}\)) with Ca\(_{\text{m}}\) load are consistent with this hypothesis. Furthermore, this hypothesis is also supported by a report showing a close relationship between mitochondrial calcium homeostasis with the MPT (22). Although less likely, the depletion of Ca\(_{\text{m}}\) could indirectly lead to apoptosis via the increase of cytoplasmic Ca\(^{2+}\). A recent study (44) has shown that increased cytoplasmic Ca\(^{2+}\) by thapsigargin treatment can lead to activation of the calcium-dependent phosphatase (calcineurin), translocation of prosapo-
ptotic protein Bad to the mitochondria, which then causes cytochrome c release and caspase activation. Mounting evidence now indicates that translocation of BH3-domain protein such as Bad, Bax, Bid, or Bik can directly induce apoptosis (32, 44–48), and Bel-2 protects the cell by inhibiting the MPT (49, 50). It is apparent that mitochondria provide the center stage for the initiation of apoptotic events. Further study on the relationship between mitochondrial calcium signaling, membrane potential, and permeability transition is required to elucidate the mechanism that initiate the release of Ca\(^{2+}\) and opening of the permeability transition pore.

Acknowledgments—We thank Dr. Shmuel Muallem for critical reading and helpful suggestions, Dr. Kevin Wang for the SH-SY5Y cells, and Richard Lorch for assistance with the laser cytometer.

REFERENCES

1. Berridge, M. J. (1995) Nature 361, 315–325
2. Clapham, D. E. (1995) Cell 80, 259–268
3. Rizzuto, R., Simpson, A. W. M., Brini, M., and Pozzan, T. (1992) Nature 358, 325–327
4. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) Science 262, 744–747
5. Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M., and Pozzan, T. (1994) J. Cell Biol. 126, 1183–1194
6. Sabatow, D. F., Herrington, J., Goodwin, P. C., Park, Y. B., and Hille, B. (1997) J. Cell Biol. 136, 833–844
7. Hoth, M., Fanger, C. M., and Lewis R. S. (1997) J. Cell Biol. 633–648
8. Landolfi, B., Cuceti, S., Debellis, L., Pozzan, T., and Hofer, A. M. (1998) J. Cell Biol. 142, 1235–1243
9. Lawrie, A. M., Rizzuto R., Pozzan, T., and Simpson A. W. M. (1996) J. Biol. Chem. 271, 10753–10759
10. Gunter, T. E., and Pfeiffer, D. R. (1990) Am. J. Physiol. 258, C755–C786
11. Gunter, T. E., Gunter, K. K., Sheu, S. S., and Gavin, C. E. (1984) Am. J. Physiol. 267, C313–C339
12. Hansford, R. G. (1994) J. Bioenerg. Biomembr. 26, 495–508
13. Nichols, B. J., and Denton, R. M. (1995) Mol. Cell. Biochem. 149/150, 203–212
14. Hajnoczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. M. (1995) Cell 82, 415–424
15. Rutter, G. A., Burnett, P., Rizzuto, R., Brini, M., Murgia, T., Pozzan, T., Tavare, J. M., and Denton, R. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5489–5494
16. Yang, E., and Korsmeyer, S. (1996) Blood 88, 386–401
17. He, H., Lam, M., MacDonald, T. S., and Distelhorst, C. W. (1997) J. Cell Biol. 138, 1219–1229
18. Kuo, T. H., Kim, H-R. C., Zhu, L., Yu, Y., Lin, H-M., and Tsang, W. (1998) Oncogene 17, 1905–1910
19. Martin, M., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggest, M., McConkey, D. J., von Eschenbach A. C., Keating, M. J., and McDonnell, T. J. (1996) Oncogene 12, 2255–2266
20. Uhlmann, E. J., Subramanian, T., Vater, C. A., Lutz, R., and Chinnadurai, G. (1990) J. Biol. Chem. 273, 17926–17932
21. Liu, B. F., Xu, X., Fridman, R., Muallem, S., and Kuo, T. H. (1996) J. Biol. Chem. 271, 5536–5544
22. Ichas, F., Jouaville, L. S., and Mazat, J.-P. (1997) Cell 89, 1145–1153
23. Jacobson, M. D., and Raff, M. C. (1995) Nature 374, 814–816
24. Loo, D. T., and Rillem, J. R. (1998) Methods Cell Biol. 57, 251–264
25. Putney, J. W., Jr. (1986) Cell Calcium 7, 1–12
26. Murphy, A. N., Bedessen, D. E., Cortopassi, G., Wang, E., and Fiskum, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9883–9888
27. Wingrove, D. E., Amatruda, J. M., and Gunter, T. E. (1984) J. Biol. Chem. 259, 9390–9394
28. Ackerman, R. O. (1978) Biochim. Biophys. Acta 502, 359–366
29. Adams, J. M., and Cory S. (1998) Science 281, 1322–1326
30. Heiskanen, K. M., Bhat, M. B., Wang, H.-W., Ma, J., and Nieminen, A.-L. (1999) J. Biol. Chem. 274, 5654–5658
31. Scaduto, R. C., Jr., and Grottyohnann, L. W. (1999) Biophys. J. 76, 469–477
32. McGinnis, K. M., Gnegy, M. E., and Wang, K. K. W. (1999) J. Neurochem. 72, 1899–1906
33. Bao, X., Hughes, F. M., Huang, Y., Cioldowsk, J. A., and Putney, J. W. (1997) Am. J. Physiol. 272, C1241–C1249
34. Preston, G. A., Barrett, J. C., Biermann, J. A., and Murphy, E. (1997) Cancer Res. 57, 537–542
35. Jayadev, S., Persank, J. G., Cheran, S. K., Biermann, J. A., Barrett, C., and Murphy, E. (1999) J. Biol. Chem. 274, 8261–8268
36. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Nature 395, 645–648
37. Rizzuto, R., Pinto, F., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tufi, A. A., and Pozzan, T. (1998) Science 280, 1763–1766
38. Duchen, M. R., Leyssens, A., and Crompton, M. (1999) J. Cell Biol. 142, 975–988
39. Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994) Exp. Cell Res. 212, 84–92
40. Furuya, Y., Lundmo, P., Short, A. D., Gill, D. L., and Isacs, J. T. (1994) Cancer Res. 54, 6167–6175
41. Zhu, W. H., and Leh, T. T. (1995) Life Sci. 57, 2091–2099
42. Krum, I. I., and Mattson, M. P. (1999) J. Neurochem. 72, 529–540
43. Green, D. R., and Reed, J. C. (1998) Science 281, 1399–1312
44. Wang, H.-G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bebo, T., Franke, T. F., and Reed, J. C. (1999) Science 284, 339–343
45. Pastorino, J. G., Chen, S. T., Tufani, M., Snyder, J. W., and Farber, J. L. (1998) J. Cell Biol. 137, 1899–1906
46. Luo, X., Budhijadri, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 481–490
47. Li, H., Zhu, H., Xu, C.-J., and Yuan, J. (1998) Cell 94, 491–501
48. Boyd, J. M., Galivo G. J., Elangovan, B., Houghton, A. B., Malstrom, S., Avery, B. J. Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., and Chinnadurai, G. (1995) Oncogene 11, 2161–2172
49. Zammazi, N., Susin, S. A., Marchetti, P., Hirsch, T., Gene-Monterrey, I., Castedo, M., and Kroemer, G. (1996) J. Exp. Med. 183, 1533–1544
50. Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) Nature 399, 483–487