Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4533–4537, May 1993
Medical Sciences

bcl-2 inhibits death of central neural cells induced by multiple agents

(apoptosis/calcium/immortalized cells/programed cell death)

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Communicated by Donald B. Lindsley, January 26, 1993

ABSTRACT **The protooncogene *bcl-2*, which has been implicated in B-cell lymphoma development, inhibits apoptosis due to growth factor withdrawal in some, but not all, hematopoietic cells. Recently we found that *bcl-2* also inhibits apoptosis in PC12 pheochromocytoma cells. We now report that *bcl-2* inhibits the death of a central neural cell line due to serum and growth factor withdrawal, the calcium ionophore A23187, glucose withdrawal, membrane peroxidation, and, in some cases, free radical-induced damage. This broad range of protective effects of BCL-2 protein suggests that BCL-2 may interact with a central step in neural cell death. Measurements of intracellular free calcium suggest that BCL-2 alters the transduction of neural death signals at a point distal to the rise in intracellular free calcium.**

The protooncogene *bcl-2* was discovered by translocation analysis of B-cell lymphomas (1). Subsequently it was reported that its protein product, BCL-2, is targeted to the inner membrane of mitochondria and that apoptosis of B cells, induced by the withdrawal of serum and growth factors, is inhibited by BCL-2 (2). The normal function of BCL-2 is unknown, as is the mechanism by which BCL-2 inhibits apoptosis. Furthermore, BCL-2 has little homology with other known proteins except the Epstein–Barr virus protein BHRF1 [for which no function is known (3)], although some homology to *ras* has been suggested (4).

Since BCL-2 inhibits apoptosis in only a subset of hematopoietic cells (5), and since *bcl-2* is not normally expressed by PC12 pheochromocytoma cells (6), it was of interest to find that vector-driven expression of *bcl-2* markedly inhibits apoptosis in PC12 cells, in the undifferentiated state and following nerve growth factor (NGF)-induced differentiation to sympathetic neuron-like cells (7). Apoptosis due to serum and growth factor withdrawal and to the calcium ionophore A23187 was inhibited by *bcl-2* expression. *bcl-2* has also been reported to inhibit the death of sympathetic neurons cultured in the absence of NGF (8). The present study examined whether central neural cells would be similarly affected by the expression of *bcl-2*, and, if so, what the range of neurological insults would be for which BCL-2 would inhibit neural cell death. This report describes the effects of *bcl-2* expression on a conditionally immortalized nigral neural cell line (9), under conditions permissive and restrictive for the immortalizing gene product. These effects include the inhibition of neural cell death due to serum and growth factor withdrawal, calcium ionophore, glucose withdrawal, membrane peroxidation, and, in some cases, free radical formation. Mechanistic studies demonstrate that the effect is unlikely to be due to BCL-2 effects on the poly(ADP-ribose) pathway (10), secreted survival factors, direct nuclear effects, or the alteration of intracellular free calcium. Rather, BCL-2 appears to alter the cellular response to such insults without exerting a direct effect on intracellular free calcium levels.

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MATERIALS AND METHODS

Cell Culture and Expression Constructs. CSM14.1 cells (9) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, heat inactivated) at 34°C or 39°C in 5% CO_2. PC12 cells were maintained as described (7). The derivation of the plasmid pBabe puro-bcl-2, control plasmid pBabe puro, and high-titer retroviral producers was also as described (7). Following infection with recombinant retroviruses, cells stably infected were selected in puromycin (7 μg/ml). Immunocytochemical detection of the expression of *bcl-2* was as described (7).

Viability in serum-free medium and in A23187 was assayed as described (7). To determine the effect of glucose starvation, cells were washed three times in Locke's solution (154 mM NaCl/5.6 mM KCl/2.3 mM $CaCl_2$/1.0 mM $MgCl_2$/3.6 mM $NaHCO_3$/5 mM Hepes) (11) before plating on 24-well plates in Locke's solution with 2% FBS (for CSM14.1 cells) or 1% horse serum plus 1% FBS (for PC12 cells). For the control group, cells were grown in the same solution with 40 mM glucose. To assess cell survival after the addition of the lipid peroxidizing agent *tert*-butyl hydroperoxide (*t*-BOOH) (12, 13), cells were grown in serum-containing medium with 0.5 μM *t*-BOOH. The *t*-BOOH was added at 8-hr intervals.

Assessment of Apoptosis. Internucleosomal fragmentation of DNA was determined as described (7). For whole-cell assessments of apoptosis, 2 μl of acridine orange (100 μg/ml) and 2 μl of ethidium bromide (100 μg/ml) were mixed on a microscope slide. Twenty microliters of cell suspension (10^6 cells per ml) was then placed on the slide, covered with a coverslip, and observed with a Zeiss Axiovert fluorescence microscope.

Intracellular Biochemical Measurements. The levels of adenosine triphosphate were determined by the firefly luciferase method, as described (14). Measurement of intracellular free calcium was as described in detail (15). For quantitation of oxygen free radical species, *in situ* fluorescence measurements of cells loaded for 15 min with 3,7-dichlorofluorescein diacetate (DCF-DA) were obtained using a Cytofluor 2300 plate scanner (Millipore). Extent of oxidation was determined using known concentrations of oxidized DCF.

RESULTS

Immunocytochemistry following selection of retrovirally infected cells (16) demonstrated expression of *bcl-2* in nearly

Abbreviations: FBS, fetal bovine serum; *t*-BOOH, *tert*-butyl hydroperoxide; DCF-DA, 3,7-dichlorofluorescein diacetate; NGF, nerve growth factor; PDGF, platelet-derived growth factor.

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every cell infected with the *bcl-2* recombinant retrovirus, with some cell-to-cell variability (Fig. 1*A*). Staining predominated in the perinuclear and cytoplasmic regions, appearing similar to that described for the expression of *bcl-2* in *Spodoptera frugiperda* cells (17). Cells that had been infected with a recombinant retrovirus also expressing the puromycin-resistance gene but lacking *bcl-2*, showed no staining with the same antibody (Fig. 1*B*).

The expression of *bcl-2* did not alter cell morphology, either in the undifferentiated or in the differentiated state (Fig. 2). Neuritic outgrowth and development of refractile somata were retained in *bcl-2*-expressing neural cells. Growth rate at the permissive temperature of 34°C was also unaffected by the expression of *bcl-2*, with the doubling time of control cells being 24 ± 3.6 hr and of *bcl-2*-expressing cells being 23 ± 1.8 hr (proliferation did not occur in either group in the differentiated state).

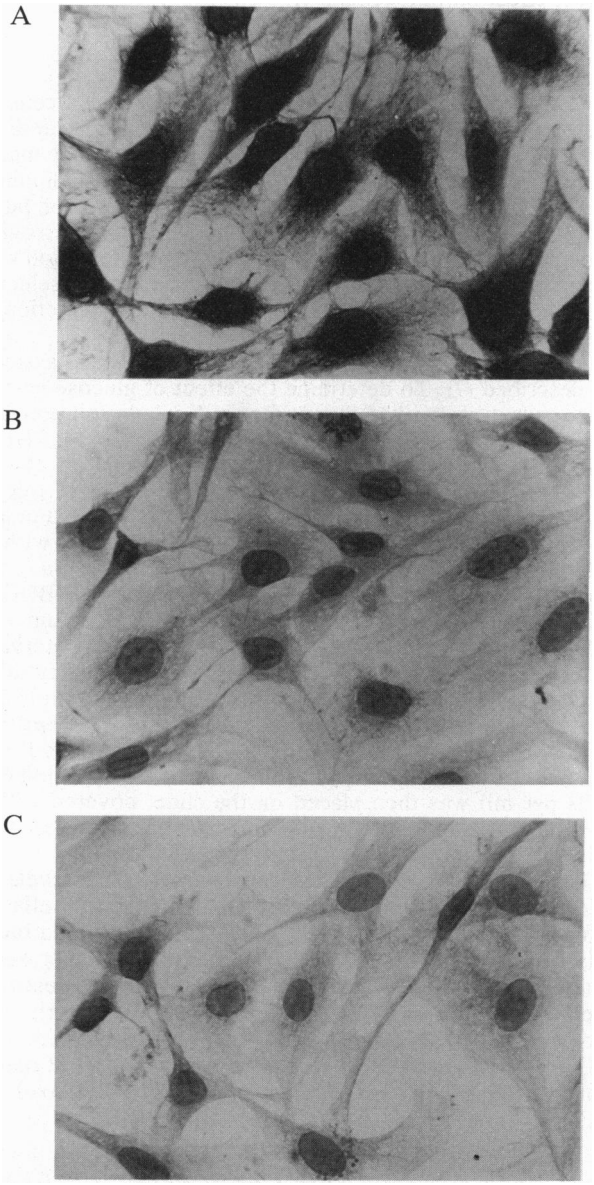

FIG. 1. Photomicrographs of immunocytochemical staining of conditionally immortalized neural cells infected with a recombinant retrovirus effecting the expression of *bcl-2* or infected with a control recombinant retrovirus. (×200; counterstained with hematoxylin.) (*A*) *bcl-2*-expressing cells; note the perinuclear and cytoplasmic staining. (*B*) Cells infected with control retrovirus; note the lack of staining except due to the nuclear counterstain. (*C*) *bcl-2*-expressing cells; primary antibody omitted.

In contrast to its lack of effect on growth or differentiation, *bcl-2* expression markedly enhanced the survival of cells that had undergone any of several different insults. Serum withdrawal led to apoptotic neural death in the control cells but not those expressing *bcl-2*; this occurred at the permissive and restrictive temperatures (Figs. 3 and 4). Mitosis virtually ceased in the *bcl-2*-expressing cells following the withdrawal of serum, but apoptosis did not occur. Similarly, the calcium ionophore A23187 induced cell death in the control cells, but little cell death occurred in *bcl-2*-expressing cells (Fig. 4). Glucose withdrawal also induced some cell death in the *bcl-2*-expressing cells but affected the control cells to a much greater extent (Fig. 4). This differential was present for PC12 pheochromocytoma cells as well as conditionally immortalized nigral neural cells [the effect of serum withdrawal, NGF withdrawal, and calcium ionophore treatment on *bcl-2*-expressing PC12 cells has been reported (7)]. When the cells were exposed to the membrane peroxidizing agent *t*-BOOH (12, 13) or to the inducer of free radical formation menadione (18), a prolongation of cell survival occurred in the *bcl-2*-expressing cells (Fig. 4).

To explore potential mechanisms for the inhibition of neural cell death by *bcl-2*, intracellular free calcium was imaged and quantitated in conditionally immortalized nigral neural cells and PC12 cells expressing *bcl-2* as well as in control cells. No differences were observed between *bcl-2* expressors and control cells in resting concentrations of free calcium or in peak or plateau levels following A23187 or ionomycin treatment (Fig. 5). Serum withdrawal did not result in a measurable change in intracellular free calcium in any of the cell groups within the first 6 hr, the time at which apoptosis is well advanced in these cells, as demonstrated by DNA fragmentation (7) [trypan blue exclusion, however, does not occur until apoptosis is complete and secondary necrosis has begun (19)].

Resting lymphocytes undergo apoptosis by a mechanism dependent on poly(ADP-ribose) synthetase (10), resulting in severe reductions in cellular NAD levels; added nicotinamide (1–5 mM) increases NAD levels and inhibits apoptosis in noncycling lymphocytes. Therefore the effect of nicotinamide on apoptosis in conditionally immortalized neural cells was evaluated. Nicotinamide at 1–15 mM imparted no resistance to apoptosis in these cells, suggesting a different underlying mechanism in the two cell types (not shown).

Since growth factors such as NGF and platelet-derived growth factor (PDGF) inhibit apoptosis in some neural cells (20) and in oligodendroglial precursors (21), respectively, it was conceivable that the effect of *bcl-2* expression might be mediated indirectly, via the secretion of a cellular survival factor. Therefore, the effect of conditioned medium from *bcl-2*-expressing cells was assayed. No significant difference was observed between the effect of conditioned medium from *bcl-2*-expressing cells and the control cells on the death of PC12 cells induced by withdrawal of serum (data not shown).

Isolated thymocyte nuclei undergo apoptosis when bathed in calcium-containing medium; this effect is blocked by calcium chelation and by endonuclease inactivation (22). These findings have led to the hypothesis that apoptosis may be mediated by a specific calcium-activated endonuclease (23). Therefore, to determine whether BCL-2 primes the nucleus to resist apoptosis (e.g., by altering the expression of calcium-activated endonuclease), nuclei were isolated from *bcl-2*-expressing cells and control cells and then exposed to 2 mM calcium in the presence or absence of EGTA. Nuclei from both cell groups demonstrated internucleosomal DNA cleavage in the presence of calcium but not in the presence of EGTA (data not shown).

The targeting of BCL-2 to the inner membrane of mitochondria (2) suggests that BCL-2 may have an effect on oxidative phosphorylation. Our previous studies showed no

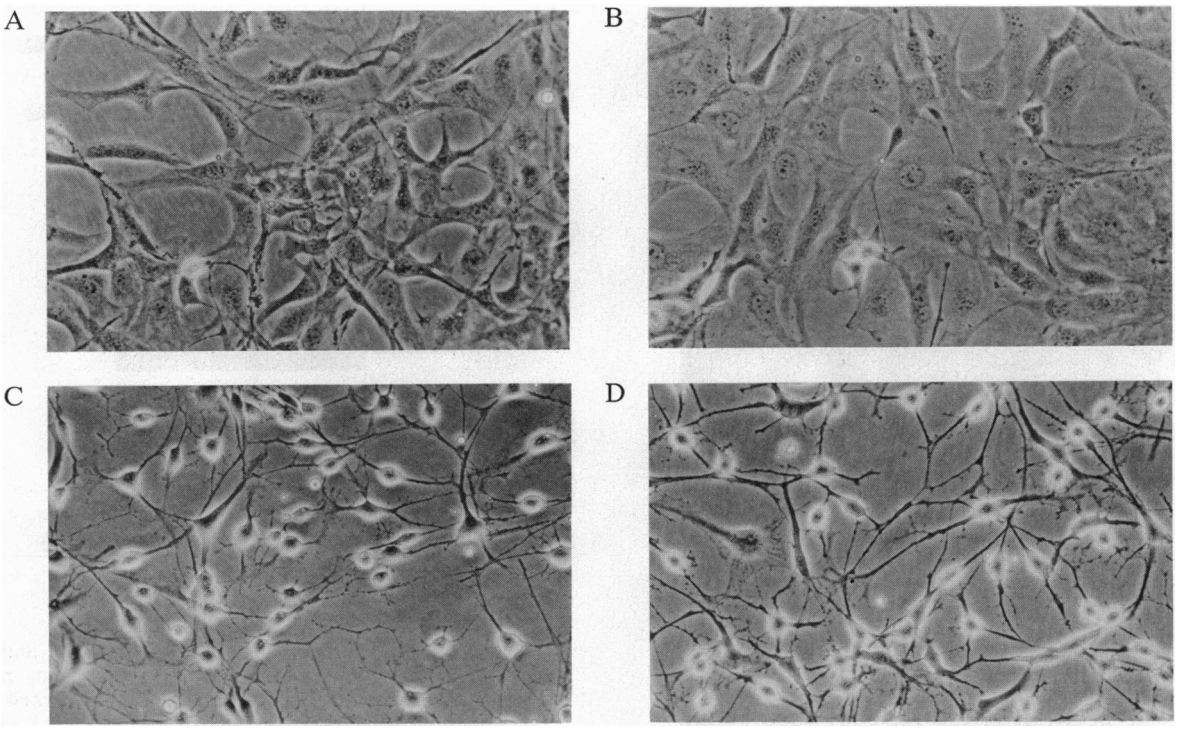

FIG. 2. Phase-contrast photomicrographs comparing conditionally immortalized neural cells expressing *bcl-2* to those expressing a control puromycin-resistance gene. (×120.) (*A*) *bcl-2*-expressing cells at the permissive temperature of 34°C. (*B*) Control cells at 34°C. (*C*) *bcl-2*-expressing cells at the restrictive temperature of 39°C. (*D*) Control cells at 39°C. Note that the *bcl-2*-expressing cells demonstrated morphological differentiation indistinguishable from that of the control cells.

significant difference between the oxygen consumption (per viable cell) of control cells and *bcl-2* expressors, prior to or following the withdrawal of serum (7). However, if BCL-2 affects the coupling of oxygen consumption to ATP formation, then ATP levels in control and *bcl-2*-expressing cells might differ markedly despite similar oxygen consumption. Therefore, levels of ATP were measured in the cells prior to and following the withdrawal of serum. ATP levels fell ≈50% in the 24 hr following serum withdrawal, with similar decrements occurring in the *bcl-2*-expressing cells and control cells (data not shown).

DISCUSSION

These studies suggest that BCL-2 inhibits a process that is central to neural cell death, whether the death is induced by serum and growth factor withdrawal, calcium ionophore, glucose withdrawal, membrane peroxidation, or at least one form of free radical-induced damage. The prevailing view of neural cell death induced by a sustained increase in intracellular free calcium is that calcium-dependent enzymes such as proteases (e.g., calpain), phospholipases, and endonucleases effect cell destruction (24). The results reported here demonstrate that two neural cells differing by the expression of a single gene may have completely different responses to the same level of rise of intracellular free calcium. This suggests that the effect of BCL-2 occurs at a step in neural cell death that follows an increase in free calcium—i.e., in the transduction of the signal. This would be somewhat surprising if numerous proteases and phospholipases were involved and would suggest that either (*i*) BCL-2 affects numerous proteases and phospholipases, either directly or indirectly, or (*ii*) BCL-2 affects one or a few molecules (e.g., an endonuclease) that are preferentially involved in neural cell death. In either case, the findings suggest that modulation of the neural cell death response at a point beyond the rise in intracellular free calcium is possible. The results presented do not exclude the possibility that the subcellular distribution of calcium may differ in the two groups of cells and that this possible alteration may not have been detected by the calcium imaging studies described. However, the marked inhibition by BCL-2 of the apoptotic response to serum withdrawal, in the absence of any change in intracellular free calcium levels, does not favor this interpretation.

The discovery that BCL-2 inhibits neural cell death due to calcium ionophore without affecting intracellular free calcium levels is compatible with the finding that BCL-2 inhibits neural cell death due to serum withdrawal, in which free calcium levels remain at the resting level at least early into the phase of DNA fragmentation. The lack of change in intracellular free calcium during this interval demonstrates that a rise in intracellular calcium is not a prerequisite for apoptosis and that BCL-2 inhibits apoptosis whether or not initiated by a rise in intracellular calcium (e.g., by ionophore).

It should be noted that, although calcium ionophores have been shown to induce apoptosis (25), controversy remains over whether some insults hypothesized to incite calcium-mediated neural death—e.g., glutamate toxicity—actually induce apoptosis, necrosis, or both. Kure *et al.* (26) reported that glutamate induces DNA fragmentation characteristic of apoptosis, in neocortical neurons in culture and following *in vivo* hippocampal injections. The endonuclease inhibitor aurintricarboxylic acid, an inhibitor of apoptosis, prevented the appearance of DNA fragmentation and neural death. Similar results were obtained for *N*-methyl-D-aspartate-induced hippocampal neuronal death by Roberts-Lewis *et al.* (27). Ignatowicz *et al.* (28), however, reported that injections of kainic acid or quinolinic acid into the hippocampus induced nonapoptotic neural cell death. McConkey *et al.* (22), studying the effects of the calcium ionophore A23187 on thymocytes, demonstrated that the distribution of apoptosis vs. nonapoptotic cell death is dependent on the intracellular free calcium level: at levels in excess of 2 μM, nonapoptotic death predominated, whereas at lower levels, apoptosis

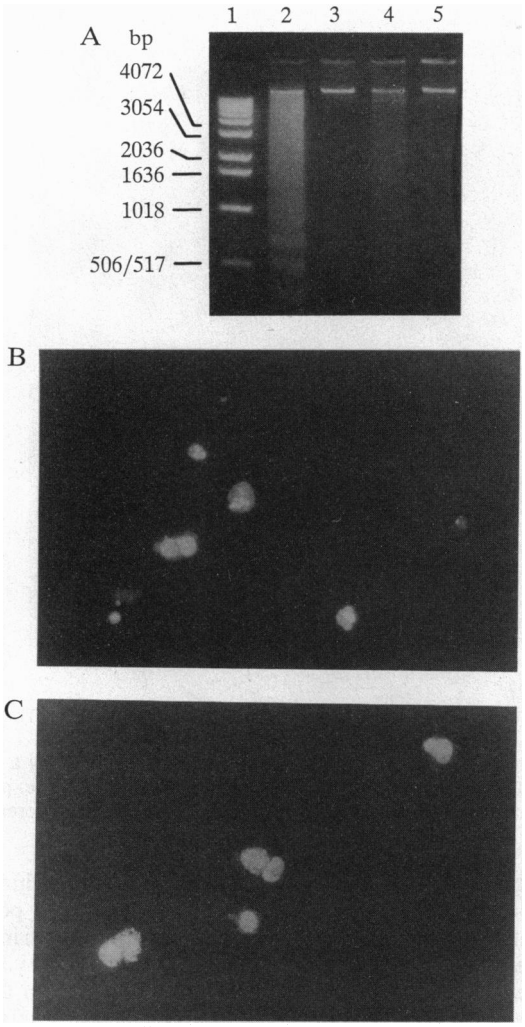

FIG. 3. Inhibition of apoptosis in *bcl-2*-expressing cells. (*A*) Agarose gel of low molecular weight DNA extracted from conditionally immortalized neural cells. Lane 1, standards, labeled in base pairs. Lane 2, control cells cultured 16 hr in serum-free medium. Note the presence of regularly spaced, low molecular weight bands of DNA. Lane 3, control cells cultured in medium containing serum. Lane 4, *bcl-2*-expressing cells cultured 16 hr in serum-free medium. Lane 5, *bcl-2*-expressing cells cultured in medium containing serum. (*B*) Acridine orange staining of control cells cultured in serum-free medium for 4 hr. Note the presence of brightly stained small fragments of nuclei characteristic of apoptosis. (*C*) Acridine orange staining of *bcl-2*-expressing cells cultured in serum-free medium for 4 hr. Note the normal appearing nuclei and cytoplasmic staining of RNA.

predominated. Thus it is possible that the intracellular free calcium level may determine to what extent apoptosis will occur and to what extent necrosis will supervene. However, the results presented here suggest that a single gene product may alter the cellular response to at least some levels of intracellular free calcium markedly.

The finding that BCL-2 inhibits apoptosis in central neural cells as well as lymphocytes is of interest since it has been reported that neural apoptosis differs from apoptosis in other cell types in its lack of increased expression of several apoptosis-associated transcripts (29). Therefore, it will be of interest to determine whether BCL-2 alters the apoptotic response of other central neural cells; our preliminary data suggest that it does. It will also be important to determine whether other gene products that inhibit apoptosis in non-neural cells—e.g., the Epstein–Barr virus latent membrane

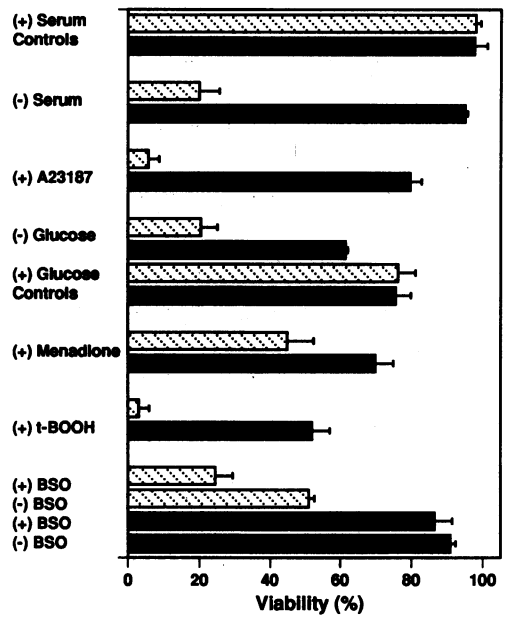

FIG. 4. *bcl-2* inhibits cell death of conditionally immortalized neural cells induced by multiple agents. Filled columns, plus *bcl-2*; striate columns, minus *bcl-2*. Cell viability was determined by trypan blue exclusion. Bars represent standard deviations. Graph is from one experiment typical of three, using duplicate or triplicate wells for each time point [only one time point is depicted for each group, due to space considerations; similarly, results from only the differentiated state of the conditionally immortalized cells are graphed except in the buthionine sulfoximine (BSO) experiment, in which undifferentiated cells were used]. In each case, the difference in viability between the cells expressing *bcl-2* and control cells was significant at $P < 0.05$ by *t* test. A23187, Ca^{2+}/Mg^{2+} salt at 2 μg/ml; menadione 50 μM; *t*-BOOH, 50 nM; BSO, 1 mM.

protein LMP1 (30) and baculovirus p35 (31, 32)—function similarly in neural cells.

The targeting of BCL-2 to the inner membrane of the mitochondrion (2) is difficult to reconcile with previous hypotheses that apoptosis may be induced directly by calcium activation of a nuclear resident endonuclease (22). If BCL-2 fails to alter intracellular free calcium levels, at what step in the proposed direct pathway might it act to inhibit induction of apoptosis? One possibility is that BCL-2 may target to subcellular locations other than the mitochondrion (17); however, we note that BCL-2 contains an N-terminal subsequence that is compatible with mitochondrial targeting

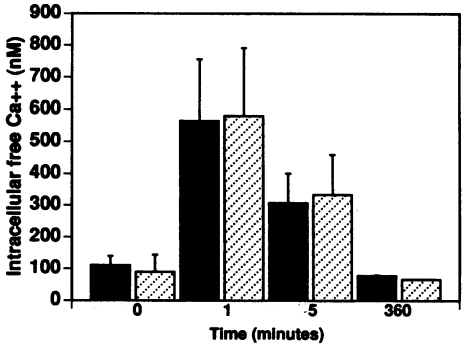

FIG. 5. Intracellular free calcium concentration in PC12 cells. Cells were cultured in the bromo form of A23187 (1 μM) (since this lacks interfering fluorescence) for the times indicated. Bars represent standard deviations. Twenty cells were evaluated from each group at each time point. Identical studies were carried out with the calcium ionophore ionomycin (1 μM) as well as with conditionally immortalized neural cells, with similar results.

(33): periodically spaced basic residues, including Arg^6, Arg^{12}, Lys^{17}, Lys^{22}, and Arg^{26}. It will therefore be important to determine whether mutation of this putative mitochondrial targeting sequence will destroy mitochondrial targeting and anti-apoptotic function.

The profile of insults for which BCL-2 inhibits cell death may be important in determining the point along the putative cell death program at which BCL-2 exerts its influence. Recently it was shown that BCL-2 fails to alter apoptosis induced by cytotoxic T cells (34). Such cells produce TIA-1 (35), which effects target cell death either by acting directly as an endonuclease or by inducing endonuclease activity. This result, coupled with the findings reported herein, suggests that BCL-2 inhibits the transduction of the death program signal (as opposed to altering a primary inducer such as calcium or lack of glucose) but does not directly inhibit the effector, a calcium/magnesium-activated endonuclease (36). Constructing such profiles for other apoptosis-inhibitory genes may aid in the reconstruction of the pathway(s) of programed cell death.

We thank H. Weiner for fellowship support to L.-t.Z., M. Cleary for the human *bcl-2* cDNA, H. Land for the pBabe retroviral vectors, D. Mason for the monoclonal antibody to BCL-2, D. Markowitz for the GP+E86 cells, M. Durand and D. Chugani for the CSM14.1 cells, R. Weiss and C. Kayalar for helpful discussions, S. Rayner for excellent technical assistance, and G. K. Moghaddami for assistance in preparation of the manuscript. These studies were supported in part by National Institutes of Health Grant 5 R29 NS27812.

1. Tsujimoto, Y., Finger, L., Yunis, J., Nowell, P. & Croce, C. (1984) *Science* **226**, 1097–1099.
2. Hockenbery, D., Nuñez, G., Milliman, C., Schreiber, R. & Korsmeyer, S. (1990) *Nature (London)* **348**, 334–336.
3. Cleary, M. L., Smith, S. D. & Sklar, J. (1986) *Cell* **47**, 19–28.
4. Haldar, S., Beatty, C., Tsujimoto, Y. & Croce, C. (1989) *Nature (London)* **342**, 195–198.
5. Nuñez, G., Loncon, L., Hockenbery, D., Alexander, M., McKearn, J. P. & Korsmeyer, S. J. (1990) *J. Immunol.* **144**, 3602–3610.
6. Zhong, L. T., Mah, S. P., Edwards, R. H. & Bredesen, D. E. (1992) *Soc. Neurosci. Abstr.* **18**, 44.
7. Mah, S. P., Zhong, L. T., Liu, Y., Roghani, A., Bredesen, D. E. & Edwards, R. H. (1993) *J. Neurochem.* **60**, 1183–1186.
8. Garcia, I., Martinou, I., Tsujimoto, Y. & Martinou, J. C. (1992) *Science* **258**, 302–304.
9. Durand, M., Chugani, D. C., Mahmoudi, M. & Phelps, M. E. (1990) *Soc. Neurosci. Abstr.* **16**, 40.
10. Carson, D., Seto, S., Wasson, D. & Carrera, C. (1986) *Exp. Cell. Res.* **164**, 273–281.
11. Cheng, B. & Mattson, M. P. (1991) *Neuron* **7**, 1031–1041.
12. Bellomo, G., Jewell, S. A., Thor, H. & Orrenius, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6842–6846.
13. Masaki, N., Kyle, M. & Farber, J. L. (1989) *Arch. Biochem. Biophys.* **269**, 390–399.
14. Verity, M. A., Sarafian, T. S., Guerra, W., Ettinger, A. & Sharp, J. (1990) *Neurotoxicity* **11**, 415–26.
15. Charles, A. C., Merrill, J. E., Dirksen, E. R. & Sanderson, M. J. (1991) *Neuron* **6**, 983–992.
16. Morgenstern, J. P. & Land, H. (1990) *Nucleic Acids Res.* **18**, 3587–3596.
17. Alnemri, E. S., Robertson, N. M., Fernandes, T. F., Croce, C. M. & Litwack, G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7295–7299.
18. Mirabelli, F., Salis, A., Perotti, M., Taddei, F., Bellomo, G. & Orrenius, S. (1988) *Biochem. Pharmacol.* **37**, 3423–3427.
19. Kerr, J. F. R. & Harmon, B. V. (1991) *Apoptosis* **3**, 321.
20. Martin, D. P., Schmidt, R. E., deStefano, P. S., Lowry, O. H., Carter, J. G. & Johnson, E. M. (1988) *J. Cell Biol.* **106**, 829–844.
21. Barres, B. A., Hart, I. K., Coles, H. S. R., Burne, J. F., Voyvodic, J. T., Richardson, W. D. & Raff, M. C. (1992) *Cell* **70**, 31–46.
22. McConkey, D. J., Hartzell, P., Nicotera, P. & Orrenius, S. (1989) *FASEB J.* **3**, 1843–1849.
23. Compton, M. M., Haskill, J. S. & Cidlowski, J. A. (1988) *Endocrinology* **122**, 2158–2164.
24. Choi, D. (1988) *Neuron* **1**, 623–34.
25. Wyllie, A. H., Morris, R. G., Smith, A. L. & Dunlop, D. (1984) *J. Pathol.* **142**, 66–77.
26. Kure, S., Tominaga, T., Yoshimoto, T., Tada, K. & Narisawa, K. (1991) *Biochem. Biophys. Res. Commun.* **179**, 39–45.
27. Roberts-Lewis, J. M., Marcy, V. R., Zhao, Y., Fedora, T., Lewis, M. E., Siman, R. & Vaught, J. L. (1992) *Soc. Neurosci. Abstr.* **18**, 44.
28. Ignatowicz, E., Vezzani, A. M., Rizzi, M. & D'Incalci, M. (1991) *Neuroreport* **11**, 651–654.
29. D'Mello, S. R. & Galli, C. (1992) *Soc. Neurosci. Abstr.* **18**, 44.
30. Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E. & Rickinson, A. (1991) *Cell* **65**, 1107–1115.
31. Friesen, P. D. & Miller, L. K. (1987) *J. Virol.* **61**, 2264–2272.
32. Clem, R. J., Fechheimer, M. & Miller, L. K. (1991) *Science* **254**, 1388–1390.
33. Heslot, H. & Gaillardin, C. (1992) *Molecular Biology and Genetic Engineering of Yeasts* (CRC, Boca Raton, FL), p. 393.
34. Vaux, D. L., Aguila, H. L. & Weissman, I. L. (1992) *Int. Immunol.* **4**, 821–824.
35. Tian, Q., Streuli, M., Saito, H., Scholssman, S. & Anderson, P. (1991) *Cell* **67**, 629–639.
36. Compton, M. M. & Cidlowski, J. A. (1987) *J. Biol. Chem.* **262**, 8288–8292.