

## Alterations of Extracellular Calcium Elicit Selective Modes of Cell Death and Protease Activation in SH-SY5Y Human Neuroblastoma Cells

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**Abstract:** The role of intracellular  $\text{Ca}^{2+}$  homeostasis in mechanisms of neuronal cell death and cysteine protease activation was investigated in SH-SY5Y human neuroblastoma cells. Cells were incubated in 2 mM EGTA to lower intracellular  $\text{Ca}^{2+}$  or 5 mM  $\text{CaCl}_2$  to raise it. Cell death and activation of calpain and caspase-3 were measured. Both EGTA and excess  $\text{CaCl}_2$  elicited cell death. EGTA induced DNA laddering and an increase in caspase-3-like, but not calpain, activity. Pan-caspase inhibitors protected against EGTA-, but not  $\text{CaCl}_2$ -, induced cell death. Conversely, excess  $\text{Ca}^{2+}$  elicited necrosis and activated calpain but not caspase-3. Calpain inhibitors did not preserve cell viability.  $\text{Ca}^{2+}$  was the death-mediating factor, because restoration of extracellular  $\text{Ca}^{2+}$  protected against cell death induced by EGTA and blockade of  $\text{Ca}^{2+}$  channels by  $\text{Ni}^{2+}$  protected against that induced by high  $\text{Ca}^{2+}$ . We conclude that the EGTA treatment lowered intracellular  $\text{Ca}^{2+}$  and elicited caspase-3-like protease activity, which led to apoptosis. Conversely, excess extracellular  $\text{Ca}^{2+}$  entered  $\text{Ca}^{2+}$  channels and increased intracellular  $\text{Ca}^{2+}$  leading to calpain activation and necrosis. The mode of cell death and protease activation in response to changing  $\text{Ca}^{2+}$  were selective and mutually exclusive, demonstrating that these are useful models to individually investigate apoptosis and necrosis. **Key Words:** Apoptosis—Necrosis—Calpain—Caspase-3— $\text{Ca}^{2+}$  homeostasis—Neuronal death.

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Alterations in intracellular calcium ( $\text{Ca}^{2+}_i$ ) homeostasis have been implicated in the onset of cell death in many cell types. However, the mode of cell death and direction of change in  $\text{Ca}^{2+}_i$  differ among cell types (Orrenius and Nicotera, 1994; McConkey and Orrenius, 1997). An overload of  $\text{Ca}^{2+}_i$  contributes directly to excitotoxicity-induced necrosis (Munir et al., 1995; Ikeda et al., 1996; Gwag et al., 1997) but can also lead to apoptosis (Furuya et al., 1994; Zhu and Loh, 1995) depending on the cell type. Chelation of extracellular  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}_o$ ) protects against apoptosis induced by specific agents in thymocytes (McConkey et al., 1989; Story

et al., 1992; Jiang et al., 1994), in human epithelial cells (Escargueil-Blanc et al., 1997), and in malignant B cells (Shan et al., 1998). Decreases in  $\text{Ca}^{2+}_i$  may lead to apoptosis. Apoptosis can be induced either by removal of  $\text{Ca}^{2+}$  from the media or blockade of  $\text{Ca}^{2+}$  channels in lymphoma cells (Waterhouse et al., 1996), pancreatic cells (Mizuno et al., 1998), and astrocytes (Chiesa et al., 1998).

The role of  $\text{Ca}^{2+}$  in neuronal cell apoptosis has not been clearly defined, but studies have consistently shown that lowered  $\text{Ca}^{2+}_i$  can lead to programmed cell death in cultured neurons (Koh and Cotman, 1992; Franklin et al., 1995; Tong et al., 1996). Apoptosis and  $\text{Ca}^{2+}$ -induced excitotoxicity have been implicated in many neurological disorders such as Parkinson's disease, Alzheimer's disease, motor neuron disease, ischemia, and stroke. Understanding the role of  $\text{Ca}^{2+}$  in neuronal cell death and the consequent biochemical sequelae may help clarify the mechanisms of neurodegenerative diseases.

One consequence of the initiation of either necrotic or apoptotic cell death is the activation of cellular cysteine proteases. We have previously demonstrated the activation of two families of cysteine proteases, caspases and calpains, in SH-SY5Y cells undergoing staurosporine-induced apoptosis (Nath et al., 1996b; Posmantur et al., 1997). Caspase-3 has been studied as a putative mediator of mammalian apoptosis (Fernandes-Alnemri et al., 1994; Casciola-Rosen et al., 1996; Posmantur et al.,

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**Abbreviations used:** Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; AM, acetoxymethyl ester; BDP, breakdown product;  $\text{Ca}^{2+}_o$ , extracellular calcium;  $\text{Ca}^{2+}_i$ , intracellular calcium; CalpInh II, calpain inhibitor II; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Z-D-DCB, carbobenzoxy-Asp- $\text{CH}_2\text{OC}(\text{O})$ -2,6-dichlorobenzene.

1997). Inhibition of caspase-3 activity attenuates apoptosis in many mammalian cell types including neuronal cells (Nath et al., 1996a; Posmantur et al., 1997). After apoptotic injury, the 32-kDa caspase-3 proenzyme is cleaved to 17- and 12-kDa fragments, which form the active heterodimer (Thornberry et al., 1992; Walker et al., 1994). Activated caspase-3 proteolytically cleaves important nuclear and cytoskeletal proteins during apoptosis (Fernandes-Alnemri et al., 1995; Nicholson et al., 1995; Tewari et al., 1995). An expanding list of caspase substrates includes poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair (Lazebnik et al., 1994), and the cytoskeletal protein nonerythroid  $\alpha$ -spectrin (Nath et al., 1996b; Vanags et al., 1996). The functional significance of these cleavages has not been determined.

Calpain is a  $\text{Ca}^{2+}$ -dependent cysteine protease with numerous substrates. Calmodulin-binding proteins are especially vulnerable to calpain cleavage (Wang et al., 1989). Calpain is activated during necrosis (Wang et al., 1996b) and in many cell types undergoing apoptosis (Sarin et al., 1993; Squier et al., 1994) including SH-SY5Y cells (Nath et al., 1996b). Calpain inhibitors have exhibited protective effects against apoptotic death in cultured cerebellar granule neurons (Nath et al., 1996a) and against excitotoxic injury to Purkinje cells in cerebellar slices (Wang et al., 1996a).

In this study we investigated the effect of manipulating extracellular  $\text{Ca}^{2+}$  on the mode of cell death in SH-SY5Y human neuroblastoma cells and the consequent activation of the calpain and caspase-3-like proteases. We found that chelation of  $\text{Ca}^{2+}_e$  in SH-SY5Y cells leads to apoptotic cell death and the rapid activation of caspase-3-like proteases with no calpain activation. Conversely, the addition of excess  $\text{Ca}^{2+}_e$  elicits necrotic cell death and mediates the activation of calpain but not of caspases.

## MATERIALS AND METHODS

### Cell culture and treatment

SH-SY5Y human neuroblastoma cells were grown on 12-well plates to confluency ( $\sim 2 \times 10^6$  cells per well) at 37°C, 5%  $\text{CO}_2$  in a humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. At the beginning of the experiment, cultures were washed three times with serum-free DMEM. To avoid artifacts in time course experiments, all conditions were washed with serum-free DMEM at time 0 and were treated at the appropriate time so that all time points were collected simultaneously. As indicated, cells were pretreated for 1 h with *N*-acetyl-Leu-Leu-Met-CHO (calpain inhibitor II; CalpInh II) or the pan-caspase inhibitor carbobenzoxy-Asp-CH<sub>2</sub>OC(O)-2,6-dichlorobenzene (Z-D-DCB; Bachem).

### Cell death measurement

Cell death was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium (25  $\mu\text{l}$  aliquots), using the Cytotox 96 colorimetric LDH assay kit (Promega) according to manufacturer's direc-

tions. Cell viability was also measured by the colorimetric monitoring of the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) to formazan (Hansen et al., 1989).

### Extraction of low molecular weight DNA for laddering

Low molecular weight DNA was extracted using a previously described method (Gong et al., 1994). The DNA was analyzed on a 1.5% agarose gel with 5  $\mu\text{g}/\text{ml}$  ethidium bromide and electrophoresed at 2 V/cm to view DNA fragmentation.

### Nuclear staining

To visualize nuclei, cells were labeled with 2  $\mu\text{g}/\text{ml}$  of the DNA dye Hoechst 33258 (bisbenzimidazole, Sigma) in phosphate-buffered saline for 10 min at room temperature. The cells were rinsed twice with phosphate-buffered saline and then visualized on a Leica DMIL fluorescence microscope with a UV2A filter.

### Protein extraction and western blotting

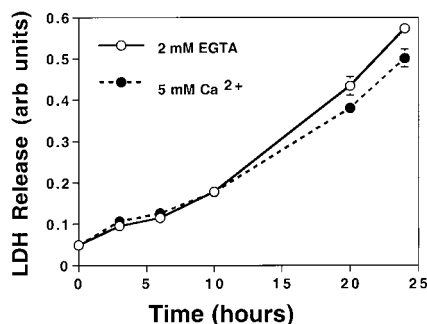
SH-SY5Y cells from three wells of a 12-well plate were collected by scraping and low-speed centrifugation. Cell pellets were washed twice in a TBS-EDTA buffer (20 mM Tris-HCl, pH 7.4, 155 mM NaCl, 1 mM EDTA) and then resuspended and lysed in a buffer containing 20 mM Tris (pH 7.4 at 4°C), 150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, and 1% (wt/vol) Triton X-100 for 1.5 h. Lysates were cleared by centrifugation and stored at -70°C in 50% glycerol until use. Protein concentration was determined by a modified Lowry method (Bio-Rad D-C protein assay kit). Equal amounts of protein were loaded on each lane and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–20% acrylamide gradient gel; Novex) with a Tris-glycine running buffer and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2  $\mu\text{M}$ ) by semidry electrotransfer for 2 h at 20 V. The blots were probed with an antibody to PARP (monoclonal, C-2-10, Biomol), anti- $\alpha$ -spectrin (monoclonal, Chemicon), or anti-caspase-3 (monoclonal, Pharmingen), a biotinylated second antibody, and avidin conjugated with alkaline phosphatase (Amersham Pharmacia). The blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Some western blots were visualized with enhanced chemiluminescence (ECL; Pierce Supersignal ECL kit).

### Caspase activity assay

SH-SY5Y cell lysates were prepared by Triton extraction as described above. Caspase-3 activity was determined by monitoring proteolysis of the fluorogenic caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-MCA; Peptide International). Cell lysates were added to a buffer containing 100  $\mu\text{M}$  peptide substrate, 100 mM HEPES, 10% glycerol, 1 mM EDTA, and 10 mM dithiothreitol. Fluorescence (excitation at 380 nm and emission at 460 nm) was measured every 30 min, to 2 h, using a Millipore Cytofluor 2300 fluorescence plate reader.

### Calcium measurement

Intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) was measured in fura-2-loaded cells by using dual wavelength spectrofluorometry as previously described (Fisher et al., 1989). After exposure to 2 mM EGTA or 5 mM excess  $\text{Ca}^{2+}_e$ , SH-SY5Y cells were incubated with 2  $\mu\text{M}$  fura-2 acetoxymethyl ester (fura-2/AM) (Molecular Probes) for 30 min at 37°C. The fura-2-loaded cells were washed twice and resuspended in DMEM with 2 mM EGTA or 5 mM excess  $\text{Ca}^{2+}_e$ . Fluorescence



**FIG. 1.** LDH release after EGTA and excess Ca<sup>2+</sup> treatment in SH-SY5Y cells. Cells were treated with 2 mM EGTA (○) or 5 mM CaCl<sub>2</sub> (●) for the indicated times. Cell viability was assessed by LDH release as described in Materials and Methods. Data are mean ± SEM values.

measurements were made on 1-ml aliquots of cells (~3 mg of protein) maintained at 37°C and constantly stirred. Changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored as changes in the fluorescence ratio of the 340/380-nm excitation wavelength in a Shimadzu RF-5000 spectrofluorometer.

### Statistics

Multiple data points were compared by one-way ANOVA with post hoc Tukey–Kramer multiple comparisons test. Student's *t* test was used to monitor significance between two data points.

## RESULTS

### Loss of cell viability as a result of altered Ca<sup>2+</sup><sub>e</sub>

We investigated the effect of changes in Ca<sup>2+</sup><sub>e</sub> on cell viability in SH-SY5Y cells treated with either 5 mM CaCl<sub>2</sub> or 2 mM EGTA. These concentrations were the lowest ones tested that consistently produced cytotoxicity (data not shown). Loss of cell viability was monitored by LDH released into the medium. Cells were exposed to serum-free medium for 24 h. Excess Ca<sup>2+</sup> or EGTA was added at appropriate times so that time 0 is a 24-h serum-free control. Treatment of the cells with either excess Ca<sup>2+</sup> or EGTA resulted in a similar time course of LDH release (Fig. 1). LDH significantly increased by 3 h after start of treatments in both EGTA- and Ca<sup>2+</sup><sub>e</sub>-treated conditions (0.049 ± 0.003 arbitrary units for control vs. 0.095 ± 0.009 for EGTA or 0.108 ± 0.008 for excess Ca<sup>2+</sup><sub>e</sub>, *p* < 0.001 for treated vs. control). LDH release continued to increase at similar rates with both treatments through 24 h. Intracellular Ca<sup>2+</sup> levels were measured using the Ca<sup>2+</sup>-selective fluorescent dye fura-2 as described (Fisher et al., 1989) after 4 h of treatment with vehicle, 2 mM EGTA, or 5 mM CaCl<sub>2</sub>. The basal [Ca<sup>2+</sup>]<sub>i</sub> was 40 nM. [Ca<sup>2+</sup>]<sub>i</sub> was reduced to unmeasurable levels after 4 h of 2 mM EGTA and elevated to 75 nM after 4 h of 5 mM CaCl<sub>2</sub>.

### EGTA, but not excess Ca<sup>2+</sup><sub>e</sub>, induces DNA laddering in SH-SY5Y cells

To determine the mechanism of cell death after treatment with EGTA or excess Ca<sup>2+</sup><sub>e</sub>, we investigated the

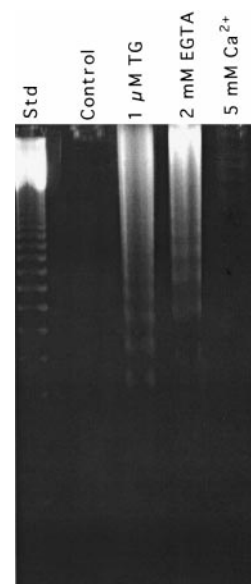
generation of one of the major apoptotic hallmarks, DNA laddering. SH-SY5Y cells were untreated or treated for 24 h with 5 mM CaCl<sub>2</sub> or 2 mM EGTA after which low molecular DNA was extracted and run on an agarose gel (Fig. 2). Thapsigargin was used as a positive control (Nath et al., 1997). In cells treated with EGTA or thapsigargin, a classic DNA ladder is readily apparent. No laddering is seen in untreated cells or in cells treated with excess Ca<sup>2+</sup><sub>e</sub>.

### Excess Ca<sup>2+</sup><sub>e</sub> elicits a response consistent with necrotic cell death

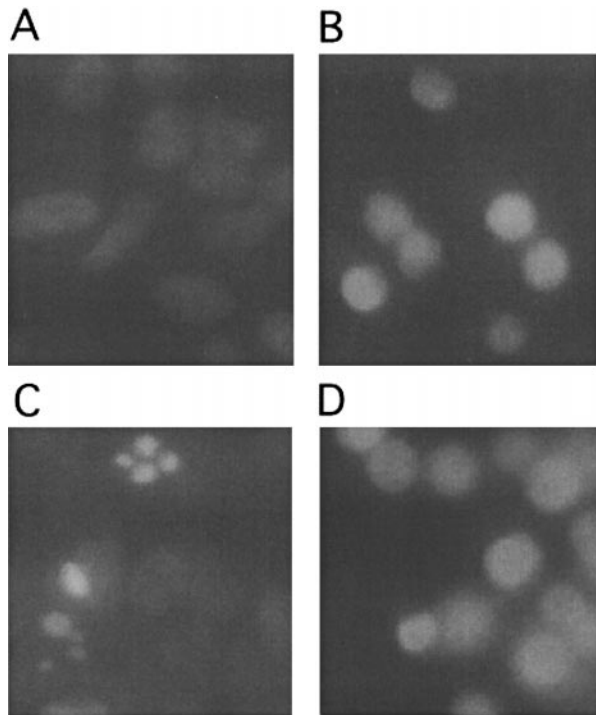
To characterize the mechanism of death in cells treated with 5 mM CaCl<sub>2</sub>, we dyed the nuclei of untreated and treated cells with bisbenzimidazole (Hoechst 33258). This technique could not be used under EGTA conditions, because in the absence of Ca<sup>2+</sup><sub>e</sub>, SH-SY5Y cells detach from the culture dish after washing. SH-SY5Y cells were untreated (Fig. 3A), or treated with 5 mM CaCl<sub>2</sub> (Fig. 3B), 2 μM thapsigargin (Fig. 3C) or 0.3 nM maitotoxin (Fig. 3D). Thapsigargin- and maitotoxin-treated cells were positive controls for apoptosis or necrosis, respectively. We have previously demonstrated that maitotoxin produces a necrotic response in SH-SY5Y cells with characteristic bright, diffuse bisbenzimidazole staining of nuclei (Fig. 3C) (Nath et al., 1996b). Thapsigargin produces an apoptotic response that is characterized by punctate staining of condensed chromatin on the margins of the nuclei (Nath et al., 1997). Cells treated with 5 mM CaCl<sub>2</sub> (Fig. 3B) displayed the diffuse DNA staining in the nuclei identical to that seen with maitotoxin (Fig. 3D), suggesting that SH-SY5Y cells die by necrosis when treated with excess Ca<sup>2+</sup><sub>e</sub>.

### EGTA, but not excess Ca<sup>2+</sup><sub>e</sub>, increases caspase-3-like activity

To characterize the activation of cysteine proteases after treatment of SH-SY5Y cells with excess Ca<sup>2+</sup><sub>e</sub> or



**FIG. 2.** DNA laddering in thapsigargin-, EGTA-, and excess Ca<sup>2+</sup><sub>e</sub>-treated SH-SY5Y cells. SH-SY5Y cells were treated for 24 h with either 1 μM thapsigargin (TG), 2 mM EGTA, or 5 mM CaCl<sub>2</sub>. DNA was extracted as described in Materials and Methods. The DNA was analyzed on a 1.5% agarose gel and visualized with ethidium bromide. A 123-bp DNA ladder was used as a standard (Std). Results shown are representative of two experiments.



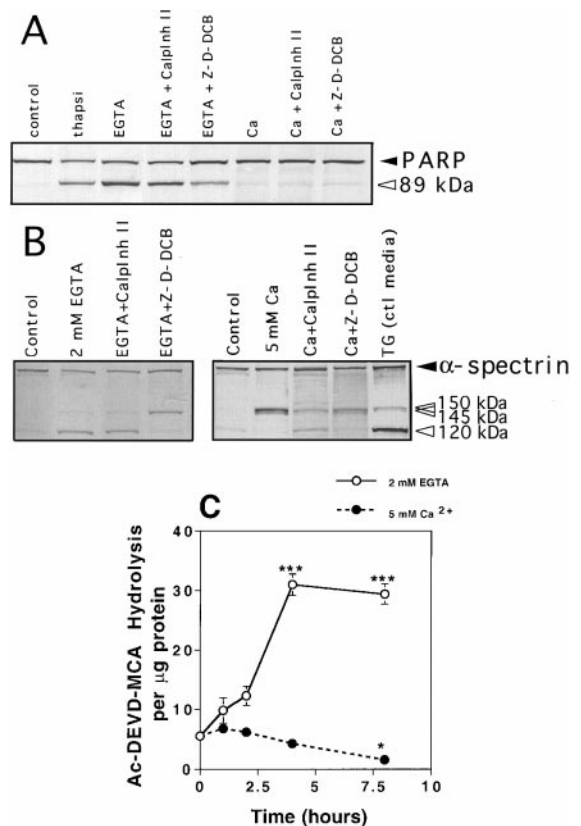
**FIG. 3.** Bisbenzimidate staining in excess  $\text{Ca}^{2+}_e$ -treated SH-SY5Y cells. Cells were untreated (**A**) or treated for 6 h with 5 mM  $\text{CaCl}_2$  (**B**) or 1  $\mu\text{M}$  thapsigargin (**C**) or for 3 h with 0.3 nM maitotoxin (**D**). Labeling with the DNA dye Hoechst 33258 was conducted as described in Materials and Methods. Classic necrotic-like bisbenzimidate staining is shown in cells treated with 5 mM  $\text{CaCl}_2$  (**B**) or 0.3 nM maitotoxin (**D**).

EGTA, we examined the activation of calpain and caspase-3-like proteases. Because EGTA treatment appeared to induce apoptosis, we suspected that caspase-3-like proteases would be activated in EGTA-, but not excess  $\text{Ca}^{2+}_e$ , treated SH-SY5Y cells. The following methods were used to examine caspase-3-like activity: (1) cleavage of the caspase-3-like protease substrates PARP and  $\alpha$ -spectrin to their caspase-3-dependent breakdown products (BDPs); (2) AC-DEVD-MCA hydrolysis, and (3) the appearance of the active caspase-3 fragments. Calpain activity is assessed by monitoring the appearance of a 145-kDa  $\alpha$ -spectrin BDP that is produced only by calpain (Nath et al., 1996b).

**Cleavage of caspase-3-like protease substrates.** We examined cleavage of PARP, an enzyme involved in DNA repair, as an early marker of caspase-3-like activity (Lazebnik et al., 1994; Nicholson et al., 1995). Caspase-3-like proteases cleave PARP at a DEVD consensus site to an 89-kDa BDP. In cells treated with 2 mM EGTA for 24 h, the 89-kDa BDP was readily apparent by western blot analysis of whole cell lysate (Fig. 4A), indicating an increase in caspase-3-like activity as a consequence of EGTA treatment. The enhanced cleavage was not influenced by pretreatment with CalpInh II but was attenuated by pretreatment with the pan-caspase inhibitor Z-D-DCB. Treatment with excess  $\text{Ca}^{2+}_e$  for 24 h did not lead

to elevated PARP fragmentation, compared with control. These data indicate that caspase-3-like proteases were not activated due to elevation of  $\text{Ca}^{2+}_e$ .

The degradation of  $\alpha$ -spectrin to BDPs of 150, 145, and 120 kDa is an established marker for the activation of both calpain and caspase-3 (Bahr et al., 1995; Bartus et al., 1995; Nath et al., 1996b). Our laboratory has established that the 120-kDa spectrin BDP is produced solely by caspase-3-like activity (Wang et al., 1998). Through cleavage at slightly different sites, the 150-kDa  $\alpha$ -spectrin BDP can be potentially produced by either calpain- or caspase-3-mediated proteolysis (Martin et al.,



**FIG. 4.** Caspase-3-like activation in SH-SY5Y cells after EGTA and  $\text{Ca}^{2+}$  challenge. **A:** PARP immunoreactivity in SH-SY5Y cells treated for 24 h with 2 mM EGTA or 5 mM  $\text{CaCl}_2$ . Whole cell lysate (20  $\mu\text{g}$ ) from treated and control cells was subjected to SDS-PAGE (4–20% Tris–glycine gel), electrotransferred to PVDF, and probed with anti-PARP antibody. Caspase-3-like activity was demonstrated by the formation of the 89-kDa PARP BDP. **B:** SH-SY5Y cells were untreated or treated for 24 h with 2 mM EGTA or 5 mM  $\text{CaCl}_2$  in the presence or absence of 50  $\mu\text{M}$  Z-D-DCB or 20  $\mu\text{M}$  CalpInh II. Treatment with 1  $\mu\text{M}$  thapsigargin (TG) was used as a positive control. Whole cell lysate (15  $\mu\text{g}$ /lane) was subjected to western blot analysis with  $\alpha$ -spectrin antibody. Caspase-3-like activity was demonstrated by the formation of the 120-kDa  $\alpha$ -spectrin BDP. **C:** Caspase-3-like activity was directly assayed in SH-SY5Y Triton extracts from cells treated for various times with 2 mM EGTA ( $\circ$ ) or 5 mM  $\text{CaCl}_2$  ( $\bullet$ ). Activity was quantified by monitoring the hydrolysis of the caspase-3-specific fluorogenic substrate Ac-DEVD-MCA. Data are mean  $\pm$  SEM values (\*\* $p < 0.001$ , \* $p < 0.05$ ; ANOVA, with post hoc Tukey–Kramer,  $n = 3$ ).

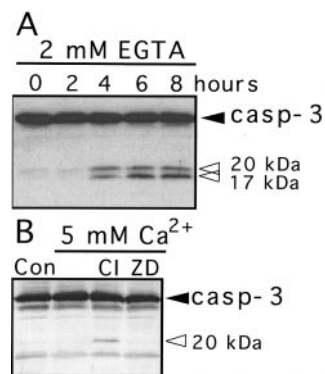
1995; Nath et al., 1996b; Wang et al., 1998). The 145-kDa  $\alpha$ -spectrin BDP is produced only by calpain (Nath et al., 1996b; Wang et al., 1998). This fragmentation pattern makes  $\alpha$ -spectrin degradation a useful tool for simultaneously investigating activation of calpain and caspase-3-like proteases.

We examined the fragmentation pattern of  $\alpha$ -spectrin using western blot analysis of whole cell lysate from EGTA- or excess Ca<sup>2+</sup><sub>e</sub>-treated SH-SY5Y cells. In control cells, only the intact  $\alpha$ -spectrin protein was seen. In some experiments, a low level of 150- and 120-kDa fragments was apparent in the control sample. EGTA treatment resulted in the appearance of predominantly the 120-kDa caspase-3-specific BDP (Fig. 4B). A low level of the 150-kDa fragment is apparent but is unlikely to be caused by calpain activity, as it was not reduced with calpain inhibitor II. Pretreatment with Z-D-DCB, the pan-caspase inhibitor, blocked the formation of the 120-kDa BDP and enhanced the formation of the 150-kDa BDP, which is a precursor for the 120-kDa BDP (Wang et al., 1998). In the presence of Z-D-DCB, this final processing is prevented. No 145-kDa BDP was formed in the presence of 2 mM EGTA, indicating that calpain was not activated.

In cells treated with excess Ca<sup>2+</sup><sub>e</sub>, the predominant  $\alpha$ -spectrin fragments were of 150 and 145 kDa. Formation of the 145-kDa BDP after excess Ca<sup>2+</sup><sub>e</sub> is much more intense than in the thapsigargin-treated apoptotic control. No 120-kDa BDP appears in Ca<sup>2+</sup><sub>e</sub>-treated cells, consistent with the absence of caspase activity. In the presence of CalpInh II, the 150-kDa BDP was ablated and the 145-kDa BDP was attenuated, suggesting that the breakdown is due to calpain activation with no caspase component. Thus, in SH-SY5Y cells exposed to 5 mM Ca<sup>2+</sup><sub>e</sub>, calpain, but not caspase-3, is active. Note that in the presence of CalpInh II the 120-kDa band is formed. CalpInh II alone shows some toxicity in these cells.

**Direct measurement of caspase-3-like activity.** Caspase-3-like activity was directly examined in cell lysates from EGTA- or Ca<sup>2+</sup><sub>e</sub>-treated SH-SY5Y cells. Activity was measured at 0, 1, 2, 4, and 8 h after treatment, using the fluorogenic caspase-3-like protease substrate Ac-DEVD-MCA (Nath et al., 1996b; Posmantur et al., 1997). An increase in caspase-3-like activity is apparent 2 h after treatment but is not significant until 4 h after start of treatment ( $5.56 \pm 0.98$  fluorescent U/ $\mu$ g of protein for control vs.  $30.9 \pm 1.8$  for EGTA treated). Caspase-3-like activity in EGTA-treated SH-SY5Y cells peaked at 4 h, followed by a slow decline (Fig. 4C). In SH-SY5Y cells exposed to excess Ca<sup>2+</sup><sub>e</sub>, no increase in caspase-3-like activity occurred. In fact, there was a gradual loss of caspase-3-like activity over 8 h.

**Processing of caspase-3.** To confirm that caspase-3 is activated in EGTA- but not excess Ca<sup>2+</sup><sub>e</sub>-challenged SH-SY5Y cells, we examined the processing of pro-caspase-3 to its active fragments. After activation, caspase-3 is proteolyzed to fragments of 17 kDa, probably corresponding to the large subunit of the active enzyme, and 11 kDa. A 20-kDa species is most likely the



**FIG. 5.** Caspase-3 processing in EGTA- and excess Ca<sup>2+</sup><sub>e</sub>-treated SH-SY5Y cells. **A:** SH-SY5Y cells were untreated or treated with 2 mM EGTA for various lengths of time. Whole cell lysate was subjected to SDS-PAGE (4–20% acrylamide, Tris-glycine gels), electrotransferred to PVDF, and probed with caspase-3 antibody. Protein was visualized with ECL. **B:** SH-SY5Y cells were untreated or treated for 24 h with 5 mM CaCl<sub>2</sub> in the presence or absence of 50  $\mu$ M Z-D-DCB or 20  $\mu$ M CalpInh II. Cells were subjected to western blot analysis as in A.

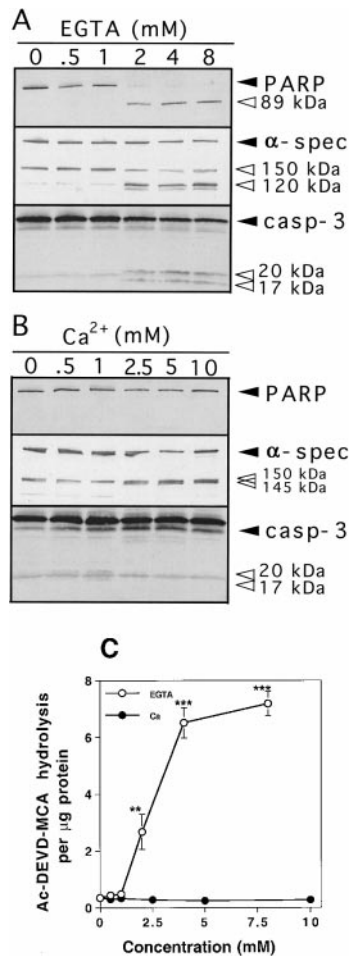
17-kDa fragment plus the precursor propiece (Fernandes-Alnemri et al., 1994; Schlegel et al., 1996). The appearance of the 20- and 17-kDa fragments by 4 h of EGTA treatment was consistent with the increase in Ac-DEVD-MCA hydrolytic activity (compare with Fig. 4C). Because overexposure of the ECL blot was required to visualize the caspase-3 fragments, the level of intact caspase-3 does not appear to decrease (Fig. 5A).

The processing of caspase-3 in Ca<sup>2+</sup><sub>e</sub>-treated SH-SY5Y cells was also examined. No caspase-3 processing in Ca<sup>2+</sup><sub>e</sub>-treated cells was detected up to 24 h of treatment (Fig. 5B). This finding is consistent with the lack of caspase-3-like activity seen with direct measurement and by monitoring PARP cleavage. Some caspase-3 processing was found in cells treated with CalpInh II, consistent with the appearance of the 120-kDa  $\alpha$ -spectrin BDP (Fig. 4B).

#### Concentration dependency of EGTA- and excess Ca<sup>2+</sup><sub>e</sub>-mediated caspase and calpain activation

We investigated the minimum concentration of each challenge required for protease activation. We examined PARP,  $\alpha$ -spectrin, and pro-caspase-3 processing after 24 h of treatment. The minimum concentration of EGTA required to activate caspase-3 is 2 mM (Fig. 6A). There was no loss of intact PARP,  $\alpha$ -spectrin, or pro-caspase-3 or formation of BDPs at EGTA concentrations of <2 mM. This is consistent with our observation that 2 mM EGTA is the minimum required concentration that is consistently cytotoxic to SH-SY5Y cells.

In cells treated with varying concentrations of Ca<sup>2+</sup><sub>e</sub>, no PARP processing was observed at any Ca<sup>2+</sup><sub>e</sub> concentration (Fig. 6B). Formation of the calpain-dependent 145-kDa  $\alpha$ -spectrin BDP began with a 2.5 mM Ca<sup>2+</sup><sub>e</sub> challenge. The activation of calpain has been correlated with necrotic death, although at concentrations of <5 mM, we did not consistently observe significant cytotox-



**FIG. 6.** Caspase-3-like activation in SH-SY5Y cells after different concentrations of EGTA and Ca<sup>2+</sup> challenge. PARP,  $\alpha$ -spectrin, and caspase-3 immunoreactivity in SH-SY5Y cells treated for 24 h with indicated concentrations of EGTA (**A**) or Ca<sup>2+</sup> (**B**). Whole cell lysate (20  $\mu$ g) from treated and control cells was subjected to SDS-PAGE (4–20% Tris–glycine gel), electrotransferred to PVDF, and probed for indicated protein. **C**: Caspase-3-like activity was directly assayed in SH-SY5Y extracts from cells treated with various concentrations of EGTA (○) or CaCl<sub>2</sub> (●). Activity was quantified by monitoring the hydrolysis of Ac-DEVD-MCA. Data are mean  $\pm$  SEM values (\*\**p* < 0.01, \*\*\**p* < 0.001 vs. control; ANOVA with post hoc Tukey–Kramer, *n* = 6).

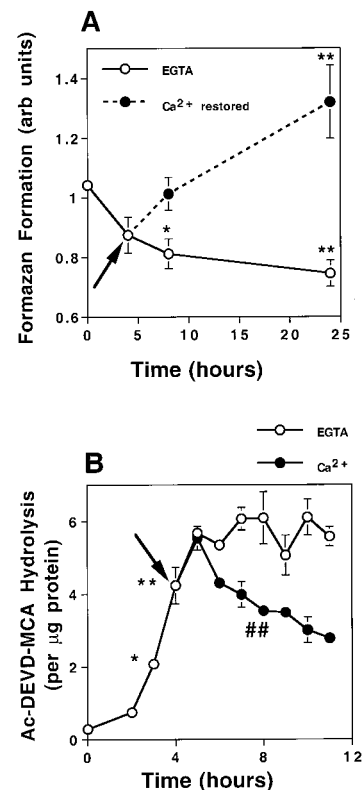
icity. It is interesting that at 0.5 and 1 mM Ca<sup>2+</sup>, there was some caspase-3 processing. This minor level of caspase-3 processing did not correlate with increased PARP or  $\alpha$ -spectrin fragmentation (Fig. 6B).

We also monitored changes in Ac-DEVD-MCA hydrolysis in cells treated with various concentrations of EGTA or Ca<sup>2+</sup>. We assayed activity after 6 h rather than 4 h of treatment to allow for potentially slower onset of caspase-3-like activity with lower concentrations of challenge (Fig. 6C). Consistent with formation of caspase-3-dependent fragments, Ac-DEVD-MCA hydrolysis was significant compared with control at 2 mM EGTA ( $2.68 \pm 0.61$  vs.  $0.35 \pm 0.01$  fluorescence U/ $\mu$ g of protein, *p*

< 0.001) and increased with higher concentrations. Excess Ca<sup>2+</sup><sub>e</sub> did not produce an increase in Ac-DEVD-MCA hydrolysis at any concentration tested.

#### Cell death is due to alterations of Ca<sup>2+</sup>

We wanted to determine whether cell death caused by alterations of Ca<sup>2+</sup><sub>e</sub> could be attenuated or blocked by reversing or preventing the changes in Ca<sup>2+</sup><sub>i</sub>. To examine whether EGTA-mediated apoptosis was caused by the loss of Ca<sup>2+</sup>, rather than another factor such as a change in pH, Ca<sup>2+</sup> was added back to the culture medium at the time of peak caspase-3-like activity. Cell viability was assessed by monitoring the conversion of tetrazolium salt (MTT) to colored formazan, which takes place in healthy mitochondria (Fig. 7A) (Hansen et al., 1989). This assay correlates mitochondrial function with



**FIG. 7.** Restoration of Ca<sup>2+</sup><sub>e</sub> in EGTA-treated SH-SY5Y cells attenuates cell death and caspase-3-like activity. **A**: Cells were untreated or treated with 2 mM EGTA (○). In some wells, 2 mM CaCl<sub>2</sub> was added after 4 h of EGTA exposure (●). Cell viability was monitored by using the MTT method as described in Materials and Methods. Arrow indicates point at which Ca<sup>2+</sup><sub>e</sub> was restored. Data are mean  $\pm$  SEM values (\**p* < 0.05; \*\**p* < 0.01 vs. time 0; ANOVA with post hoc Tukey–Kramer, *n* = 4 or 5). **B**: Hydrolysis of the caspase-3-selective fluorogenic substrate Ac-DEVD-MCA in EGTA-treated SH-SY5Y cells was monitored over time. Cells were either treated with 2 mM EGTA for up to 11 h (○) or Ca<sup>2+</sup><sub>e</sub> is restored by addition of 2 mM CaCl<sub>2</sub> at 4 h (●). Whole cell lysate (50  $\mu$ l) sampled directly from the treatment wells was incubated with 40 mM Ac-DEVD-MCA in 250  $\mu$ l final volume. Arrow indicates point at which Ca<sup>2+</sup><sub>e</sub> was restored. Data are mean  $\pm$  SEM values (\**p* < 0.05, \*\**p* < 0.01 vs. time 0; ##*p* < 0.01 vs. 5 h of EGTA; ANOVA with post hoc Tukey–Kramer, *n* = 4).

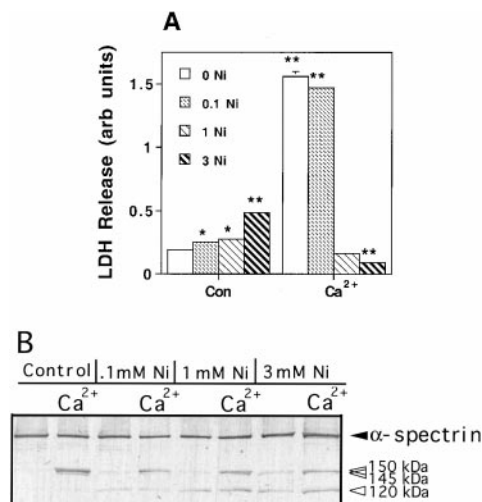
cell viability and monitors the entire population within a well. We attempted to quantify individual cell death by trypan blue exclusion, but we had an unacceptable level of false positives within the control population.

After 4 h of EGTA treatment, 2 mM CaCl<sub>2</sub> was added to the media, a concentration well below the 5 mM Ca<sup>2+</sup> used to kill the cells in other experiments. The viability of control cells with 2 mM Ca<sup>2+</sup> added was no different from untreated controls (data not shown). In EGTA-treated cells, there was a significant loss of cell viability after 8 h followed by a constant decline throughout the 24 h of incubation. Addition of 2 mM CaCl<sub>2</sub> at 4 h, however, restored cell viability to control levels within 4 h. We interpret these results to mean that with the addition of 2 mM Ca<sup>2+</sup>, mitochondrial function was restored or maintained in the subset of cells that had not progressed beyond the point-of-no-return for apoptosis, which may be loss of mitochondrial integrity and release of cytochrome c into the cytoplasm (Green and Kroemer, 1998). The very large increase in cell viability at 24 h may be attributable to increased cell number.

We then examined whether addition of Ca<sup>2+</sup> would alter the elevated caspase-3-like activity in EGTA-treated cells. Cells were treated with 2 mM EGTA for 4 h after which 2 mM CaCl<sub>2</sub> was added. In EGTA-treated cells, caspase-3-like activity was significantly elevated over untreated controls by 3 h after start of treatment (2.08 ± 0.13, EGTA-treated vs. 0.31 ± 0.05, controls, *p* < 0.05) and increased to 5 h. At 4 h of EGTA treatment, 2 mM CaCl<sub>2</sub> was added to some wells. Caspase-3-like activity increased for 1 more hour, then was significantly decreased 2 h after Ca<sup>2+</sup> addition (Fig. 7B). The caspase-3-like activity steadily declined throughout the 6 remaining hours of incubation compared with the elevated activity of the EGTA-treated cells. We interpret the decrease in caspase-3-like activity in cells treated with Ca<sup>2+</sup> to mean that the activity for the whole population declines, rather than activity of individual cells. Cells that have not yet entered the apoptotic pathway are rescued by the addition of extracellular Ca<sup>2+</sup>.

The contribution of elevated Ca<sup>2+</sup><sub>e</sub> to cell death was assessed by directly blocking Ca<sup>2+</sup> entry to the cells with Ni<sup>2+</sup>. Ca<sup>2+</sup> (5 mM) and 0.1, 1, or 3 mM Ni<sup>2+</sup> was added to the cells and LDH release was measured 12 h later. As shown in Fig. 8A, at 12 h of incubation both 1 and 3 mM Ni<sup>2+</sup>, but not 0.1 mM Ni<sup>2+</sup>, strongly protected against LDH release in cells incubated with excess Ca<sup>2+</sup><sub>e</sub>. It is interesting that although 3 mM Ni<sup>2+</sup> was toxic on its own, it produced significant protection in the presence of 5 mM excess Ca<sup>2+</sup>. The protective effect of Ni<sup>2+</sup> was overcome by 24 h of excess Ca<sup>2+</sup><sub>e</sub> challenge (data not shown).

Protease activity in cells challenged with excess Ca<sup>2+</sup><sub>e</sub> in the presence of Ni<sup>2+</sup> was assessed by monitoring α-spectrin degradation (Fig. 8B). SH-SY5Y cells were untreated or treated for 12 h with 5 mM Ca<sup>2+</sup><sub>e</sub> in the presence or absence of Ni<sup>2+</sup>. Whole cell lysate was subjected to western blot analysis. Treatment with Ni<sup>2+</sup> alone leads to the concentration-dependent formation of

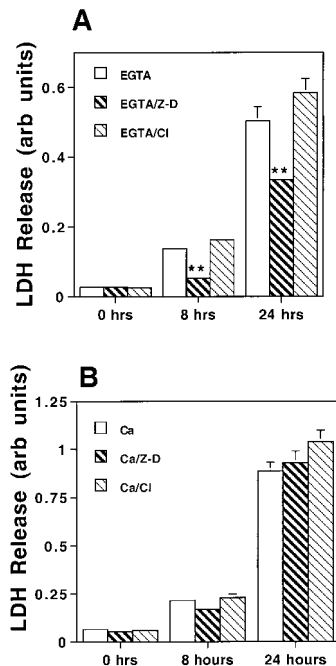


**FIG. 8.** Ni<sup>2+</sup> protects against excess Ca<sup>2+</sup>-mediated cell death. **A:** SH-SY5Y cells were treated for 12 h with 5 mM CaCl<sub>2</sub> in the absence or presence of either 0.1 mM, 1 mM, or 3 mM Ni<sup>2+</sup>. Cell death was assessed by measurement of LDH release. Data are mean ± SEM values (\**p* < 0.05, \*\**p* < 0.0001 vs. control; Student's *t* test, *n* = 6). **B:** α-Spectrin immunoreactivity in SH-SY5Y cells treated for 12 h with 5 mM CaCl<sub>2</sub> in the presence or absence of varying concentrations of NiCl<sub>2</sub>. Whole cell lysate (20 μg) from treated and control cells was subjected to SDS-PAGE, electrotransferred to PVDF, and probed for α-spectrin immunoreactivity.

the 120-kDa α-spectrin BDP. Caspase-3 activation in Ni<sup>2+</sup>-treated cells is consistent with the activation of caspase-3 that accompanies the EGTA-mediated loss of Ca<sup>2+</sup><sub>i</sub> homeostasis. In SK-N-SH cells, the parent cell line of SH-SY5Y, 3 mM Ni<sup>2+</sup> causes a decrease in Ca<sup>2+</sup><sub>i</sub> concentration (McGinnis et al., 1998). Formation of the 145-kDa α-spectrin BDP is attenuated in the presence of Ni<sup>2+</sup> in excess Ca<sup>2+</sup><sub>e</sub>-treated cells.

#### Effect of protease inhibition on LDH release in EGTA- or excess Ca<sup>2+</sup><sub>e</sub>-treated SH-SY5Y cells

Calpain was selectively increased in cells incubated in excess Ca<sup>2+</sup><sub>e</sub> and caspase-3-like activity was selectively elevated in EGTA-treated cells. We examined whether blockade of these protease activities would protect against Ca<sup>2+</sup>- or EGTA-mediated cell death. To assess the role of calpain and caspases in EGTA-mediated cell death, cells were incubated with 2 mM EGTA in the presence or absence of the pan-caspase inhibitor Z-D-DCB or the calpain inhibitor CalpInh II. Cell death was assessed by measuring LDH release. As shown in Fig. 9A, Z-D-DCB was able to significantly protect against EGTA-mediated LDH release at both 8 and 24 h of incubation. The protective effect of Z-D-DCB diminished after 24 h compared with 8 h. We have previously shown (Nath et al., 1996a) that Z-D-DCB does not produce complete inhibition of caspase-3-like proteases as evidenced by its failure to fully prevent formation of the 89-kDa PARP BDP after 24 h of EGTA exposure (Fig. 4A). As expected, calpain inhibition had no effect on EGTA-mediated cell death.



**FIG. 9.** Effect of caspase and calpain inhibition on cell death induced by EGTA or excess  $\text{Ca}^{2+}_e$ . SH-SY5Y cells were incubated with 2 mM EGTA (**A**) or 5 mM  $\text{CaCl}_2$  (**B**) in the absence or presence of 50  $\mu\text{M}$  of the caspase inhibitor Z-D-DCB or the calpain inhibitor CalpInh II. LDH release was measured at 0, 8, and 24 h. Results are given as mean  $\pm$  SEM values (\*\* $p < 0.001$  vs. EGTA-treated; Student's  $t$  test,  $n = 6$ ).

To assess the role of the proteases in excess  $\text{Ca}^{2+}_e$ -mediated cell death, cells were incubated with 5 mM  $\text{Ca}^{2+}_e$  in the presence or absence of the protease inhibitors. As shown in Fig. 9B, neither Z-D-DCB nor CalpInh II was able to protect against the  $\text{Ca}^{2+}_e$ -mediated cell death.

## DISCUSSION

Using a single cell type, we have demonstrated that either increasing or decreasing extracellular  $\text{Ca}^{2+}$  will lead directly to cell death in neuronal cells but that the mechanisms of cell death are different. We have strong evidence demonstrating that removal of  $\text{Ca}^{2+}_e$  with EGTA induces apoptotic cell death in the human neuroblastoma SH-SY5Y cells. First, incubation with EGTA led to the DNA laddering characteristic of apoptosis. Second, caspase-3-like activity was demonstrated by the caspase-specific cleavage of the substrate PARP and  $\alpha$ -spectrin, by the hydrolysis of the fluorogenic substrate Ac-DEVD-MCA and by the appearance of the active 17-kDa fragment of caspase-3. Third, caspase inhibition protected against the EGTA-mediated loss of cell viability. Conversely, high  $\text{Ca}^{2+}_e$  induced necrotic cell death in SH-SY5Y cells. Calpain activation, which has been strongly correlated with necrotic states (Wang et al., 1996b; Widdowson et al., 1997) did not occur in EGTA-treated cells but was readily apparent in cells treated with

excess  $\text{Ca}^{2+}_e$ . There was no evidence of apoptotic cell death in cells treated with excess  $\text{Ca}^{2+}_e$ . Treatment of SH-SY5Y cells with EGTA or excess  $\text{Ca}^{2+}_e$  represents relatively pure models for apoptotic and necrotic cell death, respectively, in neurons.

Although there are examples in the literature of both increases (Furuya et al., 1994; Zhu and Loh, 1995) and decreases (Waterhouse et al., 1996; Chiesa et al., 1998; Mizuno et al., 1998) in  $\text{Ca}^{2+}_e$  leading to apoptosis, in neurons it is generally decreases in cell  $\text{Ca}^{2+}$  that lead to apoptotic cell death (Koh and Cotman, 1992; Franklin et al., 1995; Tong et al., 1996). Apoptosis is elicited in cultured neurons by removing  $\text{Ca}^{2+}$  from the media (Koh and Cotman, 1992) or by removing increased  $\text{K}^+$ , which maintains high intracellular  $\text{Ca}^{2+}$  often needed for survival of cultured neurons (Franklin et al., 1995). Our experiment using restoration of  $\text{Ca}^{2+}_e$  clearly demonstrates that loss of  $\text{Ca}^{2+}_e$  is the mediator of EGTA-induced apoptosis. We have found, however, that treatment of SH-SY5Y cells with thapsigargin elicits apoptosis (Nath et al., 1997). Thapsigargin blocks the endoplasmic reticulum  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase, depleting intracellular stores of calcium and leading to an increase in intracellular  $\text{Ca}^{2+}$  (Thastrup et al., 1990). Although it seems contradictory that both EGTA and thapsigargin elicit apoptosis, the important factor may be the change in intracellular architecture caused by the depletion of intracellular stores, rather than changes in cytosolic  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  is slowly lost from the intracellular stores of cells exposed to millimolar concentrations of EGTA (Conus et al., 1998). In addition, in preliminary experiments we found that BAPTA/AM, a cell-permeant intracellular  $\text{Ca}^{2+}$  chelator, mediated concentration-dependent activation of caspase-3-like proteases and provided no protection against thapsigargin-mediated cell death in SH-SY5Y cells (unpublished data). BAPTA also depletes intracellular  $\text{Ca}^{2+}$  stores by chelating  $\text{Ca}^{2+}_i$ , which has leaked from intracellular stores and prevents refilling of the stores (Malayev and Nelson, 1995).

Depletion of endoplasmic reticulum  $\text{Ca}^{2+}$  such as in EGTA, thapsigargin, or BAPTA/AM-treated cells may lead to cytotoxicity independently of any changes in intracellular  $\text{Ca}^{2+}$  concentration.  $\text{Ca}^{2+}$  maintains the architecture of the endoplasmic reticulum. The loss of  $\text{Ca}^{2+}$  may lead to dissociation of the endoplasmic reticulum, which can be reversed in the presence of  $\text{Ca}^{2+}$  (Koch et al., 1988). Loss of endoplasmic reticulum  $\text{Ca}^{2+}$  is associated with dramatic decreases in protein synthesis (Bronstrom and Bronstrom, 1990) and has been linked to the release of a deoxyribonuclease, which may contribute to apoptosis (Peitsch et al., 1993). The loss of endoplasmic reticulum  $\text{Ca}^{2+}$  was proposed as the cause of apoptosis in HL60 leukemia cells (Zhu and Loh, 1995) and GT1-7 hypothalamic cells (Wei et al., 1998). Furthermore, in GT1-7 cells bcl-2 protects against thapsigargin-mediated apoptosis without affecting changes in  $[\text{Ca}^{2+}]_i$  (Wei et al., 1998).

We found that excessive extracellular  $\text{Ca}^{2+}$  leads to cell death by necrosis in SH-SY5Y cells. The high  $\text{Ca}^{2+}_e$

led to higher levels of intracellular Ca<sup>2+</sup>, which activated the Ca<sup>2+</sup>-dependent protease calpain and initiated necrosis. Our finding that blocking Ca<sup>2+</sup> channels with the nonspecific Ca<sup>2+</sup> channel inhibitor Ni<sup>2+</sup> protects against high Ca<sup>2+</sup><sub>e</sub>-mediated cell death supports this contention. In addition, in previous studies we found that treatment of cells with the Ca<sup>2+</sup> channel activator maitotoxin led to necrosis (Wang et al., 1996b) as shown in Fig. 2. Physiologically, an excessive increase in intracellular Ca<sup>2+</sup> can result from activation of excitatory amino acid receptors, especially glutamate receptors. Continued activation of these receptors leads to persistent Ca<sup>2+</sup> entry and resultant cell death. Although there may be an apoptotic component, evidence suggests that cell death from excitatory amino acids is primarily necrotic (Ikeda et al., 1996; Gwag et al., 1997) and attributable to increased intracellular Ca<sup>2+</sup> levels (Mattson, 1990; Clementi et al., 1996).

An interesting result of this study is that the cysteine proteases caspase-3 and calpain were selectively activated by the alterations in Ca<sup>2+</sup><sub>e</sub>. Caspases have been identified as mediators in apoptotic cell death in many types of cells, so it was not surprising that we found EGTA treatment increased caspase-3-like activity and the caspase inhibitor could protect against EGTA-mediated cell death. Activation of calpain during apoptosis, however, is more cell-type specific. Calpain is activated in staurosporine-induced apoptosis in SH-SY5Y cells and cerebellar granule cells in which apoptosis is induced by removal of high K<sup>+</sup>. It is interesting that calpain inhibitors were able to provide protection against low K<sup>+</sup>-induced apoptosis in cerebellar granule cells but provided only minimal protection against staurosporine-induced apoptosis in SH-SY5Y cells (Nath et al., 1996a). This suggests that a role for calpain in mediating or contributing to apoptotic cell death is likely cell-type specific. Calpain is triggered during necrosis induced by the Ca<sup>2+</sup> channel activator maitotoxin (Wang et al., 1996b), which concurs with our finding of calpain activation resulting from excess Ca<sup>2+</sup><sub>e</sub>. However, calpain inhibition had no protective effect in the excess Ca<sup>2+</sup><sub>e</sub>-induced cell death in SH-SY5Y cells. Calpain inhibition can, however, attenuate hypoxic/hypoglycemic injury to cerebrotical neurons in culture and excitotoxic injury to Purkinje cells in cerebellar slices (Wang et al., 1996a).

Our studies support the growing amount of evidence suggesting that perturbation of intracellular Ca<sup>2+</sup> homeostasis may be a common and early step in the development of cytotoxicity especially in neurons and immune cells (Orrenius and Nicotera, 1994). We have shown that removal of Ca<sup>2+</sup><sub>e</sub> from the neuroblastoma culture medium leads to apoptotic cell death with activation of caspase-3-like protease activity. On the contrary, excessive Ca<sup>2+</sup><sub>e</sub> led to cell death that was likely necrotic with calpain activation and no indication of apoptosis. These models could be highly useful in the investigation of mechanisms of apoptotic and necrotic cell death in neurons and the selective activation of the cysteine proteases calpain and caspase-3.

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