Evidence for Activation of Caspase-3-Like Protease in Excitotoxin- and Hypoxia/Hypoglycemia-Injured Neurons

*Rathna Nath, *Albert Probert, Jr., †Kim M. McGinnis, and *†Kevin K. W. Wang

*Laboratory of Neuro-biochemistry, Department of Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Warner-Lambert Company; and †Department of Pharmacology, University of Michigan School of Medicine, Ann Arbor, Michigan, U.S.A.

Abstract: Caspase activation has been shown to be a critical step in several models of neuronal apoptosis such as staurosporine treatment of human neuroblastoma SH-SY5Y cells and potassium deprivation of rat cerebellar granule neurons. One common event is the appearance of caspase-mediated 120-kDa nonerythroid α-spectrin breakdown product (SBDP120). Second, inhibitors of the caspase family are effective blockers of such neuronal death. In this study, we report the appearance of caspase-mediated SBDP120 in excitotoxin-challenged fetal rat cerebrocortical neurons [N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainate] and rat cerebellar granule neurons (NMDA and kainate). A general caspase inhibitor, carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-D-DCB), blocked the formation of SBDP120 under these conditions and attenuated the observed NMDA-induced lactate dehydrogenase (LDH) release in both cell types. Furthermore, hydrolytic activity toward a caspase-3-preferred synthetic peptide substrate, acetyl-DEVD-7-amido-4-methylcoumarin, was significantly elevated in NMDA-treated granule neurons. Lastly, oxygen–glucose deprivation (OGD)-challenged cerebrocortical cultures also showed the appearance of SBDP120. Again, Z-D-DCB blocked the SBDP120 formation as well as attenuated the LDH release from the OGD-challenged neurons. Taken together, the presence of caspase-specific SBDP120 and the neuroprotective effects of Z-D-DCB strongly suggest that caspase activation contributes at least in part to excitotoxin- and OGD-induced neuronal death. **Key Words:** Caspase—ICE-like—Excitotoxicity—Neuronal death—Protease inhibitor.
J. Neurochem. **71,** 186–195 (1998).

Apoptosis is a form of programmed cell death characterized by (a) cytoplasmic shrinkage, (b) nuclear DNA cleavages at the nucleosome linkage regions giving rise to the "DNA laddering" phenomenon when chromosomal DNA extract is electrophoresed on an agarose gel, and (c) chromatin condensation along the inside surface of the nuclear envelope (as a result of DNA fragmentation). Linnik et al. (1993) were the first group to report evidence for apoptosis in a rat

global focal model by showing the presence of DNA laddering. Since then numerous reports have appeared documenting similar findings in either focal ischemia or kainate toxicity models (Héron et al., 1993; MacManus et al., 1993; Filipkowski et al., 1994; Li et al., 1995; Chen et al., 1997). On the other hand, several research groups found little or no evidence of classic apoptosis (Dessi et al., 1993; Scott and Hegyi, 1997; MacManus et al., 1997). Terminal deoxyribonucleotidyl transferase-mediated biotin-16-dUTP nick end-labeling (TUNEL) is one method that has been misused for positive identification of apoptosis as it detects only the increase in content of fragmented DNA, which occurs in both apoptosis and necrosis (Charriaut-Marlangue and Ben-Ari, 1995). Regarding excitotoxicity, Bonfoco et al. (1995) reported that high and low levels of excitotoxin challenge to neuronal cultures will result in necrosis and apoptosis, respectively (based on nuclear morphology). Yet, other research groups found little evidence for apoptosis when cerebrocortical cultures or cerebellar granule neurons were challenged with excitotoxins (Dessi et al., 1993; Héron et al., 1993; MacManus et al., 1997; Portera-Cailliau and Price, 1997). It is clear that this area of research will benefit from a more definitive, preferably biochemical marker to define the presence of apoptosis.

In the last few years, an emerging theme in apoptosis is the activation of the caspase protease family (Nich-

olson and Thornberry, 1997). For example, caspase-3 (previously called CPP32, Yama, or apopain) is found to be almost universally activated in numerous cell types when subjected to various apoptotic challenges. Three biochemical events are observed during caspase-3 activation: (a) processing of intact 32-kDa caspase-3 to the 17- and 12-kDa dimeric form (Nicholson et al., 1995; Posmantur et al., 1997), (b) fragmentation of the 113-kDa poly(ADP-ribose) polymerase (PARP) to an 89-kDa form (Lazebnik et al., 1994), and (c) processing of nonerythroid α-spectrin (α-spectrin; 280 kDa) into a 120-kDa spectrin breakdown product (SBDP120) (Martin et al., 1995). In fact, recently caspase-3, PARP, and α-spectrin processing have been reported in several neuronal apoptosis models, such as cultured neuroblastoma or neuronal cells exposed to staurosporine as well as cerebellar granule neurons subjected to potassium deprivation (Koh et al., 1995; Nath et al., 1996a; Wiesner and Dawson, 1996; Armstrong et al., 1997; Ni et al., 1997). In our previous study, we reported that although both calpain and caspase are capable of fragmenting α-spectrin, only caspase produces the distinctive SBDP120 (Nath et al., 1996a). We also found that the SBDP120 was observed only in apoptotic neurons but not in necrotic neurons (Nath et al., 1996a), which is consistent with the fact that caspase is only activated in apoptosis but not in necrosis (Armstrong et al., 1997). Using SBDP120 as a marker for apoptosis has the added advantage that commercial anti-α-spectrin antibodies react with rat, e.g., cerebellar granule neurons, and human, e.g., SH-SY5Y cells, α-spectrin (Martin et al., 1995; Nath et al., 1996a). Pharmacologically, several recently available caspase-specific inhibitors [such as carbobenzoxy-Asp-CH₂OC(O)-2,6-dichlorobenzene (Z-D-DCB), acetyl-YVAD-chloromethyl ketone, and carbobenzoxy-Val-Ala-Asp(OMe)-fluoromethyl ketone] are found to be excellent neuronal apoptosis inhibitors (Loddick et al., 1996; Nath et al., 1996a,b; Schulz et al., 1996; Hara et al., 1997). Also, they were ineffective against necrosis (Armstrong et al., 1997). Therefore, these agents are also useful in defining the "apoptotic" component in a given form of neuronal injury.

In this study using both the fetal rat cerebrocortical cultures and rat cerebellar granule neurons, we examine the evidence for a caspase-mediated apoptotic component in excitotoxicity and hypoxic/hypoglycemic injury by monitoring the presence of SBDP120 and the effects of a cell-permeable caspase inhibitor, Z-D-DCB.

MATERIALS AND METHODS

Excitotoxicity in fetal rat cerebrocortical mixed cultures

All procedures described in this study were carried out in compliance with the NIH *Guide for the Care and Use of Laboratory Animals*, guidelines from the Society for Neuro-

science, and the Parke-Davis Animal Use Committee. Cerebrocortical cells were harvested from fetal rats (Sprague–Dawley) on day 18 of gestation and cultured with Dulbecco's modified Eagle's medium/F12 medium containing 10% horse and 6% fetal bovine serum (heat-inactivated) in 96-well poly-L-lysine-coated plates as described previously (Hajimohammadreza et al., 1995). Nonneuronal cell division was halted 3 days into culture by adding 35 μg/ml uridine and 15 μg/ml 5-fluoro-2'-deoxyuridine. On day 17 postplating, the cultures were washed three times with serum-free medium. Caspase inhibitor (Z-D-DCB) (Dolle et al., 1994) (made in-house at Parke-Davis) or calpain inhibitor I was added at this point for a 1-h preincubation, if desired. The cultures were then challenged with various excitotoxins as follows: 200 μM N-methyl-D-aspartate (NMDA) for 15 min (unless stated otherwise) before replacing with normal serum-free medium for 16 h, 200 μM α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or 200 μM kainate in serum-free medium for 16 h, or 1 nM maitotoxin or 0.5 μM staurosporine for 3 h before replacing with normal serum-free medium for 16 h. All cultures were maintained in a 37°C oxygenated incubator (21% O_2, 8% CO_2, and 71% N_2) except during medium changes. Various concentrations of NMDA have also been included. Under these conditions, we and others have reported that the neuronal population is selectively vulnerable to the applied excitotoxins. Neuronal death was assessed by measuring activity of the cytosolic enzyme lactate dehydrogenase (LDH) released into the medium as described previously (Koh and Choi, 1987; Hajimohammadreza et al., 1995). Cellular proteins were extracted at 16 h as described previously (Wang et al., 1996a). We chose this time point for protein extraction to standardize better our comparison of proteolysis patterns with different neurotoxic challenges.

Excitotoxicity in cerebellar granule neurons

Cerebellar granule neurons were isolated from 7-day-old rat pups (Sprague–Dawley; Charles River Laboratories). The meninges were removed from the cerebellum, and the minced tissue was trypsinized (0.25 mg/ml) for 15 min at 37°C. The trypsinized tissue was then washed in 0.4 mg/ml DNase I for 5 min at 1,000 rpm. The tissue was triturated three times in 0.4 mg/ml DNase I. The combined supernatant was divided into four centrifuge tubes, and 3 ml of 4% (wt/vol) bovine serum albumin/1 mM MgSO₄ was layered underneath the supernatant. The cells were then centrifuged at 600 g and washed in HEPES-buffered salt solution (pH 7.3; 20 mM HEPES and 0.59 mM EDTA) for 5 min at 600 g. The pellet was resuspended and cultured (about one cerebellum per 12-well plate) in 2 ml of feeding medium (Dulbecco's modified Eagle's medium plus 30 mM KCl, 10% fetal bovine serum, 5 mg/ml insulin, and 100 U/ml penicillin/streptomycin) in a 37°C humidified 5% CO_2 incubator. Half of the medium was replaced with fresh medium containing 20 μM cytosine arabinoside after 24 h. More than 95% population of cells in these cultures are neurons, based on routine inspection. At the beginning of an experiment, 7-day-old cultures were washed three times with serum-free Dulbecco's modified Eagle's medium without serum and pretreated with calpain inhibitor II (N-acetyl-Leu-Leu-Met-H; Calbiochem) or the caspase inhibitor Z-D-DCB for 2 h (if desired). The cultures were then washed with serum-free Dulbecco's modified Eagle's medium containing 30 mM KCl and challenged with 200 μM NMDA, AMPA (with 30 μM cyclothiazide), or kainate or 5 mM KCl (low K⁺) (Nath

et al., 1996*b*). Cultures were maintained for 16 h, at which time cell viability was monitored and protein extraction was performed. For cell viability here, owing to the lower plating density compared with the cerebrocortical cultures, we used the CytoTox 96 cytotoxicity kit (Promega) to measure LDH release into the medium.

Oxygen–glucose deprivation (OGD) in cerebrocortical cultures

Cerebrocortical cells were harvested from fetal rats (Sprague–Dawley) on day 18 of gestation and cultured with Dulbecco's modified Eagle's medium/F12 medium containing 10% horse and 6% fetal bovine serum (heat-inactivated) in 96-well poly-L-lysine-coated plates as described previously (Hajimohammadreza et al., 1995). Nonneuronal cell division was halted 3 days into culture by adding 35 μg/ml uridine and 15 μg/ml 5-fluoro-2'-deoxyuridine. On day 17 postplating, the cultures were washed three times with serum-free medium. Z-D-DCB (30–100 μM), carbobenzoxy-Val-Ala-Asp(OEt)-CH$_2$OC(O)-2,6-dichlorobenzene [Z-VAD(OEt)-DCB] (30 μM), calpain inhibitor I (10 μM), or the NMDA receptor antagonist ($\pm$)-3-(2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid (CPP; 100 μM) was added at this point for a 1-h preincubation. The cultures were then challenged with hypoxia–hypoglycemia (90–300 min with an exposure atmosphere in a gas incubator of 1% O$_2$, 8% CO$_2$, and 91% N$_2$ in an exposure medium of Hanks' balanced salt solution containing 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}, and 0.2 g/L D-glucose) in the presence of the same inhibitor. The plates were then reconstituted to normal serum-free medium (with calpain inhibitor) and then placed in an oxygenated incubator (21% O$_2$, 8% CO$_2$, and 71% N$_2$) until 24 h after the experiment initiation. Normoxic/normoglycemic cultures with the same number of medium changes were used as controls. Under these conditions, we found that only the neuronal population was selectively vulnerable to the challenge, whereas the more resistant astrocytes and microglial cells were spared. Neuronal death was then assessed by measuring the activity of the cytosolic enzyme LDH released into the medium (25-μl samples) as described earlier (Koh and Choi, 1987; Hajimohammadreza et al., 1995). For protein extraction, the medium was aspirated, and the attached cells were washed twice with 20 mM Tris-HCl (pH 7.4, room temperature), 155 mM NaCl, and 1 mM EDTA. Cells were lysed with sodium dodecyl sulfate lysis buffer, and cellular proteins were extracted as described before (Wang et al., 1996*a,b*).

Protein sample analysis

Protein concentration in tissue extract were estimated with a modified Lowry assay (Bio-Rad). Samples (15 μg of protein) were electrophoresed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% acrylamide) with a Tris-glycine running buffer system and then transferred onto a PVDF membrane with a Tris-glycine buffer system using a semidry electrotransferring unit (Bio-Rad) at 20 mA for 1.5–2 h. The blots were probed with an anti-α-spectrin (monoclonal; Chemicon) antibody. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Densitometric analysis of western blots was performed using a color scanner (Umax UC630) and the NIH program Image 1.5.

Caspase-3-like activity in NMDA-treated granule neurons

To assay for caspase-3-like protease, 200 μM NMDA-treated granule cells from three wells (20 h) were scraped

off and centrifuged for 5 min at 4°C. Cell pellets were lysed in a buffer containing 20 mM Tris-HCl (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 h. The cleared lysates were mixed with 50% (vol/vol) glycerol. Cell lysates were assayed with 100 μM acetyl-DEVD-7-amido-4-methyl-coumarin (Ac-DEVD-MCA; Bachem Bioscience), 100 mM HEPES, 10% glycerol, 1 mM EDTA, 10 mM dithiothreitol, and 10 μM Z-D-DCB (optional). Fluorescence (excitation, 380 $\pm$ 15 nm; emission, 460 $\pm$ 15 nm) was measured at 60 min with a Cytofluor 2300 apparatus.

RESULTS

Evidence for caspase-mediated SBDP120 in excitotoxin-challenged cerebrocortical cultures

We have previously established that exposure of mixed cerebrocortical cultures to high levels of excitotoxins results in neuronal death (Hajimohammadreza et al., 1995). The mode of cell death is presumably necrotic, but recently such a notion is being challenged, as evidence for apoptosis has been reported in several in vivo neurodegeneration models that involves excitotoxicity and hypoxia (MacManus et al., 1997; Portera-Cailliau and Price, 1997). Our previous work (Nath et al., 1996*b*) has established that (a) although both caspase and calpain can give rise to an SBDP of ~150 kDa (SBDP150), calpain can produce an additional SBDP of 145 kDa (SBDP145), whereas caspase selectively produces SBDP of 120 kDa (SBDP120), and (b) calpain-mediated SBDPs occur in both neuronal apoptosis and necrosis paradigms, whereas caspase-mediated spectrin breakdown only occurs in apoptosis. Therefore, we examined the spectrin breakdown pattern after cerebrocortical cultures were exposed to toxic levels of three excitotoxins (NMDA, AMPA, and kainate). In the control cultures, α-spectrin was found to be mostly in the intact 280-kDa form, with some minor background signal of its fragments (Fig. 1A). On 200 μM NMDA challenge (15 min in the absence of Mg^{2+} followed by a 16-h recovery in normal medium), the intact spectrin signal was reduced, whereas SBDP150 and SBDP145 were observed, along with the appearance of an SBDP120. We noted that the same spectrin breakdown pattern (including the presence of SBDP120) was observed when the cultures were challenged with 500 μM NMDA in the presence of 0.8 mM Mg^{2+} (data not shown). In the case of 200 μM AMPA (for 16 h), the level of SBDP120 was weaker but significant, and with 200 μM kainate (for 20 h), all three SBDPs were again observed. We quantified the level of SBDP120 on excitotoxin challenges. In all three cases, the increase was significant (Fig. 1C). Whereas an SBDP of 150 kDa has been reported previously under similar conditions (Arai et al., 1991; Lee et al., 1991; Manev et al., 1991; Wang et al., 1996*a,b*), this is the first report of the presence of lower-molecular-weight SBDP suggestive of caspase attack. We also performed a time course experiment of NMDA challenge (200 μM) and showed that the
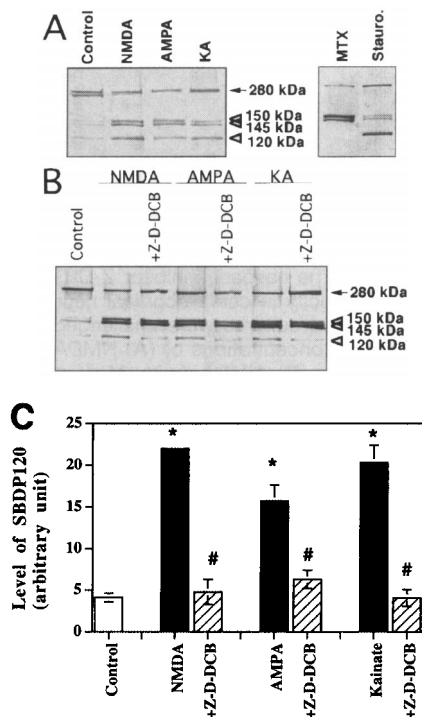
FIG. 1. Excitotoxins induce caspase-mediated α-spectrin breakdown in cerebrocortical neurons. **A:** Cerebrocortical cultures were challenged with 200 μM NMDA (in Mg^{2+}-containing medium), 200 μM AMPA (with 30 μM cyclothiazide), or 200 μM kainate (KA) in serum-free medium for 16 h or 1 nM maitotoxin (MTX) or 0.5 μM staurosporine (Stauro.) for 3 h before replacing with normal serum-free medium for 16 h. **B** and **C:** The same cultures were subjected to excitotoxin and challenge with 50 μM Z-D-DCB (1-h preincubation). The solid arrow indicates the intact α-spectrin protein, whereas the open triangles indicate the SBDPs. **C:** Quantification of apoptosis-specific SBDP120. Data are mean ± SEM (bars) values (n = 3). Data significantly different from control alone are indicated: $*p < 0.05$ by Tukey–HSD ANOVA analysis. Data significantly different from respective excitotoxin alone are indicated: $#p < 0.05$ by Tukey–HSD ANOVA analysis.

SBDP120 appeared as early as 6 h after initiation of NMDA challenge (data not shown).

To confirm that the SBDP120 observed here was derived from caspase activation, we took advantage of our previous finding that a selective caspase inhibitor, Z-D-DCB, is cell-permeable and particularly effective in blocking the SBDP120 in two cell-based neuronal apoptosis models (Nath et al., 1996a). Cultures were preincubated with Z-D-DCB (for 1 h) before excitotoxin challenges. As predicted, the presence of Z-D-DCB completely abolished the formation of SBDP120 associated with all three excitotoxin treatments (Fig. 1B and C). As reported before, SBDP145 and SBDP150 were not inhibited by Z-D-DCB because calpain also contributes to these fragmentations.
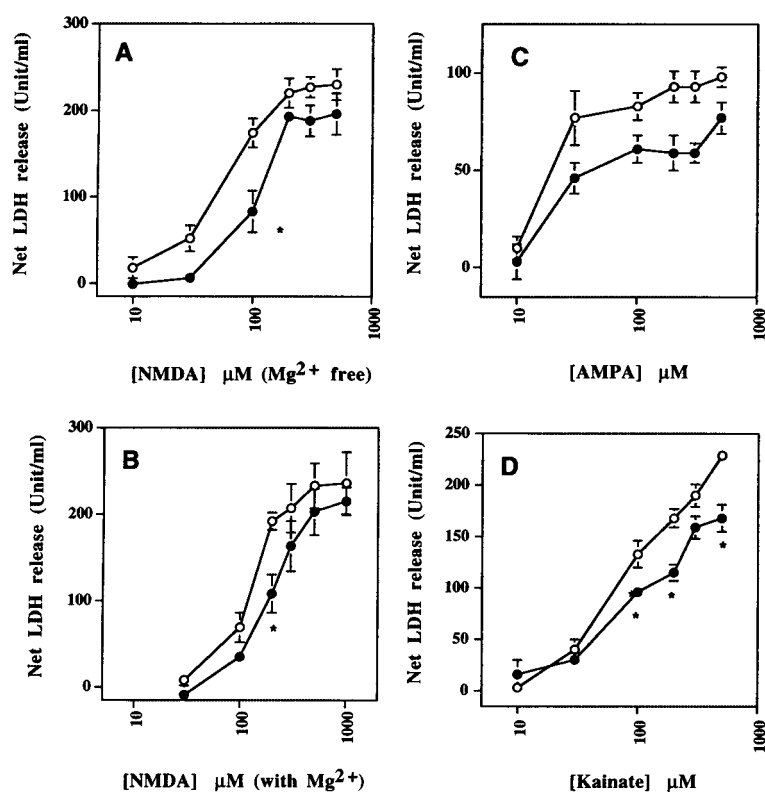
Caspase inhibitor attenuated excitotoxicity in cerebrocortical cultures

As caspase activation was observed in excitotoxin-challenged neurons, we examined if caspase activity

contributes to neuronal death under these excitotoxic conditions. NMDA challenge was done either in the absence (Fig. 2A) or in the presence of 0.8 mM Mg^{2+} (Fig. 2B). Because Mg^{2+} is a voltage-dependent blocker of the NMDA receptor, in the presence of Mg^{2+}, higher concentrations of NMDA were needed to provide the same levels of toxicity as in its absence (compare Fig. 2B with 2A). In both cases, we found that the presence of Z-D-DCB (30 μM) significantly attenuated neuronal death at 100 μM NMDA (Mg^{2+}-free) and 200 μM NMDA (with Mg^{2+}), respectively (Fig. 2A and B). In parallel, we found that kainate toxicity (at 100, 200, and 500 μM) was both partially and significantly attenuated by 30 μM Z-D-DCB. Although there appears to be a trend of attenuation toward AMPA toxicity, no statistical significance was achieved. We suspected that higher concentrations of Z-D-DCB (50–100 μM) might provide further protection against these excitotoxin challenges. We also performed glutamate receptor binding assays [N-[1-(2-thienyl)cyclohexyl]-3,4-[piperidyl-3,4-^{3}H(N)]piperidine ([^{3}H]TCP), [^{3}H]AMPA, and [^{3}H]kainate] in the presence of various concentrations of Z-D-DCB (Hajimohammadreza et al., 1995). We found that 30 μM Z-D-DCB did not significantly inhibit glutamate agonist binding (data not shown). Thus, the attenuation of excitotoxicity was unlikely to be an effect of direct glutamate receptor antagonism.

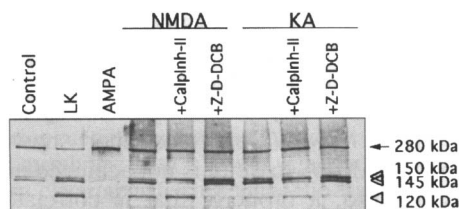## Caspase activation in cerebellar granule neurons challenged with NMDA and kainate

To examine if caspase activation also occurs during excitotoxicity in other neuronal cell types, we used another culture model: cerebellar granule neurons. It has been shown previously that glutamate treatment induces α-spectrin breakdown and cell injury in this cell type (Siman and Noszek, 1988). This model has the added advantage that spontaneous apoptosis can be invoked by simply lowering the extracellular potassium from 30 to 5 mM (Nath et al., 1996a), which can be used as a positive apoptosis control. Again, we first examined the α-spectrin breakdown patterns in excitotoxin-challenged granule neurons (Fig. 3). Only the results for NMDA and kainate are reported here because AMPA challenge did not significantly induce toxicity or α-spectrin breakdown in this model. The lack of AMPA toxicity in granule neurons 7 days in vitro is consistent with a previous report showing that half-maximal AMPA toxicity is not achieved until 13–16 days in vitro, a property that is correlated with GluR1 receptor expression (Hack et al., 1995). In the present study, in control cells (maintained in 30 mM potassium), we noted that besides the intact α-spectrin, there are detectable levels of SBDPs, most likely a result of background apoptosis (Fig. 3). Both NMDA (200 μM for 20 h) and kainate (200 μM for 20 h) resulted in SBDP150 and SBDP145. SBDP120 was also present with NMDA challenge and to a lesser extent with kainate challenge (Fig. 3). It is important

FIG. 2. The caspase inhibitor Z-D-DCB attenuated excitotoxicity in cerebrocortical neurons. Rat cerebrocortical cultures were challenged for 24 h with various concentrations of (**A**) NMDA (in Mg^{2+}-free medium), (**B**) NMDA (with Mg^{2+}-containing medium), (**C**) AMPA, or (**D**) kainate, in the absence (O) or presence (●) of 30 μM Z-D-DCB, as described in Materials and Methods. Data are mean ± SEM (bars) values (n = 5). Data significantly different from excitotoxin challenge alone are indicated: *p < 0.05 by Tukey–HSD ANOVA analysis.

FIG. 3. Appearance of caspase-mediated 120-kDa SBDP in NMDA- and kainate (KA)-treated cerebellar granule neurons. Rat cerebellar granule neuron cultures were challenged with low extracellular potassium exposure (LK; 5 mM KCl), 200 μM AMPA (with 30 μM cyclothiazide), 200 μM NMDA (in Mg^{2+}-containing medium), or 200 μM KA, in the absence or presence of 50 μM Z-D-DCB. The solid arrow indicates the intact α-spectrin protein, whereas the open triangles indicate the three SBDPs. Results are typical of three experiments. CalpInh-II, calpain inhibitor II.

to point out that the levels of SBDP120 in NMDA/kainate challenges were much lower than that in low potassium-treated cultures (Fig. 3). As in their cerebrocortical culture counterpart, the caspase inhibitor Z-D-DCB (50 μM) fully blocked SBDP120 in NMDA- and kainate-challenged cells. In contrast, calpain inhibitor II only attenuated the calpain-mediated SBDP145 while having no effect on SBDP120.

Another advantage of this model is that this culture system contains >95% neurons, with little glial contamination. We reasoned that we could attempt to detect caspase-3-like activity using a fluorogenic sub-

strate. Previously, we developed a simple method to assay for caspase-3-like activity in Triton X-100 cell extracts. Using low potassium as the apoptotic challenge, we found that Ac-DEVD-AMC hydrolytic activity was drastically elevated, presumably owing to the reported processing and the associated activation of caspase-3 (Nath et al., 1996b). In the present study, we prepared cell extracts from control or NMDA-treated cerebellar granule neurons. We found that NMDA treatment significantly increased the caspase-3-like activity, although it is much lower than that observed in low potassium-treated cells, by comparison (Fig. 4A). As expected, Z-D-DCB (30 μM) but not calpain inhibitor II (10 μM) significantly blocked the NMDA-induced increase of Ac-DEVD-AMC-hydrolytic activity (Fig. 4A). As a measurement of cell death, NMDA-induced LDH release from cerebellar granule cells was also monitored (Fig. 4B). We found that the NMDA-mediated increase in LDH release was again partially but significantly reduced by 50 μM Z-D-DCB (Fig. 4B). These data suggest that caspase activation also contributes to NMDA-mediated excitotoxicity in cerebellar granule neurons.

Evidence for caspase activation during hypoxia–hypoglycemia in cerebrocortical cultures

Previous studies have strongly implicated glutamate toxicity as mediating hypoxia–hypoglycemia-induced neuronal injury both in vitro and in vivo (Goldberg et al., 1987; Kaku et al., 1993). Similarly, we (Hajimo-

A

B
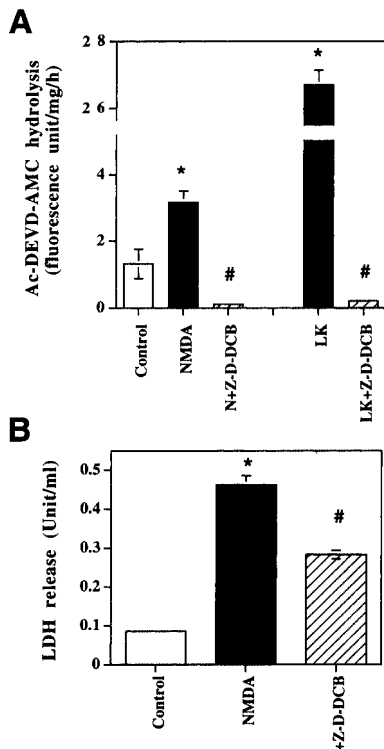
FIG. 4. Increase of caspase-3-like Ac-DEVD-AMC hydrolytic activity and protective effects of Z-D-DCB during NMDA toxicity in granule neurons. **A:** Granule neurons were exposed to 200 μM NMDA in the absence (solid column) or presence of 30 μM Z-D-DCB (hatched column) for 6 h or low potassium (LK; 5 mM) in the absence (shaded column) or the presence of 30 μM Z-D-DCB (hatched column) (as indicated). Protein extracts from different time points (using the Triton X-100 method) (15 μg) were assayed with Ac-DEVD-AMC peptide substrate for caspase-3 in vitro. Data are mean ± SEM (bars) values (n = 3). Data significantly different from control alone are indicated: *p < 0.05 by ANOVA test. Data significantly different from NMDA alone or low potassium alone are indicated: #p < 0.05 by ANOVA test. **B:** LDH release was monitored with the CytoTox 96 cytotoxicity kit (Promega) 24 h on treatment with 200 μM NMDA in the absence (solid column) or the presence (hatched column) of 50 μM caspase inhibitor Z-D-DCB. Data are mean ± SEM (bars) values (n = 5). Data significantly different from control alone are indicated: *p < 0.05 by Tukey–HSD ANOVA analysis. Data significantly different from NMDA alone: #p < 0.05 by Tukey–HSD ANOVA analysis.

hammadreza et al., 1995; Wang et al., 1996a,b) have reported α-spectrin fragmentation occurred in cerebrocortical cultures subjected to hypoxic–hypoglycemic challenges. As we have reported previously, under our experimental conditions, 165–225 min of OGD induced irreversible cell injury and death, despite subsequent recovery in normal oxygen and glucose levels (up to 24 h) (Hajimohammadreza et al., 1995). Under these conditions, we now carefully reexamine the pattern of α-spectrin breakdown. In control cells under normoxic and normoglycemic conditions, spectrin was essentially in its intact form (Fig. 5A and B). On increasing duration of OGD (165–195 min) the levels

of SBDP150 and SBDP145 dramatically increased (measured at 20 h after initiation of OGD challenge and recovery). Concomitantly, lower but significant levels of SBDP120 were clearly detected with 165–195 min of OGD challenge (Fig. 5A and B). Again, whereas calpain inhibitor II (10 μM) attenuated the level of calpain-mediated SBDP145 in OGD-treated cells, Z-D-DCB drastically reduced the OGD-induced SBDP120 signals at all three time points examined (Fig. 5A and B). It is interesting that when the level of calpain-mediated SBDP145 versus caspase-mediated SBDP120 was plotted against OGD time, it appears that calpain activation parallels caspase activation (Fig. 5C).

Next, we examined the potential neuroprotective effects of Z-D-DCB against OGD-induced cell death. As we have shown previously (Hajimohammadreza et al., 1995), increasing duration of OGD progressively increased LDH release from these cultures measured ~20 h after deprivation initiation (Fig. 6). Under these conditions, we found that Z-D-DCB dose-dependently (30 and 100 μM) and significantly attenuated the OGD-induced LDH release. Consistent with this, another caspase inhibitor, Z-VAD(OEt)-DCB (30 μM), again provided a similar level of protection (Fig. 6A). When compared with an NMDA receptor antagonist, CPP (100 μM), we found that the caspase inhibitors provided lower levels of neuroprotection. We also note that slightly higher concentrations of Z-D-DCB were needed to reduce LDH release than those needed to inhibit SBDP120 (Fig. 6B). We suspected that to achieve significant cytoprotection, one needs higher concentrations of Z-D-DCB to preserve most of its cellular proteins. Furthermore, there are likely other non-caspase pathways for cell death also occurring in parallel.

DISCUSSION

In this report, we show, for the first time, direct evidence for caspase activation during excitotoxicity and OGD in cultured neurons by using two criteria: (a) increase in content of the caspase inhibitor-sensitive SBDP120 (Figs. 1, 3, and 5) and (b) attenuation of the associated neuronal death by a caspase inhibitor (Figs. 2, 4B, and 6). We also illustrated that the role of caspase in excitotoxicity is model-independent as we have essentially the same findings using either rat cerebrocortical mixed cultures (Figs. 1 and 2) or rat cerebellar granule neurons (Figs. 3 and 4). In the latter model, because the large majority of cells were neurons, we were also able to detect an increase of caspase-3-like activity in cells on NMDA challenge (Fig. 4A). Recently, others have reported evidence of apoptosis in cultured neurons challenged with excitotoxins (Bonfoco et al., 1995) and hypoxia–hypoglycemia (Gwag et al., 1995; Gottron et al., 1997), based on morphological criteria or the presence of DNA laddering. Because we used a biochemical marker that
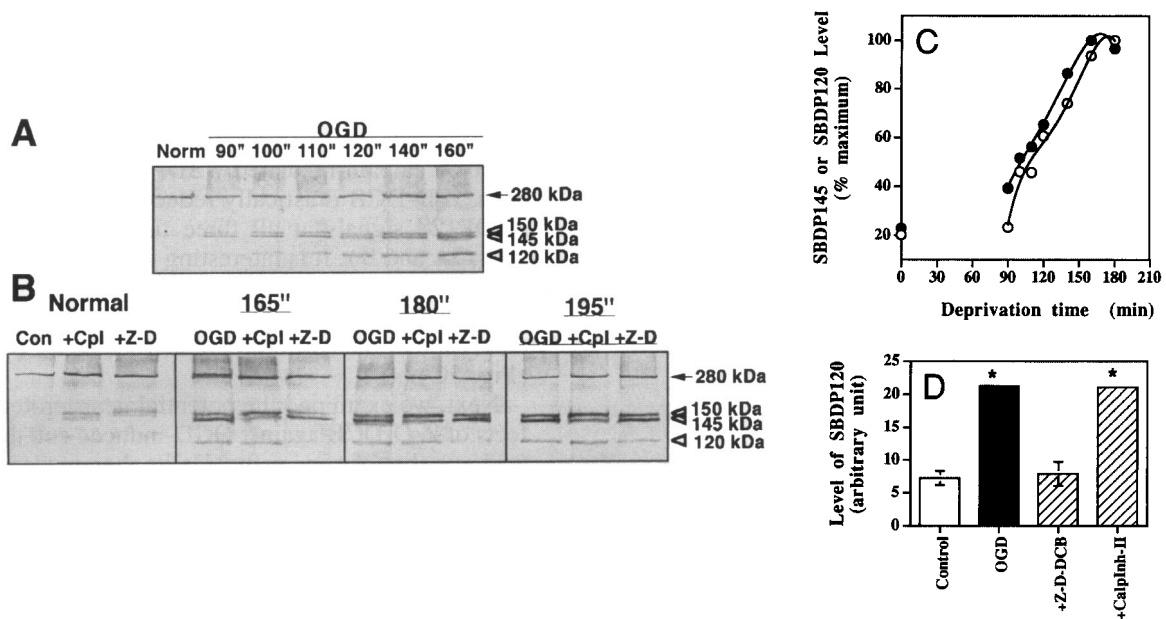
FIG. 5. Appearance of caspase-mediated 120-kDa SBDP in oxygen–glucose-deprived cerebrocortical cultures. **A:** Cerebrocortical cultures were subjected to normoxic–normoglycemic medium (Norm; serum-free) for 24 h or 90–160 min of OGD (see Materials and Methods). The plates were then returned to normal serum-free medium in an oxygenated incubator until 24 h after the experiment initiation. **B:** Cultures were subjected to normoxic–normoglycemic medium or to 165, 180, or 195 min of OGD, in the absence or the presence of either 10 μM calpain inhibitor I (CpI) or 50 μM Z-D-DCB (Z-D). Con, control. The protein extracts were analyzed for spectrin breakdown. The solid arrow indicates the intact α-spectrin protein, whereas the open triangles indicate the SBDPs. **C:** SBDP145 level (●) and SBDP120 level (○) parallel each other with the time course of OGD. **D:** The level of SBDP120 at 165 min is quantified as in B. CalpInh-II, calpain inhibitor II. Data are mean ± SEM (bars) values (n = 3). Data significantly different from control alone are indicated: *$p < 0.05$ by Tukey–HSD ANOVA analysis.

earmarks an apoptosis-linked event (caspase activation), our evidence is more compelling and unequivocal. In the previous study by Bonfoco et al. (1995), apoptosis was more evident with lower concentrations of NMDA. In our study, the SBDP120 is found at low (50 and 100 μM) as well as high (200–500 μM) NMDA concentrations but is more prominent at higher NMDA concentrations, which might simply reflect the higher levels of injury (Figs. 1 and 3 and data not shown). However, in terms of LDH release, consistent with Bonfoco et al. (1995), we found significant protection at 100 μM NMDA but not at 200, 300, or 500 μM (Mg^{2+}-free; Fig. 2). The previous study did not address kainate toxicity; however, we found that the protections provided by Z-D-DCB were significant at higher concentrations of kainate. Lastly, with OGD, it was obvious that Z-D-DCB protection is inversely related to the duration of deprivation. It is important to note again that in all excitotoxicity models we examined, the level of caspase activation was much lower than that observed when a strict apoptosis inducer was used (staurosporine in cortical cultures and potassium deprivation in granule neurons) (Figs. 1A and 3). Likewise, Z-D-DCB only provided a partial protection against excitotoxic and hypoxic–hypoglycemic challenges (Figs. 2, 4B, and 6).

A simplistic interpretation of these data is that apoptosis is only a partial component in these forms

of neuronal injury. Maybe under these neurotoxic conditions, the injured neurons are subjected to a mixture of cell death signals (MacManus et al., 1997; Portera-Cailliau and Price, 1997). It is possible that while necrotic events are occurring, the apoptosis machinery is also being activated, e.g., caspase activation. Following this hypothesis, one would expect to find evidence for both necrosis and apoptosis, depending on the degree and the duration of the challenge and other factors. Also, if both necrosis and apoptosis are occurring, their distinctive features may not be as easily recognized. For example, chromosomal DNA may be fragmented by an apoptosis-linked endonuclease(s) at the nucleosome linker regions, producing discrete DNA laddering patterns. However, these fragments could be further degraded by the less discriminative necrosis-linked endonuclease activities under a mixed apoptosis–necrosis condition. This could potentially explain the lack of evidence for apoptosis during glutamate toxicity recently reported by several groups (Dessi et al., 1993; Heron et al., 1993; MacManus et al., 1997; Portera-Cailliau and Price, 1997).

The use of SBDPs as markers of neurodegeneration is not a novel idea. An increase of the SBDP150 content has been reported in vivo in models for excitotoxicity (Vartanian et al., 1996), focal ischemia (Lee et al., 1991), and global ischemia (Roberts-Lewis et al., 1994) and in cell cultures subjected to excitotoxins
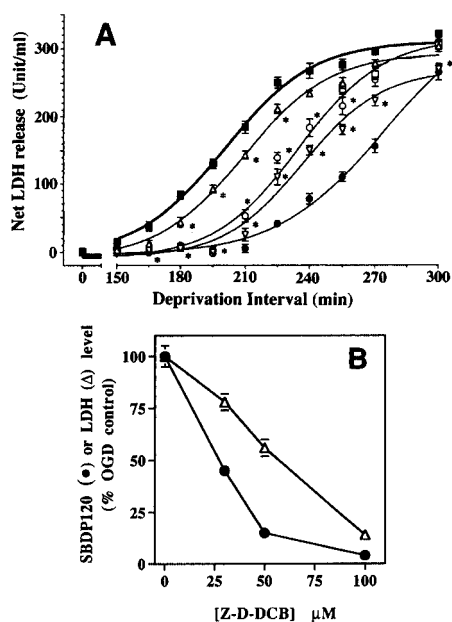
FIG. 6. Caspase inhibitor Z-D-DCB attenuated OGD-induced injury in cerebrocortical neurons. **A:** Cerebrocortical cultures were subjected to 150–300 min of OGD (see Materials and Methods). The plates were then returned to normal serum-free medium in an oxygenated incubator until 24 h after the experiment initiation. The conditions are OGD alone (■) or OGD in the presence of 30 μM Z-D-DCB (△), 100 μM Z-D-DCB (▽), 30 μM Z-VAD(OEt)-DCB (○), or 100 μM CPP (●). Neuronal death was then assessed by measuring the activity of the cytosolic enzyme LDH released into the medium. Data are mean ± SEM (bars) values (n = 7–8). Some error bars were smaller than the symbols and thus not visible. Data significantly different from hypoxia-hypoglycemia alone are indicated: *p < 0.05 by Tukey–HSD ANOVA test. **B:** Dose–response effects of Z-D-DCB on the level of LDH release (△; n = 5) and SBDP120 level (●; n = 2). LDH release and protein extraction for SBDP analysis were measured from cultures 24 h after challenge with 210 min of OGD.

(Siman and Noszek, 1988) and OGD (Wang et al., 1996a,b). However, those reports preceded the recent discovery that α-spectrin is a substrate for caspases (Nath et al., 1996a; Cryns et al., 1996; Vanags et al., 1996). Thus, it was presumed that neurodegeneration-linked α-spectrin fragmentation was only mediated by calpain. Our recent study clearly demonstrates that calpain can produce both SBDP150 and SBDP145, whereas caspase also produces a distinct SBDP150 and SBDP120. We have recently confirmed these findings by identifying all four cleavage sites (K. K. W. Wang et al., manuscript submitted). SBDP120 is easily distinguished from other fragments because of its lower molecular weight and its high sensitivity to the caspase inhibitor Z-D-DCB (Nath et al., 1996a) (Figs. 1, 3, and 5). In principle, PARP cleavage is an equally well-established marker for caspase-3 activation and apoptosis (Lazebnik et al., 1994). However, the shortcoming is that the commercially available mouse monoclonal antibody only detects the human PARP (clone C-2-10; SA250; BioMol), whereas a rabbit

polyclonal antibody against bovine PARP antibody (SA252; BioMol) cross-reacts with PARP in rats or mice only weakly. In fact, it is extremely difficult to demonstrate quantitatively the small increases in level of the 89-kDa PARP breakdown product using this antibody, e.g., in excitotoxicity (data not shown). On the other hand, as we noted earlier, the two commercially available anti-α-spectrin antibodies (MAB1622 from Chemicon and FG6090 from Affiniti; raised against chicken α-spectrin) cross-react with α-spectrin from the brain in many species (human, rat, and mouse) (Nath et al., 1996a). Beside the intact α-spectrin, these anti-spectrin antibodies readily detect the SBDP120. Thus, we would like to propose that the SBDP120 is an excellent marker for neuronal apoptosis.

Based on the appearance of the caspase-specific SBDP120 and the neuroprotective effects of a caspase inhibitor, Z-D-DCB, in excitotoxin challenges in two neuronal cell types (cerebrocortical neurons and cerebellar granule neurons), we conclude that caspase activation is triggered under excitotoxic conditions. Similarly, OGD also triggers caspase activation in cerebrocortical cultures. Therefore, it is tempting to suggest the potential therapeutic values of caspase inhibitors in neurodegenerative conditions where glutamate toxicity is implicated. In support of that, Hara et al. (1997) recently reported that a caspase inhibitor, carbobenzoxy-Val-Ala-Asp(OMe)-fluoromethyl ketone, was neuroprotective in a rat model of focal ischemic and excitotoxic brain damage.

REFERENCES

Arai A., Vanderklish P., Kessler M., Lee K., and Lynch G. (1991) A brief period of hypoxia causes proteolysis of cytoskeletal proteins in hippocampal slices. *Brain Res.* **555,** 276–280.

Armstrong R. C., Aja T. J., Hoang K. D., Gaur S., Bai X., Alnemri E. S., Litwack G., Karanewsky D. S., Fritz L. C., and Tomaselli K. J. (1997) Activation of the CED3/ICE-related protease CPP32 in cerebellar granule neurons undergoing apoptosis but not necrosis. *J. Neurosci.* **17,** 553–562.

Bonfoco E., Krainc D., Ankarcrona M., Nicotera P., and Lipton S. A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* **92,** 7162–7166.

Charriaut-Marlangue C. and Ben-Ari Y. (1995) A cautionary note on the use of TUNEL stain to determine apoptosis. *Neuroreport* **7,** 61–64.

Chen J., Jin K., Chen M., Pei W., Kawaguchi K., Greenberg D. A., and Simon R. P. (1997) Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death. *J. Neurochem.* **69,** 232–245.

Cryns V. L., Bergeron L., Zhu H., Li H., and Yuan J. (1996) Specific cleavage of { alpha } -fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1 { beta } -converting enzyme/Ced-3 protease distinct from the poly-(ADP-ribose) polymerase protease. *J. Biol. Chem.* **271,** 31277–31289.

Dessi F., Charriaut-Marlangue C., Khrestchatisky M., and Ben-Ari Y. (1993) Glutamate-induced neuronal death is not a programmed cell death in cerebellar culture. *J. Neurochem.* **60,** 1953–1955.

D'Mello S. R., Galli C., Ciotti T., and Calissano P. (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc. Natl. Acad. Sci. USA 90, 10989–10993.

Dolle R. E., Hoyer D., Prasad C. V. C., Schmidt S. J., Helaszek C. T., Miller R. E., and Ator M. A. (1994) P1 aspartate-based peptide—((2,6-dichlorobenzoyl)oxy)methyl ketones as potent time-dependent inhibitors of interleukin-1-converting enzyme. J. Med. Chem. 37, 563–564.

Filipkowski R. K., Hetman M., Kaminska B., and Kaczmarek L. (1994) DNA fragmentation in rat brain after intraperitoneal administration of kainate. Neuroreport 5, 1538–1540.

Goldberg M. P., Weiss J. H., Pham P. C., and Choi D. W. (1987) N-Methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture. J. Pharmacol. Exp. Ther. 243, 784–791.

Gottron F. J., Ying H. S., and Choi D. W. (1997) Caspase inhibition selectively reduces the apoptotic component of oxygen–glucose deprivation-induced cortical neuronal cell death. Mol. Cell Neurosci. 9, 159–169.

Gwag B. J., Lobner D., Koh J. Y., Wie M. B., and Choi D. W. (1995) Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen–glucose deprivation in vitro. Neuroscience 68, 615–619.

Hack N. J., Sluiter A. A., and Balazs R. (1995) AMPA receptors in cerebellar granule cells during development in culture. Brain Res. 87, 55–61.

Hajimohammadreza I., Probert A. W., Coughenour L. L., Borosky S. A., Boxer P. A., Marcoux F. W., and Wang K. K. W. (1995) Inhibition of calmodulin dependent protein kinase-II attenuates excitotoxic amino acid mediated neuronal death. J. Neurosci. 15, 4093–4101.

Hara H., Friedlander R. M., Gagliardini V., Ayata C., Fink K., Huang Z., Shimizu-Sasamata M., Yuan J., and Moskowitz M. A. (1997) Inhibition of interleukin 1beta converting enzyme family proteases reduce ischemic and excitotoxic neuronal damage. Proc. Natl. Acad. Sci. USA 94, 2007–2012.

Héron A., Pollard H., Dessi F., Moreau J., Lasbennes F., Ben-Ari Y., and Charriaut-Marlangue C. (1993) Regional variability in DNA fragmentation after global ischemia evidenced by combined histological and gel electrophoresis observations in the rat brain. J. Neurochem. 61, 1973–1976.

Hill I. E., MacManus J. P., Rasquinha I., and Tuor U. I. (1995) DNA fragmentation indicative of apoptosis following unilateral cerebral hypoxia–ischemia in the neonatal rat. Brain Res. 676, 398–403.

Jacobson M. D., Weil M., and Raff M. C. (1996) Role of Ced-3/ICE-family in staurosporine induced programmed cell death. J. Cell Biol. 133, 1041–1051.

Kaku D. A., Giffard R. G., and Choi D. W. (1993) Neuroprotective effects of glutamate antagonists and extracellular acidity. Science 260, 1516–1518.

Koh J. Y. and Choi D. D. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J. Neurosci. Methods 20, 83–90.

Koh J. Y., Wie M. B., Sensi S. L., Canzoniero L. M., Demaro L., Cernansky C., and Choi D. W. (1995) Staurosporine-induced neuronal apoptosis. Exp. Neurol. 135, 153–159.

Kuida K., Zheng T., Na S., Kuan C., Yang D., Karasuyama H., Rakic P., and Flavell A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384, 368–370.

Lazebnik Y. A., Kaufman S. H., Desnoyers S., Poirier G. G., and Earnshaw W. C. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371, 346–347.

Lee K. S., Frank S., Vanderklish P., Arai A., and Lynch G. (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. Proc. Natl. Acad. Sci. USA 88, 7233–7237.

Li Y., Chopp M., Jiang N., and Zaloga C. (1995) In situ detection of DNA fragmentation after focal cerebral ischemia in mice. Brain Res. Mol. Brain Res. 28, 164–168.

Linnik M. D., Zobrist R. H., and Hatfield M. D. (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 24, 2002–2008.

Loddick S. A., MacKenzie A., and Rothwell N. J. (1996) An ICE inhibitor, z-VAD-DCB attenuates ischaemic brain damage in the rat. Neuroreport 7, 1465–1468.

MacManus J. P., Buchan A. M., Hill I. E., Rasquinha I., and Preston E. (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci. Lett. 164, 89–92.

MacManus J. P., Rasquinha I., Black M. A., Laferriere N. B., Monette R., Walker T., and Morley P. (1997) Glutamate-treated rat cortical neuronal cultures die in a way different from the classical apoptosis induced by staurosporine. Exp. Cell Res. 233, 310–320.

Manev H., Favaron M., Siman R., Guidotti A., and Costa E. (1991) Glutamate neurotoxicity is independent of calpain I inhibition in primary cultures of cerebellar granule cells. J. Neurochem. 57, 1288–1295.

Martin S. J., O'Brien G. A., Nishioka W. K., McGahon A. J., Mahboubia T., Saido T. C., and Green D. (1995) Proteolysis of fodrin during apoptosis. J. Biol. Chem. 270, 6425–6428.

Nath R., Raser K. J., Stafford D., Hajimohammadreza I., Posner A., Allen H., Talanian R. V., Yuen P.-W., Gilbertsen R. B., and Wang K. K. W. (1996a) Nonerythroid alpha-spectrin breakdown by calpain and ICE-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. Biochem. J. 319, 683–690.

Nath R., Raser K. J., McGinnis K., Staford D., and Wang K. K. W. (1996b) Effects of calpain and ICE-like protease inhibitors on neuronal apoptosis. Neuroreport 8, 249–256.

Ni B., Wu X., Du Y., Su Y., Hamilton-Byrd E., Rockey P. K., Rosteck P. Jr., Poirier G. G., and Paul S. M. (1997) Cloning and expression of a rat brain interleukin-1beta-converting enzyme (ICE)-related protease (IRP) and its possible role in apoptosis of cultured cerebellar granule neurons. J. Neurosci. 17, 1561–1569.

Nicholson D. W. and Thornberry N. A. (1997) Caspases: killer proteases. Trends Biochem. Sci. 22, 299–306.

Nicholson D. W., Ambereen A. N., Thornberry N. A., Vallancourt J. P., Ding C. K., Gallant M., Gareau Y., Griffin P. R., Labelle M., Lazebnik Y. A., Munday N. A., Raju S. M., Smulson M. E., Yamin T.-T., Yu V. L., and Miller D. K. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.

Portera-Cailliau C. and Price D. L. (1997) Excitotoxic neuronal death in the immature brain is an apoptosis–necrosis morphological continuum. J. Comp. Neurol. 378, 70–87.

Portera-Cailliau C., Hedreen J. C., Price D. L., and Koliatsos V. E. (1995) Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. J. Neurosci. 15, 3775–3787.

Posmantur R., McGinnis K., Nadimpalli R., Gilbertsen R. B., and Wang K. K. W. (1997) Characterization of CPP32-like protease activity following apoptotic challenge in SH-SY5Y neuroblastoma cells. J. Neurochem. 68, 2328–2337.

Roberts-Lewis J. M., Savage M. J., Marcy V. R., Pinsker L. R., and Siman R. (1994) Immunolocalization of calpain-I-mediated spectrin degradation to vulnerable neurons in ischemic gerbil brain. J. Neurosci. 14, 3934–3944.

Schulz J. B., Weller M., and Klockgether T. (1996) Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirement for new mRNA and protein synthesis, ICE-like protease activity, and reactive oxygen species. J. Neurosci. 16, 4696–4706.

Scott R. J. and Hegyi L. (1997) Cell death in perinatal hypoxic-ischaemic brain injury. Neuropathol. Appl. Neurobiol. 23, 307–314.

Siman R. and Noszek J. C. (1988) Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. Neuron 1, 279–287.

Siman R., Baudry M., and Lynch G. (1984) Brain fodrin: substrate

for calpain I, an endogenous calcium-activated protease. *Proc. Natl. Acad. Sci. USA* **81,** 3572–3576.

Vanags D. M., Porn-Ares M. I., Coppola S., Burgess D. H., and Orrenius S. (1996) Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J. Biol. Chem.* **271,** 31075–31081.

Vartanian M. G., Cordon J. J., Kupina N. C., Schielke G., Posner A., Raser K. J., Wang K. K. W., and Taylor C. P. (1996) Phenytoin pretreatment prevents hypoxic–ischemic brain damage in neonatal rats. *Dev. Brain Res.* **95,** 169–175.

Wang K. K. W., Posner A., and Hajimohammadreza I. (1996a) Improved method of total protein extraction from cultured cells

for use in protein electrophoresis and Western blotting. *Biotechniques* **20,** 662–668.

Wang K. K. W., Nath R., Avigail P., Raser K. J., Buroker-Kilgore M., Ye Q., Takano E., Hajimohammadreza I., Hatanaka M., Maki M., Marcoux F. W., Caner H., Collins J. L., Fergus A., Lee K. S., Lunney E. A., Hays S. J., and Yuen P. W. (1996b) Mercaptoacrylic acid derivatives are selective non-peptide and calcium-binding domain targeting calpain inhibitors. *Proc. Natl. Acad. Sci. USA* **93,** 6687–6692.

Wiesner D. and Dawson G. (1996) Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway. *J. Neurochem.* **66,** 1418–1425.