Genetic and Metabolic Status of NGF-deprived Sympathetic Neurons Saved by an Inhibitor of ICE Family Proteases

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Abstract. Sympathetic neurons undergo programmed cell death (PCD) when deprived of NGF. We used an inhibitor to examine the function of interleukin-1β–converting enzyme (ICE) family proteases during sympathetic neuronal death and to assess the metabolic and genetic status of neurons saved by such inhibition. Boc-aspartyl(OMe)-fluoromethylketone (BAF), a cell-permeable inhibitor of the ICE family of cysteine proteases, inhibited ICE and CPP32 ($IC_{50} \sim 4$ μM) in vitro and blocked Fas-mediated apoptosis in thymocytes ($EC_{50} \sim 10$ μM). At similar concentrations, BAF also blocked the NGF deprivation–induced death of rat sympathetic neurons in culture. Compared to NGF-maintained neurons, BAF-saved neurons had markedly smaller somas and maintained only basal levels of protein synthesis; readdition of NGF restored growth and metabolism. Although BAF blocked apoptosis in sympathetic neurons, it did not prevent the fall in protein synthesis or the increase in the expression of *c-jun*, *c-fos*, and other mRNAs that occur during neuronal PCD, implying that the ICE-family proteases function downstream of these events during PCD. NGF and BAF rescued sympathetic neurons with an identical time course, suggesting that NGF, in addition to inhibiting metabolic and genetic events associated with neuronal PCD, can act posttranslationally to abort apoptosis at a time point indistinguishable from the activation of cysteine proteases. Both poly-(ADP ribose) polymerase and pro-ICE and Ced-3 homolog-1 (ICH-1) appear to be cleaved in a BAF-inhibitable manner, although the majority of pro-CPP32 appears unchanged, suggesting that ICH-1 is activated during neuronal PCD. Potential implications of these findings for anti-apoptotic therapies are discussed.

MULTICELLULAR organisms regulate their cell numbers not only by cell proliferation but also through the elimination of cells by programmed cell death (PCD)[1] (Glücksmann, 1951). Dying cells exhibit characteristic morphological changes of apoptosis, which include shrinking of the cytoplasm, plasma membrane blebbing, nuclear chromatin condensation, and fragmentation of genomic DNA into oligonucleosomal units (Wyllie et al., 1980). Extensive PCD occurs in the developing mammalian nervous system. Neurons are produced in excess and are dependent upon limiting amounts of trophic factor secreted by target or other cells for survival. Competition for this trophic factor is thought to be the mechanism that matches the size of the target cell population with the number of innervating neurons (reviewed by Oppenheim, 1991).

Sympathetic neurons from the embryonic rat superior cervical ganglion (SCG) are dependent on NGF for survival and undergo PCD upon removal of NGF in vivo (Levi-Montalcini and Booker, 1960) as well as in vitro (Martin et al., 1988; Edwards et al., 1991). NGF deprivation–induced death of sympathetic neurons in vitro is morphologically apoptotic, occurs within 24–48 h, and is prevented by inhibitors of macromolecular synthesis, such as actinomycin D or cycloheximide (Martin et al., 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). During PCD, neurons exhibit characteristic changes in several metabolic and genetic events. These include a decrease in glucose uptake, a transient increase in reactive oxygen species, and decreases in the rates of protein and RNA synthesis (Deckwerth and Johnson, 1993; Greenlund et al., 1995*a*). Despite an overall reduction in total RNA and protein, mRNA levels of a few genes, such as

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1. *Abbreviations used in this paper*: BAF, boc-aspartyl(OMe)-fluoromethylketone; BTF, Boc-threonine(OMe)-fluoromethylketone; CHX, cycloheximide; ICE, interleukin-1β-converting enzyme; ICH-1, ICE and Ced-3 homolog-1; IL-1β, interleukin-1β; JNK, jun kinases; PCD, programmed cell death; PARP, poly-(ADP ribose) polymerase; RT-PCR, reverse transcriptase-polymerase chain reaction; SCG, superior cervical ganglion.

c-jun, *c-myb*, *c-fos*, *fosB*, *NGFI-A*, and *Cyclin D1*, are increased in dying neurons (Estus et al., 1994; Freeman et al., 1994). Because neuronal PCD depends on protein synthesis, expression of these genes is thought to be important for cell death. The c-jun protein is apparently required for the death of sympathetic neurons. Microinjection of either c-jun antibody or a dominant negative *c-jun* construct into sympathetic neurons blocks the NGF deprivation–induced death of these neurons (Estus et al., 1994; Ham et al., 1995). Likewise, microinjection of an antibody that neutralizes proteins of the Fos-family (c-fos, FosB, Fra1, and Fra2) also prevents PCD of sympathetic neurons (Estus et al., 1994).

Recently, the interleukin 1β–converting enzyme (ICE) family of cysteine proteases have received considerable attention in cell death because they are the mammalian homologues of the *Caenorhabditis elegans ced-3* gene (Henkart, 1996; Vasilakos and Shivers, 1996). The *ced-3* gene is required for cell death; mutations that inactivate ced-3 block all naturally occurring cell death in *C. elegans* (Ellis et al., 1991). ICE, which was identified as the first mammalian homologue of *ced-3* (Yuan et al., 1993), is a cysteine protease that cleaves the 31-kD pro interleukin 1β (IL-1β) to its active 17-kD mature form (Thornberry et al., 1992). Additional members of this gene family include *Nedd2/Ich-1* (Kumar et al., 1994; Wang et al., 1994), *CPP32/apopain/Yama* (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995), *ICH-2/ICE$_{rel}$II/TX* (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), *ICE$_{rel}$III* (Munday et al., 1995), *Mch2* (Fernandes-Alnemri et al., 1995*a*), and *ICE-LAP3/CMH-1/Mch3* (Fernandes-Alnemri et al., 1995*b*; Duan et al., 1996; Lippke et al., 1996). These ICE family proteins are all cysteine proteases that cleave only after aspartic acid residues (Martin and Green, 1995).

ICE itself may not be important in cell death since mice in which the ICE gene has been deleted, although defective in processing pro IL-1β and partially defective in Fas-mediated apoptosis, have no other gross defects in PCD (Kuida et al., 1995; Li et al., 1995). However, two kinds of studies suggest that the ICE family proteases are functionally important in mammalian PCD. First, the ICE family proteases, which are inactive when translated because they contain a prodomain, are cleaved and activated during cell death (Darmon et al., 1995; Chinnaiyan et al., 1996; Duan et al., 1996; Schlegel et al., 1996). Since overexpression of the active form alone is sufficient to induce apoptosis in various mammalian cell lines (Miura et al., 1993; Fernandes-Alnemri et al., 1994; Kumar et al., 1994; Wang et al., 1994; Alnemri et al., 1995; Faucheu et al., 1995; Fernandes-Alnemri et al., 1995*a*; Kamens et al., 1995; Munday et al., 1995; Duan et al., 1996; Lippke et al., 1996), it is generally believed that once the ICE family proteases are activated, they initiate a cascade of events that cause apoptosis. Second, viral proteins such as crmA (Ray et al., 1992) and p35 (Clem et al., 1991), which inhibit the ICE family of cysteine proteases, inhibit cell death in a variety of experimental models. For example, crmA and p35 each block neuronal death after trophic factor deprivation (Rabizadeh et al., 1993; Gagliardini et al., 1994; Martinou et al., 1995) and Fas- or TNF-induced apoptosis (Beidler et al., 1995; Enari et al., 1995; Los et al., 1995; Miura et al., 1995; Tewari and Dixit, 1995); expression of p35 also inhibits apoptosis in *C. elegans* (Sugimoto et al., 1994; Xue and Horvitz, 1995) and *Drosophila* (Hay et al., 1994; White et al., 1996). Peptide inhibitors that inhibit the ICE family of cysteine proteases also inhibit PCD in several cell death paradigms, including Fas (Enari et al., 1995; Schlegel et al., 1996) or staurosporine (Jacobson et al., 1996) -induced death, apoptosis of mammary epithelial cells (Boudreau et al., 1995), and the death of motoneurons in vitro and in vivo (Milligan et al., 1995).

In this paper, we addressed five questions. Are ICE family proteases required for NGF deprivation–induced PCD of rat sympathetic neurons? Where do they function in the pathway of PCD? What is the metabolic state of neurons in which neuronal death was blocked by inhibiting the activity of ICE family proteases? Do these neurons remain responsive to subsequent readdition of trophic factor? And, which known proteases might be important in neuronal PCD? To address these questions, we chose to examine the effect of Boc-aspartyl(OMe)-fluoromethylketone (BAF), a cell-permeable inhibitor of ICE family proteases on neuronal death. BAF blocked the NGF deprivation–induced death of rat sympathetic neurons in culture, indicating that the ICE family proteases are important in neuronal death. Both poly-(ADP ribose) polymerase (PARP) and ICE and Ced-3 homolog-1 (ICH-1) appear to be cleaved in a BAF-inhibitable manner, although the majority of CPP32 appears unchanged. BAF-saved neurons were atrophic. However, readdition of NGF restored growth and metabolism. Although BAF blocked cell death, it did not prevent the fall in protein synthesis or the increase in the levels of specific mRNAs that occur during neuronal PCD, indicating that the ICE family proteins function after these events during PCD. Additionally, we found that the time course of rescue of sympathetic neurons with NGF addition was identical to that of BAF rescue, suggesting that NGF and BAF act at a similar time to inhibit apoptosis. Much of this work has been reported in abstract form (Deshmukh, M., J. Vasilakos, T.L. Deckwerth, P.A. Lampe, B. Shivers, and E.M. Johnson, Jr. 1996. *Soc. Neurosci. Abs.* 22:566).

Materials and Methods

ICE Family Inhibitors and Reagents

BAF, Z-VAD-fluoromethylketone (Z-VAD-FMK), and Z-DEVD-FMK were purchased from Enzyme Systems Products (Dublin, CA). Ac-YVAD-CHO, Ac-DEVD-CHO, Ac-YVAD-pNA, and Ac-DEVD-pNA were purchased from BACHEM Biosciences Inc. (King of Prussia, PA).

In Vitro ICE and CPP32 Assays

Enzyme activity was measured in spectrophotometric assays with purified recombinant enzymes and paranitroanilide substrates (pNA) in 96-well plates as described previously (Bump et al., 1995). The ICE (50 nM) assay was performed with the Ac-YVAD-pNA (50 μM) substrate while the CPP32 (10 nM) assay was performed with the Ac-DEVD-pNA (100 μM) substrate. A range of concentrations of the ICE family inhibitors was added to the enzymatic reaction. The reaction buffer for both enzymes contained 100 mM Hepes, pH 7.5, 0.5 mM EDTA, 20% glycerol, and 0.05% BSA; buffer for the ICE enzyme assay also included 10 mM DTT. The plates were incubated for 45 min at 37°C for the ICE assay or at 30°C for the CPP32 assay, before measuring the colorimetric readout at 405 nm.

Percent inhibition was calculated as [100 − (experimental treatment −

minimal activity)/(maximum activity − minimum activity)] × 100. The maximum and minimum activities were assayed by using enzyme plus substrate alone or substrate alone, respectively.

Fas-mediated Apoptosis

Thymocytes from C57B1/6 mice were cultured for a total of 29 h at 37°C, 5% CO_2, in a 96-well plate in RPMI medium containing 10% FCS. Cells were preincubated with the ICE family inhibitors for 1 h at 37°C before adding the anti-Fas antibody (Jo2, final concentration of 5 μg/ml; PharMingen, San Diego, CA). After 20 h, the viability indicator alamarBlue (Alamar Biosciences, Sacramento, CA) was added to each well and the plates were incubated for an additional 8 h at 37°C. Viability was assessed by fluorometric analysis as described by the manufacturer.

DNA Fragmentation

Thymocytes from C57B1/6 mice were pretreated with the ICE family inhibitor and subjected to Fas-mediated apoptosis as described above. 12 h after adding the anti-Fas antibody, genomic DNA was isolated as described previously (Schulze et al., 1994). DNA was subjected to electrophoresis on a 1.8% agarose gel and visualized after staining with ethidium bromide (0.5 μg/ml).

Primary Neuronal Cultures

Primary cultures of sympathetic SCG neurons were prepared from embryonic day 21 rats by using a modification (DiStefano et al., 1985) of a previously published procedure (Johnson and Argiro, 1983). Neuronal cultures to be used for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis had an additional preplating step of incubating the cells on Primaria plates (Becton Dickinson Immunocytometry Sys., San Jose, CA) for 1 h to minimize the number of nonneuronal cells. Depending on the experiment, between 2,000 and 15,000 cells were plated in the center of collagen-coated, 35-mm dishes (Corning Glass, Corning, NY) or chamber slides (Nunc, Inc., Naperville, IL) and maintained in NGF-containing medium (AM50). This medium contained Eagle's minimum essential medium with Earle's salts (Life Technologies Inc., Gaithersburg, MD) with the addition of 50 ng/ml of 2.5 S NGF (prepared by the method of Bocchini and Angeletti, 1969), 10% FCS, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin; 20 μM fluorodeoxyuridine, 20 μM uridine, and 3.3 μg/ml aphidicolin were also included to reduce the number of nonneuronal cells. After 5 d, the neurons were deprived of NGF by incubating in the same medium, but without NGF (AM0) and containing a neutralizing goat polyclonal anti–mouse NGF antiserum.

Viability of neurons was assessed by fixing the neuronal cultures with 4% paraformaldehyde and staining with crystal violet as described earlier (Deckwerth and Johnson, 1993).

Hoechst 33258 Staining

Neuronal cultures were fixed with 4% paraformaldehyde and stained with bisbenzimide (Hoechst 33258; Molecular Probes, Inc., Eugene, OR) as described previously (Deckwerth and Johnson, 1993).

Protein Synthesis

5-d-old SCG cultures were labeled for 4 h at 35°C with 20 μCi/ml L-[4,5-^{3}H]leucine (151 Ci/mmol; Amersham Corp., Arlington Heights, IL) in AM50 or AM0 containing 10 μM of unlabeled leucine (instead of the normal 400 μM leucine). Cultures were washed once with PBS and lysed with 500 μl of lysis buffer (0.5% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5). After addition of 10 μg of BSA to each sample, the protein was precipitated with 10% TCA for 1 h on ice and retained by filtration through a 0.45-μm nitrocellulose filter (BA-85; Schleicher & Schull, Inc., Keene, NH). The filter was washed twice with cold 10% TCA and its radioactivity measured in a liquid scintillation counter.

cDNA Preparation and RT-PCR Analysis

Our methods for cDNA preparation from primary SCG cultures and RT-PCR analysis have been described previously (Estus et al., 1994; Estus, 1996). Multiple 35-mm dishes containing equal numbers of SCG neurons (~15,000 neurons per dish) were maintained in AM50 for 5 d. After neuronal cultures were deprived of NGF for various times, poly A+ mRNA was isolated by using an oligo-dT-cellulose mRNA purification kit (QuickPrep Micro Kit; Pharmacia LKB Biotechnology, Piscataway, NJ) as described by the manufacturer. Half the mRNA was converted into cDNA by reverse transcription with 200 U Superscript II (Life Technologies Inc., Gaithersburg, MD) by using random hexamers (16 μM) as primers. The 30-μl reaction (RT) contained 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 500 μM of each of dATP, dTTP, dCTP, dGTP, and 20 U RNasin (Promega Corp., Madison, WI). The random hexamers were first mixed with the cDNAs for 2 min at 95°C. After adding the rest of the RT mixture, the samples were incubated for 10 min at 20°C and then for 50 min at 42°C; the reaction was terminated by heating for 2 min at 95°C.

Semiquantitative PCR amplification was performed in a 50-μl reaction with specific primer pairs (1 μM each), cDNA equivalent of about 150 cells, 1 U of Taq polymerase in 1× Taq buffer, 1.5 mM $MgCl_2$, 100 μM each of dATP, dGTP, and dTTP, 50 μM dCTP, and 10 μCi [α-^{32}P]dCTP. The cycle parameters were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 15–25 cycles followed by a final 10-min incubation at 72°C. 10 μl of each reaction were electrophoresed on a 12.5% polyacrylamide gel, and the dried gel was analyzed on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Control experiments were performed to determine the range of PCR cycles over which the amplified PCR product was directly proportional to the amount of input cDNA. The validity of this procedure for semiquantitative analysis is described in detail elsewhere (Estus, 1996). The cyclophilin forward (5′-ATGGTCAACCCCACCGTGTT-3′) and reverse (5′-CGTGTGAAGTCACCACCCT-3′) primers generate a 206-bp fragment. The c-fos forward (5′-AATAAGATGGCTGCAGCCAA-3′) and reverse (5′-TTGGCAATCTCGGTCTGCAA-3′) primers generate a 115-bp fragment. The c-jun forward (5′-ACTCAGTTCTTGTGCCCCAA-3′) and reverse (5′-CGCACGAAGCCTTCGGCGAA-3′) primers generate a 65-bp fragment.

Immunohistochemistry

Neurons were immunostained by using a previously described procedure (Greenlund et al., 1995*b*) with the following modifications. Cells were incubated in the primary anti–c-fos antibody (catalog No. SC52; 0.2 μg/ml, final concentration; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature and with a Cy3-conjugated donkey anti–rabbit secondary antibody (1.5 μg/ml, final concentration; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature. The fluorescent signal was detected with a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY).

Western Blots

Extracts from an equal number of cells plated at the start of the experiment were prepared by washing the cells several times with PBS and lysing them in sample buffer (2% SDS, 100 mM dTT, 60 mM Tris, pH 6.8, 0.001% bromophenol blue). The samples were boiled for 5 min before subjecting them to electrophoresis on 10% SDS-PAGE gels; extracts from an equivalent of 40,000 cells were loaded in each lane. After transferring the proteins onto polyvinyl difluoride membrane (Millipore Corp., Bedford, MA), the membrane was incubated in blocking buffer (1× PBS, 0.1% Tween-20, 5% nonfat dry milk) for 1 h at room temperature and then in the primary antibody solution (1× PBS, 0.05% Tween-20, 5% BSA) overnight at 4°C. The membrane was washed three times in blocking buffer, incubated in the secondary antibody solution (in blocking buffer) for 1 h at room temperature, and washed again in blocking buffer as before. The blot was then developed with the CDP-star chemiluminescence system (Tropix, Bedford, MA). The antibodies used were anti-PARP (No. 422; Enzymes Systems Products), anti-CPP32 (No. 06-529; Upstate Biotechnology Inc., Lake Placid, NY), anti-ICH-1 (No. 129120; Transduction Labs, Lexington, KY), and anti-MAPK (New England Biolabs, Beverly, MA).

Results

ICE Family Protease Inhibitor, BAF, Inhibits Both ICE and CPP32 In Vitro

Based on its structure, BAF is predicted to inhibit ICE family proteases irreversibly (Graybill et al., 1994; Boudreau et al., 1995; Mashima et al., 1995; Thornberry and

Molineaux, 1995). This sulfhydryl-reactive aspartate derivative lacks the molecular determinants that a priori target specific ICE family proteases and thus has the potential to inhibit a number of them. We tested whether BAF inhibited the activity of ICE and CPP32 in vitro. Purified recombinant ICE or CPP32 was incubated with its cleavable tetrapeptide substrate, Ac-YVAD-pNA or Ac-DEVD-pNA, respectively, in the presence of increasing concentrations of BAF. Enzyme activity was assayed spectrophotometrically by measuring the extent of cleavage of the peptide substrate. BAF inhibited the activity of ICE and CPP32 in vitro with a 50% inhibitory concentration (IC_{50}) of 3–4 μM for either enzyme under these conditions; the activity of either enzyme was reduced by >75% with 10 μM BAF (Fig. 1, *A* and *B*). Boc-threonine(OMe)-fluoromethylketone (BTF), which has a structure similar to BAF but contains a threonine instead of an aspartate amino acid, did not inhibit either ICE or CPP32 (Fig. 1, *A* and *B*). BAF (200 μM) did not inhibit calpain I or II, cysteine proteases that do not cleave after aspartic acid residues, indicating that BAF was not a general inhibitor of cysteine proteases (data not shown). Furthermore, BAF did not inhibit global protein degradation in sympathetic neurons (Franklin, J., personal communication). Although the Ac-YVAD-CHO and Ac-DEVD-CHO tetrapeptide inhibitors inhibited ICE and CPP32, respectively, at lower concentrations than BAF (Fig. 1, *A* and *B*), BAF inhibited both enzymes with comparable potency and therefore appeared to be a more suitable inhibitor of PCD if multiple ICE family proteases act in redundant pathways to affect cell death.

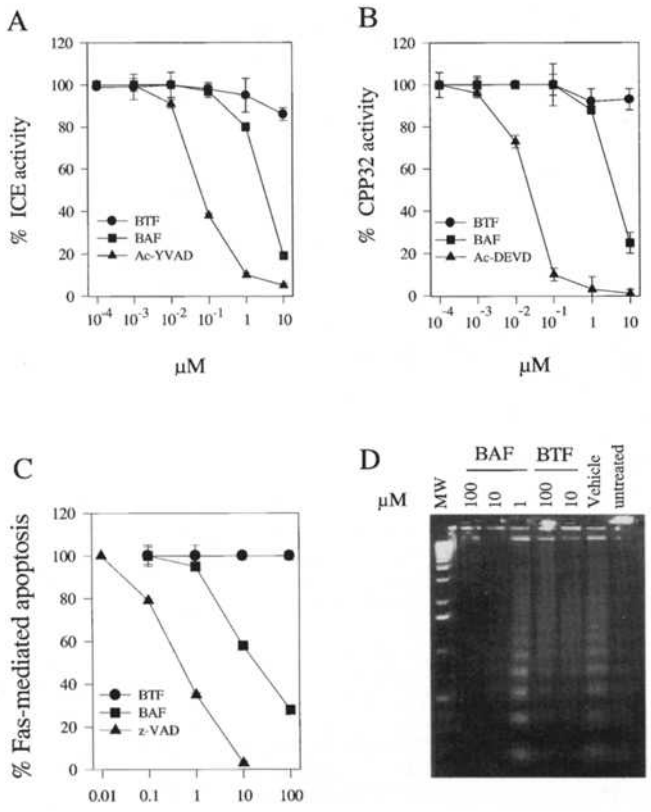

Figure 1. BAF inhibits ICE and CPP32 in vitro and Fas-mediated apoptosis in thymocytes. (*A*) Activity of recombinant ICE measured spectrophotometrically by its ability to cleave Ac-YVAD-pNA in the presence of increasing concentration of BTF (*circles*), BAF (*squares*), and Ac-YVAD-CHO (*triangles*). (*B*) Activity of recombinant CPP32 measured spectrophotometrically by its ability to cleave Ac-DEVD-pNA in the presence of increasing concentration of BTF (*circles*), BAF (*squares*), and Ac-DEVD-CHO (*triangles*). (*C*) Extent of apoptosis in mice thymocytes incubated with an anti-Fas antibody in the presence of increasing concentration of BTF (*circles*), BAF (*squares*), and Z-VAD-CH_2F (*triangles*). Cell viability was measured with the alamarBlue indicator. (*D*) Agarose gel showing genomic DNA fragmentation during Fas-mediated apoptosis of mice thymocytes in the presence of increasing concentrations of BAF and BTF inhibitors.

To determine whether BAF inhibits cell death in intact cells, we tested whether BAF inhibited Fas-mediated apoptosis in mouse thymocytes. Binding of the Fas ligand or an anti-Fas antibody to the Fas receptor on thymocytes triggers apoptosis by a pathway that requires the activity of ICE-like proteases (Enari et al., 1995; Los et al., 1995; Schlegel et al., 1996). Mouse thymocytes were preincubated for 1 h with various concentrations of BAF before the addition of an anti-Fas antibody and the extent of apoptosis was measured 20 h later by examining viability of the culture using the alamarBlue indicator (Fig. 1 *C*). BAF protected thymocytes from Fas-mediated apoptosis in a dose-dependent manner, with a 50% inhibition observed with 10 μM BAF; BAF was a less potent inhibitor of Fas-mediated apoptosis than Z-VAD-FMK, another ICE family protease inhibitor (Fig. 1 *C*). The control compound, BTF, did not block Fas-mediated apoptosis. Since cleavage of genomic DNA into oligonucleosomal fragments is an event that typically accompanies apoptosis, we also examined whether BAF prevented DNA fragmentation in these cells. Addition of 10 μM BAF prevented DNA fragmentation in thymocytes initiated to undergo Fas-induced apoptosis; BTF did not have any effect even at 100 μM (Fig. 1 *D*). The results of BAF inhibiting Fas-mediated apoptosis in thymocytes, which require ICE- and CPP32-like protease activity for death (Enari et al., 1996), are consistent with the observation that BAF was an effective inhibitor of at least two, and probably more, ICE-like proteases in vitro.

BAF Protects Rat Sympathetic Neurons from PCD Induced by NGF Deprivation

Sympathetic neurons from embryonic day 21 old rats are dependent on NGF for survival; removal of NGF triggers PCD and results in their apoptotic death over 24–48 h (Martin et al., 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). To determine if cysteine proteases are involved in sympathetic neuronal death, cultures of rat SCG neurons were grown for 5 d in the presence of NGF and then deprived of NGF to initiate PCD in the presence of an increasing concentration of BAF. BAF blocked the NGF deprivation–induced PCD of rat sympathetic neurons in culture (Fig. 2). Neuroprotection was dose-dependent: 3 d after NGF removal, >80% of the neurons were protected with 30 μM BAF and all were protected with 100 μM BAF (Fig. 2). In contrast, all neurons were dead in untreated, control cultures within 48 h after NGF removal. Even 9 d after NGF removal, 50% of neurons remained alive in 100 μM BAF, indicating that

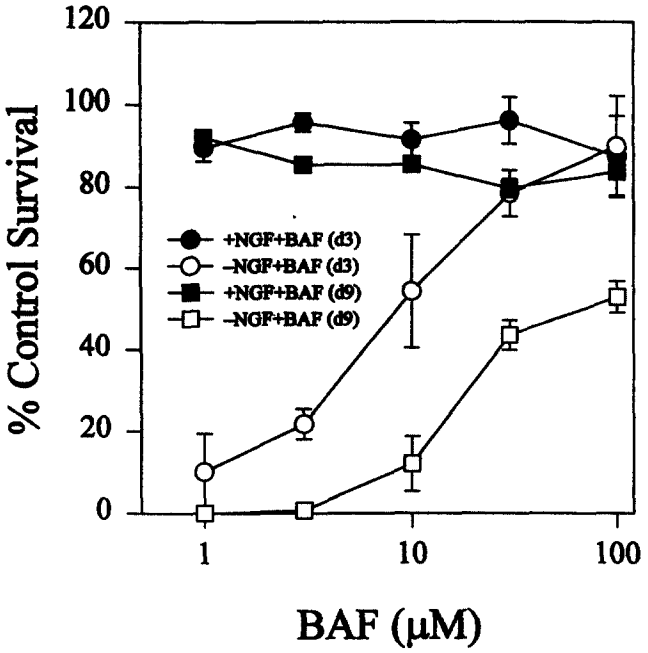

Figure 2. The ICE family protease inhibitor, BAF, promotes the survival of rat sympathetic neurons deprived of NGF. SCG neurons from embryonic day 21 rats were maintained for 5 d in the presence of NGF and then incubated in either NGF-containing (*filled symbols*) or NGF-deprived (*open symbols*) medium containing increasing concentrations of BAF. 3 (*circles*) or 9 (*squares*) d after treatment, the number of viable neurons (crystal violet stained) was determined. For the 9-d paradigm, the medium was changed once with fresh BAF inhibitor added 5 d after treatment. The number of surviving neurons for various conditions are represented as percentage of the number of neurons in NGF-maintained cultures. Results (mean ± SD) for the 3-d paradigm represent two experiments ($n = 2$) and for the 9-d paradigm represent one experiment ($n = 2$). Approximately 1,500 cells were counted for each sample point.

protection with BAF was long term (Fig. 2). 100 μM of the control compound, BTF, did not block neuronal death (data not shown). No evidence of toxicity was observed in NGF-maintained neurons with similar concentrations of BAF. At concentrations of >500 μM, however, BAF was toxic to sympathetic neurons and inhibited protein synthesis to ~65% of control levels in these neurons (data not shown). Like BAF, Z-VAD-FMK also blocked NGF deprivation–induced death of sympathetic neurons. However, the concentration of Z-VAD-FMK required to inhibit neuronal death exceeded 100 μM and thus was higher than that required for protection by BAF (data not shown).

Rat sympathetic neurons lose their phase-bright appearance and show fragmentation of neurites during cell death (Deckwerth and Johnson, 1993). Neurons protected by BAF maintained intact neurites and phase-bright cell bodies (Fig. 3 *C*). The only visible difference between the NGF-maintained and the NGF-deprived BAF-saved neurons was that the cell bodies were smaller in BAF-saved neurons (Fig. 3, compare *A* with *C*); the Z-VAD-FMK–saved neurons likewise had smaller soma (data not shown). To examine these changes in soma size, 5-d-old sympathetic cultures were either deprived of NGF in the presence of BAF or maintained in NGF, and the soma diameter was measured at various times after treatment. Within 3 d after NGF removal, the average diameter of BAF-saved neurons decreased from 19 to 15 μm (assuming that the cell bodies are spherical, this corresponds to a 48% decrease in somal volume); no further decrease in the diameter was observed even when measured 18 d after NGF removal (Fig. 4). Neurons in the presence of NGF continued to increase in size such that by comparison, cells that were deprived of NGF but maintained in BAF for 13 d had a 50% reduction in diameter (87% decrease in somal volume). To determine whether readdition of NGF restores growth, NGF was added back to neurons that had been protected with BAF for 5 d. Readdition of NGF caused an increase in somal diameter such that 13 d after NGF addition, these cells were indistinguishable from the NGF-maintained control neurons (Fig. 4). Thus, BAF-saved neurons survive long-term and retain their capacity to resume growth upon readdition of trophic support.

During cell death, the chromatin in intact spherical nuclei first becomes marginated, then condenses into smaller bead-like shapes, and ultimately disappears, leaving behind "ghost cells." Nuclei of NGF-deprived BAF-saved neurons were stained with bisbenzimide to examine whether any of these nuclear changes occur in neurons saved with BAF. The nuclei of BAF-saved neurons appeared intact, spherical, and uniformly stained and were indistinguishable from the NGF-maintained control neurons (Fig. 3, compare *E* with *G*). Thus, BAF blocks the apoptosis program before the start of any visible nuclear changes in neurons.

BAF Does Not Block the Decrease in Protein Synthesis Occurring during NGF Deprivation–induced PCD

Although NGF-deprived neurons saved with BAF did not exhibit any of the morphological changes that are associated with apoptosis, these neurons were smaller than the NGF-maintained control neurons, suggesting that although BAF blocks neuronal death, it did not prevent the initiation of PCD. We have previously described several biochemical and genetic events that are associated with NGF deprivation–induced death of rat sympathetic neurons (Deckwerth and Johnson, 1993; Estus et al., 1994; Freeman et al., 1994; Greenlund et al., 1995*a*). Knowledge of which events occur and which do not in NGF-deprived BAF-saved neurons is useful in determining the site of action of ICE family proteases in the cascade of events during PCD after trophic factor withdrawal. The rate of protein synthesis falls rapidly after NGF removal in sympathetic neurons (Deckwerth and Johnson, 1993). To determine whether BAF acts upstream or downstream of this event, we measured the rate of protein synthesis in NGF-deprived sympathetic neurons in the presence of BAF. After NGF deprivation, protein synthesis rates decreased to 20% of NGF-maintained control levels within 12 h, both in the presence and absence of BAF (Fig. 5). Thereafter, protein synthesis was maintained at 13% of control levels in BAF-saved cultures. In NGF-deprived cultures without BAF, protein synthetic rates fell down to <5% of control levels as the cells died. Thus, BAF did not block the decrease in protein synthesis that occurs after NGF removal.

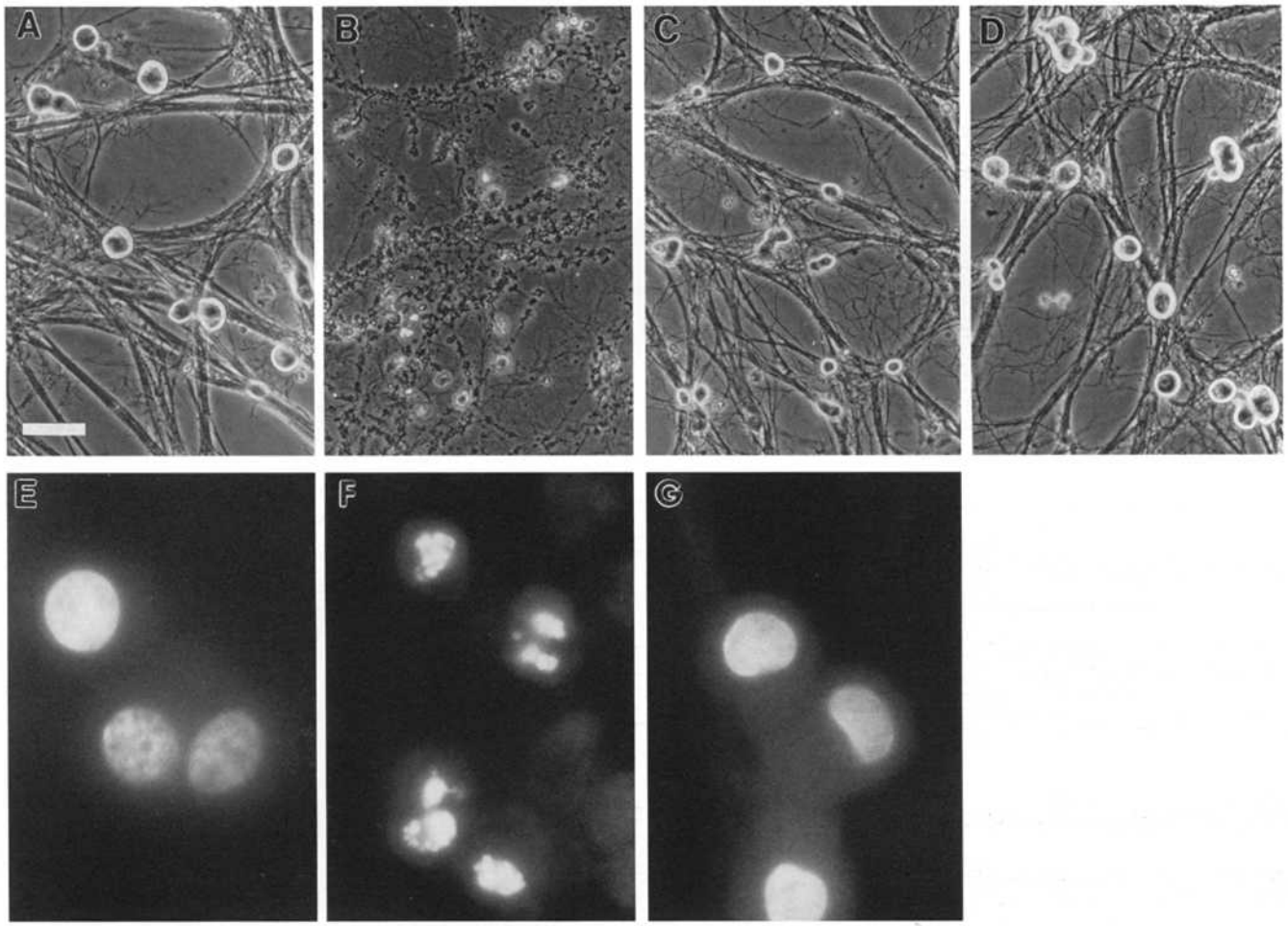

Figure 3. BAF-saved SCG neurons are phase bright and have intact neurites and normal nuclei. Shown are phase-contrast images (*A–D*) or photographs of bisbenzimide-stained nuclei (*E–G*) of dissociated rat SCG neurons maintained in NGF for 5 d and treated as follows: (*A*) Neurons maintained in NGF for an additional 5 d, (*B*) neurons deprived of NGF for 5 d, (*C*) neurons deprived of NGF for 5 d but in the presence of 30 μM BAF, and (*D*) neurons treated as in *C* and then rescued with readdition of NGF for 8 additional days. (*E*) Nuclei of neurons maintained in NGF for an additional 3 d, (*F*) nuclei of neurons deprived of NGF for 3 d, and (*G*) nuclei of neurons deprived of NGF for 3 d but in the presence of 30 μM BAF. Bars: (*A–D*) 50 μm; (*E–G*) 10 μm.

We also determined whether BAF affected protein synthesis in the presence of NGF. Since PCD in sympathetic neurons is blocked by inhibitors of protein synthesis (Martin et al., 1988), it was necessary to establish that BAF was not blocking neuronal death by inhibiting protein synthesis. Consistent with a lack of discernible toxicity at the saving concentrations of BAF, 30 μM BAF did not inhibit protein synthesis in sympathetic neurons (Fig. 5). This result excludes the possibility that BAF protects sympathetic neurons by inhibiting protein synthesis.

BAF Does Not Block the Increase in c-jun and c-fos Associated with Neuronal PCD

Although the mRNA levels of most genes decrease as sympathetic neurons die, the expression of a few genes is increased. These include *c-jun*, *c-myb*, *mkp-1*, and *cyclinD1*, which are induced by 5 h and show maximum expression 12–18 h after NGF removal, and *c-fos*, *fos B*, and *NGFI-A*, which are induced not earlier than 10 h and show maximum expression 15–20 h after NGF removal (Estus et al., 1994; Freeman et al., 1994; Ham et al., 1995). To determine whether the ICE family proteases function before or after the increase in these genes, we examined whether *c-jun* and *c-fos*, examples of genes that are induced early and late after NGF removal, respectively, are induced in BAF-saved neurons. mRNAs were isolated from two sets of sympathetic cultures, containing an equal number of neurons, at various times after NGF deprivation. BAF was added to only one set of cultures at the time of NGF deprivation to block death, and the changes in expression of these genes were determined by RT-PCR analysis. Results for the expression pattern of *c-jun*, *c-fos*, and *cyclophilin* are shown in Fig. 6. *Cyclophilin* mRNA decreases as sympathetic neurons undergo PCD; this pattern of expression is representative of most genes in dying neurons (Estus et al., 1994). In BAF-saved neurons, the amount of cyclophilin mRNA decreased but the level was sustained at 20% of NGF-maintained control neurons (Fig. 6 *A*), consistent with the observation that BAF-saved neurons appeared smaller in diameter and maintained a very reduced level of metabolism (Figs. 4 and 5). Both *c-jun* and *c-fos* were increased in BAF-saved neurons upon NGF deprivation (Fig. 6, *B* and *C*). In fact, mRNAs of

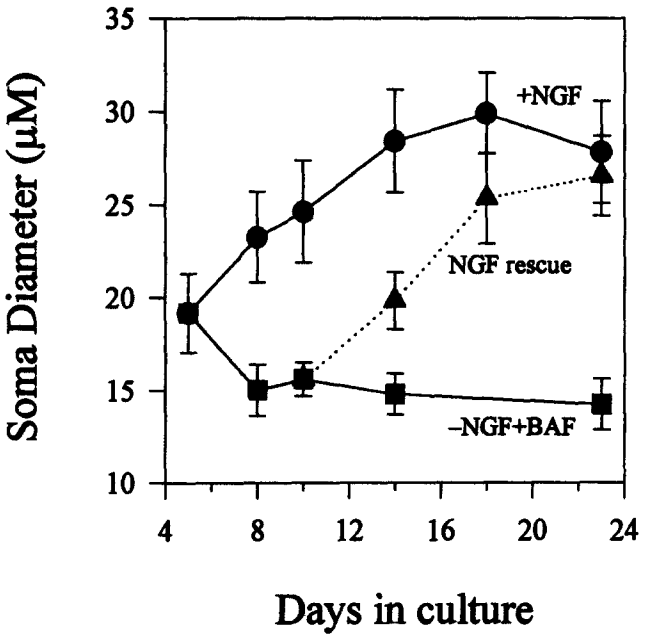

Figure 4. BAF-saved neurons appear smaller but resume growth upon readdition of NGF. Soma diameter of rat sympathetic neurons was measured on the indicated days after various treatments. Neurons were maintained in NGF for 5 d and then either continued in NGF (*circles*), deprived of NGF in the presence of 30 μM BAF (*squares*), or deprived of NGF in the presence of 30 μM BAF for 5 d and then rescued with readdition of NGF (*triangles*). Fresh medium was added to the cultures every 5 d. Soma diameter was calculated by taking phase contrast photographs of neurons, enlarging the image, and measuring the diameter of spherical-looking neurons. Results are the mean ± SD of at least 100 neurons for each time point.

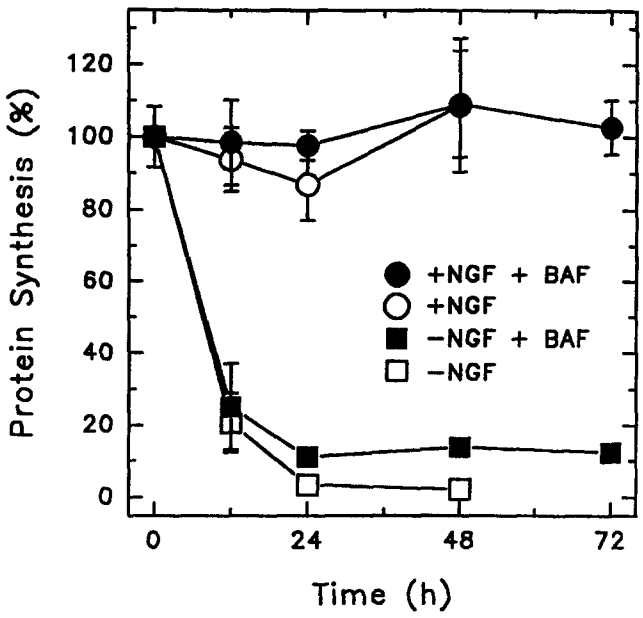

Figure 5. Time course of the rate of protein synthesis in NGF-deprived neurons exposed to BAF. Sympathetic neuronal cultures were deprived of NGF and treated with 30 μM BAF (*solid squares*). Control cultures lacking BAF (*open symbols*) or maintained in the presence of NGF (*circles*) were set up in parallel. The rate of protein synthesis was measured in the absence of BAF during a 4-h labeling period centered around the time indicated on the abscissa. Treatment with BAF had no effect on the rate of protein synthesis in NGF-maintained cultures, nor did BAF attenuate the fall of protein synthesis in NGF-deprived cultures during the first 12 h. During the next 60 h, NGF-deprived neurons prevented from dying by BAF maintained a basal rate of protein synthesis at around 13% of NGF-maintained cultures. Mean ± SD of triplicate determinations.

both these genes were increased several-fold higher in NGF-deprived neurons in the presence of BAF; induction of *c-jun* was 4.5-fold higher and that of *c-fos* was 6-fold higher in BAF-saved neurons as compared to neurons undergoing apoptosis without BAF. Expression of both *c-jun* and *c-fos* is then reduced to baseline levels after this period of increased expression. Control experiments showed that BAF alone did not induce the expression of these genes in NGF-maintained neurons (data not shown). Thus, although BAF blocked apoptosis, it did not prevent the increase of *c-jun* and *c-fos* that occurs during PCD. Expression of other NGF deprivation–induced genes, such as *cyclin D1* and *fosB*, was similarly increased in BAF-saved neurons (data not shown).

Transient Accumulation of c-fos in the Nucleus Occurs Before Chromatin Condensation during Neuronal Apoptosis

Previous studies have demonstrated that c-fos accumulates in the nucleus of sympathetic neurons during NGF deprivation–induced apoptosis. However, <1% of sympathetic neurons in the dying population are positive for c-fos at any given time and virtually all the c-fos–positive neurons also have condensed nuclei (Estus et al., 1994; Ham et al., 1995). Thus, it was unclear whether the induction of c-fos immediately precedes chromatin condensation or whether chromatin condensation induces c-fos.

To determine whether c-fos was induced before or after chromatin condensation, we determined whether the c-fos protein accumulated in the nuclei of NGF-deprived BAF-saved neurons in which PCD was initiated but was blocked before the condensation of chromatin. We deprived sympathetic cultures of NGF for 20 h in the presence of BAF and examined the pattern of c-fos expression by immunostaining with an anti–c-fos antibody. The neuronal nuclei were visualized by counterstaining with bisbenzimide. c-fos was induced in NGF-deprived sympathetic neurons undergoing apoptosis but was observable in very few neurons at any given time (Fig. 7, *A* and *B*). The chromatin of these c-fos–positive nuclei was either marginated or condensed (Fig. 7, *C* and *D*), as previously described (Estus et al., 1994; Ham et al., 1995). In contrast, ~15% of the neurons saved by BAF showed nuclear staining for c-fos (Fig. 7, *E* and *F*) and none of these had condensed chromatin (Fig. 7, *G* and *H*). These data separate the induction of c-fos from chromatin condensation and strongly suggest that the transient accumulation of c-fos in the nucleus occurs before chromatin condensation.

BAF and NGF Block Apoptosis at a Similar Time during Neuronal PCD

Depending on the site of action during the progression of

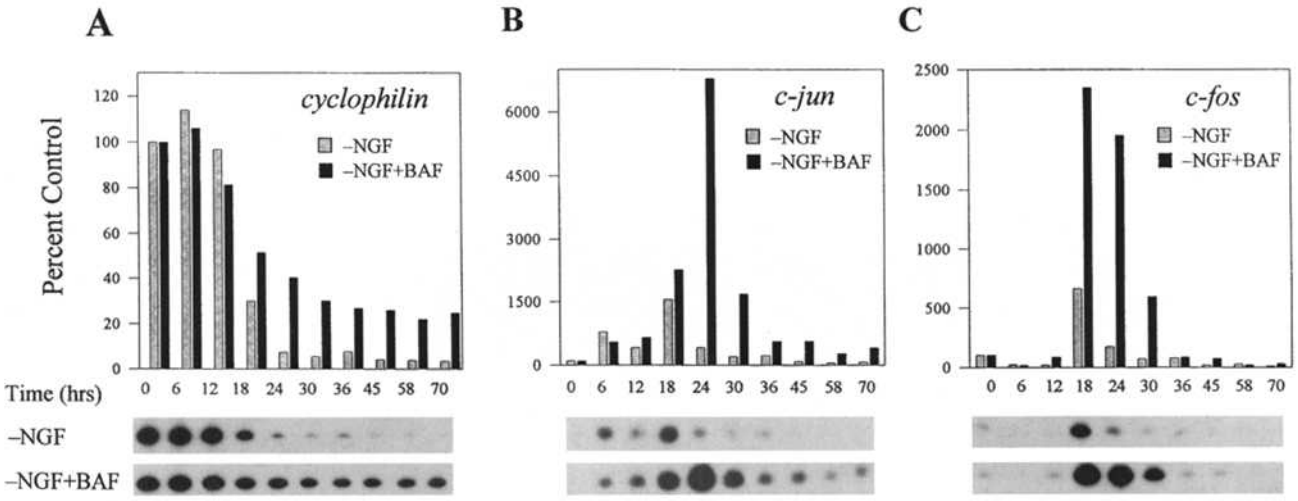

Figure 6. BAF does not block the increase in *c-jun* and *c-fos* mRNAs during NGF deprivation–induced neuronal death. Preplated SCG cultures were maintained in the presence of NGF (lanes marked *0*) or deprived of NGF for 6, 12, 18, 24, 30, 36, 45, 58, or 70 h either without (*−NGF, gray bars*) or with 30 μM BAF (*−NGF+BAF, black bars*). Poly (A)+ RNA was purified from each culture and reverse transcribed to cDNA. PCR analyses were performed with specific oligonucleotide pairs corresponding to the sequences for *Cyclophilin* (*A*; 16 cycles), *c-jun* (*B*; 21 cycles), and *c-fos* (*C*; 22 cycles). The amplified DNA products were separated on 8% polyacrylamide gels and visualized by autoradiography. The changes in amounts of these mRNAs were calculated as relative to the amount of that mRNA expressed at the 0 time point (presence of NGF). These data represent results from one experiment; similar results were obtained in a separate experiment (data not shown).

PCD, different inhibitors of the cell death process may prevent progression of PCD at different times after the onset of NGF deprivation. We refer to the time at which a neuroprotective reagent acts as the "commitment point" of the agent and measure it by determining the time after NGF removal at which 50% of neurons can no longer be rescued from death by the addition of that agent. The commitment points may be different for various agents. For example, the commitment point of cycloheximide rescue is 16 h, whereas that of NGF rescue is 22 h, indicating that the rate-limiting step inhibited by cycloheximide occurs ~6 h before the NGF-inhibitable rate-limiting step during neuronal PCD (Deckwerth and Johnson, 1993).

To determine the time point at which BAF acts to arrest PCD, neuronal cultures were deprived of NGF for various times and BAF was added thereafter for 2 d. After this rescue period, the number of living neurons was counted. As an internal standard, the time course of rescue with cycloheximide and with NGF was determined in parallel cultures. BAF and NGF had virtually indistinguishable time courses of rescue; each lost the ability to protect 50% of the neurons after 22 h of NGF deprivation (Fig. 8). As observed previously, the commitment point of cycloheximide was 6 h before that time (Edwards et al., 1991; Deckwerth and Johnson, 1993). These data indicate that the ICE family proteases acted late during PCD and that NGF was able to block death up to a very similar time during neuronal PCD.

Status of CPP32 and ICH-1 during Neuronal Apoptosis

One of the substrates of the ICE family proteases is PARP; PARP cleavage is an indicator of the activation of the ICE family proteases during apoptosis (Kaufmann et al., 1993; Nicholson et al., 1995; Tewari et al., 1995; Jacobson et al., 1996). We determined whether PARP got cleaved during NGF deprivation–induced apoptosis and whether BAF prevented this cleavage. PARP levels decreased dramatically by 24 h after NGF deprivation (Fig. 9), consistent with our observation that the ICE family proteases were activated in neurons by this time. Cleavage of PARP did not occur in BAF-saved neurons (Fig. 9), indicating that BAF inhibited the PARP-cleaving ICE family protease in neurons.

We also examined whether CPP32 and ICH-1 became activated during neuronal apoptosis. Levels of pro–ICH-1 decreased by 24 h after NGF deprivation, suggesting its apparent activation by cleavage into its active subunits (Fig. 9). BAF prevented the apparent activation of ICH-1 since ICH-1 levels did not change in BAF-saved neurons. Since the antibodies to detect the active subunits of rat ICH-1 or peptide substrates of ICH-1 are not currently available, we could not directly examine whether ICH-1 becomes activated during neuronal apoptosis. In contrast, the levels of pro-CPP32 remained constant, even 48 h after NGF deprivation when all the neurons are dead (Fig. 9, data not shown), indicating that the majority of CPP32 did not get activated during neuronal apoptosis.

Discussion

BAF Inhibits Programmed Cell Death

We have used a cell-permeable inhibitor of the ICE family, BAF, to examine the function of ICE family proteases in neuronal PCD. Since the ICE family consists of multiple proteins, some of which may perform redundant functions, we used a compound capable of inhibiting multiple proteins in the ICE family. BAF is a reactive derivative of as-

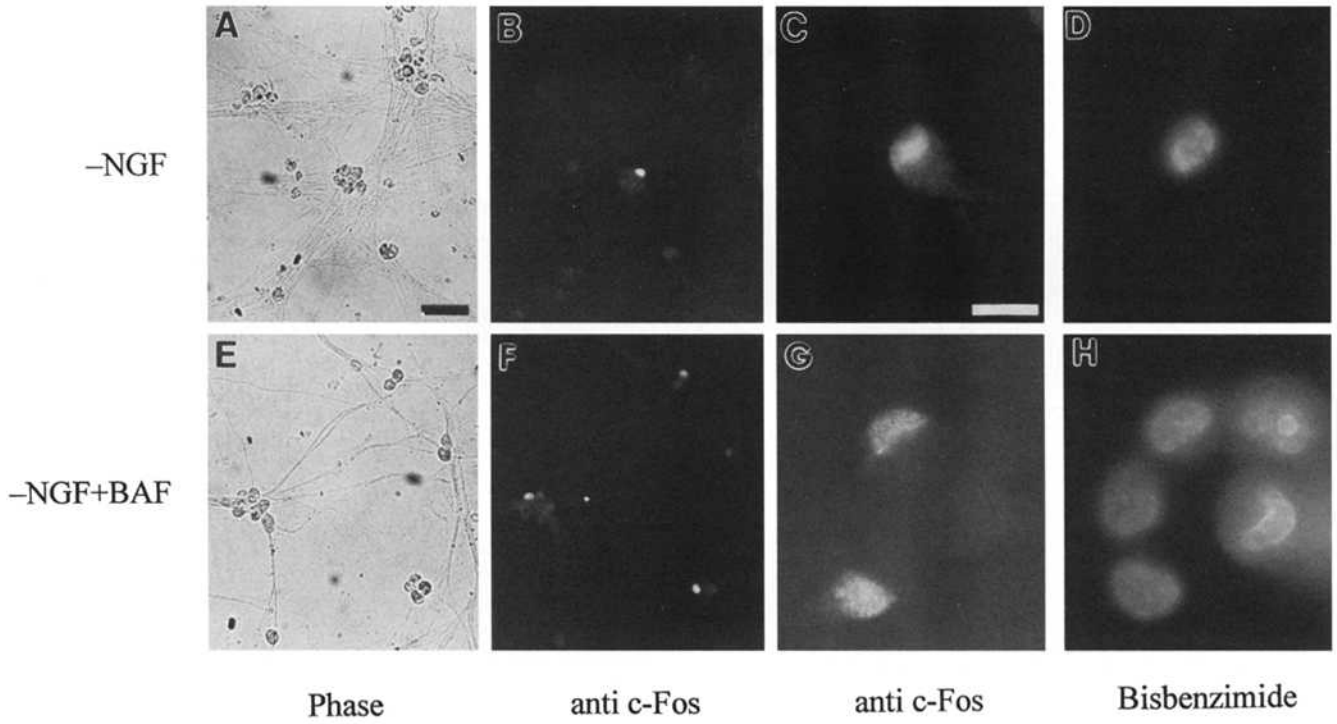

Figure 7. c-fos accumulates in the nucleus before chromatin condensation during neuronal apoptosis. Dissociated SCG neurons were maintained in the presence of NGF for 5 d, then deprived of NGF either without (*A–D*) or with 30 μM BAF (*E–H*) for 20 h, and immunostained with an anti–c-fos primary antibody and a Cy3-conjugated, secondary antibody. Shown are a representative field of neurons in phase-contrast microscopy (*A* and *E*) and the corresponding expression of c-fos in these neurons (*B* and *F*). Higher magnification shows the nuclear accumulation of c-fos (*C* and *G*) and the morphology of the corresponding nuclei visualized by staining with bisbenzimide (*D* and *H*). Bars: (*A*, *B*, *E*, and *F*) 100 μm; (*C*, *D*, *G*, *H*) 20 μm.

partic acid, the amino acid after which ICE family proteases cleave. The lack of other amino acids NH_2-terminal of the aspartate diminishes the potential for a high degree of specificity (Graybill et al., 1994; Thornberry and Molineaux, 1995). This was reflected by our observation that BAF inhibited both ICE and CPP32 in vitro with an IC_{50} of 3–4 μM for both enzymes (Fig. 1, *A* and *B*), while the tetrapeptide inhibitors Ac-YVAD-CHO or Ac-DEVD-CHO have vastly different K_i's for ICE and CPP32 (Thornberry et al., 1992; Nicholson et al., 1995). Because of its small size, neutral charge, and its relative hydrophobicity, BAF is membrane permeable and, thus, able to inhibit ICE and CPP32, among other proteases, in living cells. Consistent with this, BAF inhibited Fas-mediated death in intact thymocytes where both ICE and CPP32 are reported to be functionally important (Kuida et al., 1995; Enari et al., 1996; Schlegel et al., 1996) (Fig. 1, *C* and *D*). The spread between the IC_{50} of BAF for the inhibition of the isolated enzymes in vitro and its EC_{50} for the inhibition of PCD is much less than that for any of the tetrapeptide inhibitors, which require a considerably higher concentration for the inhibiting PCD than for the inhibition of ICE family proteases in vitro (Thornberry et al., 1992; Enari et al., 1995; Milligan et al., 1995; Nicholson et al., 1995). This observation is consistent with the suggestion that BAF is more membrane permeable than the tetrapeptide inhibitors.

The inhibition of neuronal apoptosis by BAF indicates that the ICE family proteins are required in the NGF deprivation–induced PCD of rat sympathetic neurons (Figs. 2 and 3). While BAF may inhibit ICE family proteases other than ICE and CPP32, it is not a general inhibitor of cysteine proteases as it did not inhibit calpain I or II. Also, a similar inhibitor, Z-Asp-CH_2DCB, inhibits ICE but does not inhibit cathepsin B in vitro (Dolle et al., 1994). Inhibitors of other serine, aspartyl, and lysosomal proteases have no effect on trophic factor deprivation–induced neuronal death, indicating that these proteases are not required for neuronal death (Martin et al., 1992). ICE family proteases have been implicated in other neuronal deaths as well. PCD in chicken dorsal root ganglion and rat sympathetic neurons is blocked by microinjection of the viral *crmA* and *p35* genes, respectively, which inhibit ICE family proteases (Gagliardini et al., 1994; Martinou et al., 1995). Transfection of *p35* also prevents PCD induced by glucose withdrawal, exposure to calcium ionophore, or serum withdrawal in a mammalian neural cell line (Rabizadeh et al., 1993). Naturally occurring cell death of motoneurons is also inhibited by the Ac-YVAD-CHO tetrapeptide ICE inhibitor both in vitro and in vivo (Milligan et al., 1995).

The apparent cleavage of PARP in dying neurons and its inhibition by BAF (Fig. 9) is also consistent with the finding that ICE family proteases are activated during neuronal apoptosis. That we did not detect the accumulation of the cleaved PARP product in dying neurons may be because the cleaved product was degraded during neuronal apoptosis since death occurs over a protracted period of 24–48 h. Since most ICE family proteases are capa-

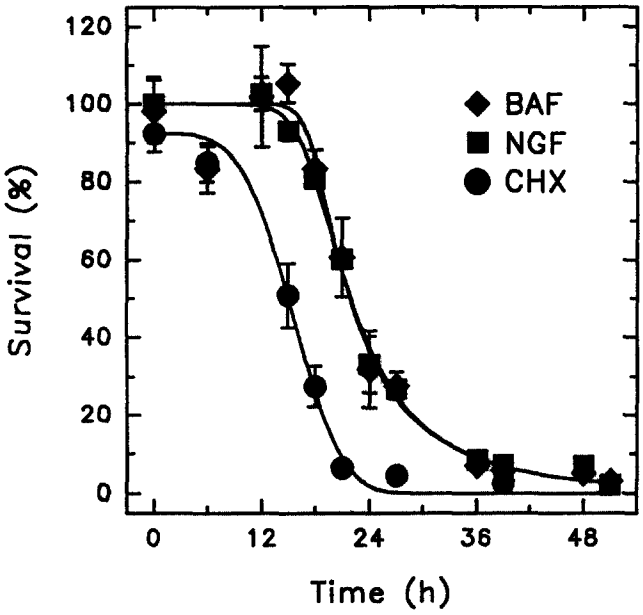

Figure 8. Comparison of the time courses of rescue with BAF, NGF, and cycloheximide (*CHX*). Sympathetic neuronal cultures were deprived of NGF for the duration indicated on the abscissa and then rescued with either 50 ng/ml NGF, 1 μg/ml CHX, or 30 μM BAF for 48 h. The time courses of rescue with BAF and NGF are identical with half of the neurons remaining rescuable after 22 h of NGF deprivation. In contrast, half of the neurons rescued with CHX are committed to die after 15 h. The number of viable neurons for each time point and rescue paradigm is expressed relative to the mean number of neurons in the NGF-maintained cultures. Data from three independent experiments with duplicate or triplicate samples each were averaged and are expressed as mean ± SD. The smooth curves were generated by fitting the survival data to asymmetric logistic equations (for details, see Deckwerth and Johnson, 1993).

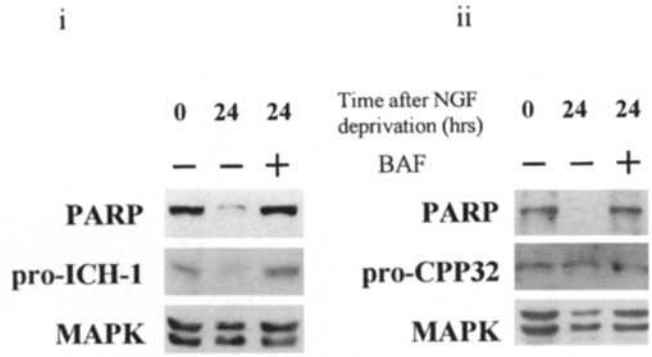

Figure 9. Apparent cleavage of PARP and ICH-1 but not CPP32 during neuronal PCD. Extracts from SCG neurons deprived of NGF for 24 h with or without BAF (30 μM) and control, untreated neurons were subjected to SDS-PAGE and Western analysis. Shown are the protein bands corresponding to PARP (116 kD), pro–ICH-1 (48 kD), pro-CPP32 (32 kD), and MAPK (44, 42 kD); MAPK serves as a loading control. Multiple proteins were detected on the same blot by stripping one antibody and subsequent reprobing with another; results from two experiments are shown (*i* and *ii*) because the signal to background ratio decreased considerably after three probes. Identical results were obtained in multiple experiments.

ble of cleaving PARP (Nicholson et al., 1995; Tewari et al., 1995; Gu et al., 1995; Fernandes-Alnemri et al., 1995*a*,*b*; Lippke et al., 1996), we have not identified any single ICE family protease as being the key mediator of neuronal apoptosis after trophic factor deprivation. Indeed, no such absolute requirement for any specific ICE family protease in PCD has yet been reported. Z-VAD-FMK inhibited Fas-mediated death of thymocytes at a lower concentration than did BAF (Fig. 1 *C*), whereas BAF inhibited sympathetic neuronal death at a lower concentration than did Z-VAD-FMK. This suggests that the repertoire of ICE family proteases involved in neuronal death is different than in Fas-induced death. ICE is expressed in thymocytes but not in sympathetic neurons (data not shown); sympathetic neurons from ICE-deficient mice undergo normal PCD upon NGF deprivation (Spiegel, K., personal communication). Therefore, ICE itself is unlikely to be required for sympathetic neuronal death. Consistent with this expectation, the ICE-specific inhibitor Ac-YVAD-CHO did not block the NGF deprivation–induced death of sympathetic neurons in culture (Greenlund, L., unpublished observation). Both CPP32 and ICH-1 are expressed in rat SCG neurons (Fig. 9). However, our results indicate that little, if any, CPP32 was activated during neuronal PCD, implying that CPP32 may not be important in neuronal death. Consistent with this, we found that the cell-permeable DEVD-FMK inhibitor, which inhibits CPP32-like proteases, did not inhibit sympathetic neuronal death (data not shown). However, we cannot exclude the possibility that a small amount of CPP32 is activated and that this small amount is enough to execute the death program. In contrast, the apparent activation of ICH-1 suggests its importance in neuronal death (Fig. 9). This hypothesis can be tested once peptide inhibitors of ICH-1 become available.

Order of Events During Neuronal Death: Where Do the ICE Family Protease Function?

One of our goals is to determine the biochemical and genetic events that are necessary for neuronal PCD. A flowchart of the temporal sequence of events during PCD in NGF-deprived sympathetic neurons is shown in Fig. 10. Protein synthesis decreased at an identical rate in the first 12 h after NGF removal in sympathetic cultures either with or without BAF (Fig. 5), consistent with the expectation that BAF prevents the execution (apoptosis) but not the initiation of PCD. Other molecules that block neuronal death by inhibiting the cell death pathway, such as superoxide dismutase and Bcl-2, likewise do not prevent the fall in protein synthesis (Greenlund et al., 1995*a*,*b*).

c-jun and *c-fos* mRNAs are induced in sympathetic neurons undergoing NGF deprivation–induced PCD (Estus et al., 1994). Both these genes are induced even in BAF-saved neurons (Fig. 6). Assuming that the neuronal PCD pathway upon trophic factor withdrawal is linear (Fig. 10), our results indicate that the ICE family proteases function downstream of the events that cause the increase in the steady state levels of *c-jun* and *c-fos* during PCD. Several experiments suggest that the events that cause an increase in *c-jun* mRNA are essential for neuronal PCD. Increase in *c-jun* mRNA transcription is mediated by the c-jun pro-

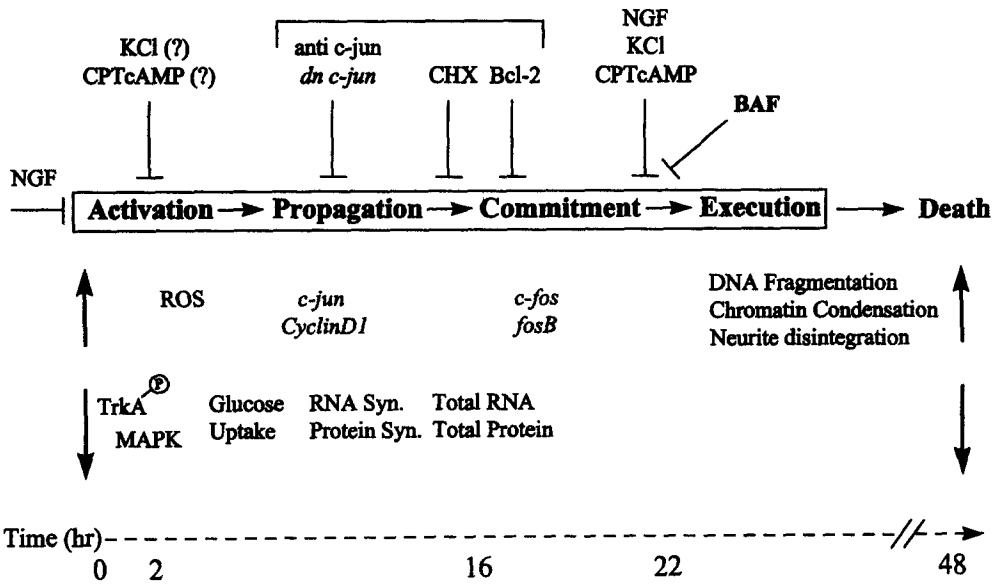

Figure 10. Temporal sequence of events during PCD in NGF-deprived rat sympathetic neurons. The pathway of PCD has been divided into four stages and the known events are shown in the approximate temporal sequence in which they occur after NGF removal. The step inhibited by microinjection of neutralizing antibodies against c-jun (*anti c-jun*) or a dominant negative *c-jun* construct (*dn c-jun*) is shown coincident with the observed *c-jun* increase. However, whether this step occurs before or after the CHX inhibitable event is unknown. Likewise, the relative position of Bcl-2 function is unknown but is shown to occur after the CHX inhibitable event because Bcl-2 inhibits apoptosis in models of apoptosis that are either protein synthesis dependent or independent and suppression of PCD by Bcl-2 overexpression does not prevent the fall in protein synthesis or the c-jun expression associated with PCD in sympathetic neurons. These data are compiled from this and previous work (Edwards et al., 1991; Garcia et al., 1992; Deckwerth and Johnson, 1993; Estus et al., 1994; Freeman et al., 1994; Franklin et al., 1995; Greenlund et al., 1995*a*,*b*; Ham et al., 1995; Virdee and Tolkovsky, 1995; Creedon et al., 1996). *ROS*, reactive oxygen species; *MAPK*, map kinase.

tein after it becomes phosphorylated by the activated jun kinases (JNK) (Karin, 1994; Smeal et al., 1994). Microinjection of either an anti–c-jun neutralizing antibody or a dominant negative *c-jun* construct, which inhibits the activity of c-jun protein, inhibits NGF deprivation–induced PCD in rat sympathetic neurons (Estus et al., 1994; Ham et al., 1995). Neuronally differentiated PC12 cells show an increase in JNK activity when deprived of NGF (Xia et al., 1995; Park et al., 1996); expression of a dominant-interfering mutant of JNK inhibited apoptosis in these cells (Xia et al., 1995).

The importance of increased *c-fos* expression in sympathetic neurons undergoing PCD is less clear. While the involvement of Fos family proteins is strongly suggested by the observation that microinjection of an antibody, which neutralizes several Fos family proteins (c-fos, Fos B, Fra 1, Fra 2), blocks NGF deprivation–induced death of rat sympathetic neurons (Estus et al., 1994), the requirement of individual Fos family proteins has not been resolved. Our results indicate that c-fos increases before chromatin condensation during trophic factor deprivation–induced PCD and is consistent with the model in which ICE family proteases function downstream of the event that causes c-fos induction but upstream of chromatin condensation.

The increase in both *c-jun* and *c-fos* mRNAs after NGF removal was severalfold higher in BAF-saved neuronal cultures as compared to cultures without BAF (Fig. 6). The most likely explanation for this result is that in cultures without BAF, the actual level of increase of these mRNAs may not have been apparent since at a population level, the mRNA increase in some neurons occurred when other neurons had already undergone apoptosis. In cultures maintained with BAF, a much greater increase was detected in the population since the increase in mRNA signal was not attenuated by any neuronal loss.

A particularly informative method of assessing where the ICE family proteases function during PCD is by examining the commitment point of rescue with BAF. Our results show that 50% of neurons could no longer be rescued with BAF 22 h after NGF removal (Fig. 8), indicating that the ICE family proteases functioned at this time during neuronal death. This timing is again consistent with the ICE family proteases acting downstream of the increases in *c-jun* and *c-fos* mRNAs (Fig. 10). An important observation is that the time courses of rescue of NGF–deprived sympathetic neurons with BAF or readdition of NGF are identical (Fig. 8). NGF prevents the initiation of the pathway at the very beginning, and it also blocks apoptosis posttranslationally by modifying some later, as yet unidentified, step in the pathway (Edwards et al., 1991; Deckwerth and Johnson, 1993). Our results show that NGF prevented apoptosis by blocking an event that functions at, or very near, the time at which the ICE family proteases were required for progression of PCD. NGF could posttranslationally inactivate either the ICE family proteases or act on a molecule functioning just before or after the ICE family protease activation. The time course of rescue with either KCl or CPTcAMP is also identical to that of NGF, indicating that these two saving agents may also block apoptosis by acting at a similar step (Edwards et al., 1991; Deckwerth and Johnson, 1993).

Comparison of BAF-saved Sympathetic Neurons with Those from the BAX-deficient Animals

The inability of BAF-saved sympathetic neurons to undergo apoptosis is strikingly similar to recent observations in neurons from *Bax*-deficient mice. BAX, a death-promoting member of the BCL-2–related family, is a protein in whose absence sympathetic and facial motor neurons

survive deprivation of NGF and disconnection from their targets by axotomy, respectively (Deckwerth et al., 1996). In both cases, where neuronal apoptosis is prevented by blocking the function of proteins in the PCD pathway, surviving neurons appear atrophic but remain viable for an extended period of time upon trophic factor deprivation, and respond to readdition of trophic factor with neurite outgrowth and soma hypertrophy. The similarity in the phenotype suggests that both BAX and the ICE family proteases function very distally and potentially in close proximity in the pathway of PCD and indicates that inhibiting the function of these cell death genes is an effective way of preventing neuronal death.

Implications for the Use of Antiapoptotic Strategies in Therapy

The observations of the morphological, genetic, and metabolic status of neurons prevented from undergoing terminal execution (apoptosis) by inhibitors of ICE-like proteases or in the absence of BAX may have therapeutic implications. Our results, summarized in Fig. 10, suggest that although inhibition of ICE-like proteases or BAX function prevents apoptosis, neither has an impact on the many events that occur subsequent to NGF removal that lead the cell to the point where it commits to undergo apoptosis. Such NGF-deprived cells are atrophic, fail to maintain growth, have a dramatically altered pattern of gene expression, and are metabolically hypoactive. It is hard to envision that such neurons maintain anything approaching normal function. However, such cells are capable of responding to reexposure to NGF, reinitiating somal and process growth, and presumably resuming a more normal level of function.

Although extrapolating from this model system to pathological conditions is tenuous, we feel that our observations have implications. Recent data have implicated apoptosis as being involved in several situations of neuronal death, such as in mechanical trauma, stroke, and in chronic neurodegenerative disease (Linnik et al., 1993; Ferrer et al., 1994; MacManus et al., 1994; Su et al., 1994; Yoshiyama et al., 1994; LaFerla et al., 1995). To the extent that apoptosis occurs in these conditions, the terminal apoptotic events occur after a period in which the cell undergoes a series of changes leading to apoptosis. These changes could be considered analogous to the changes seen in our paradigm (Fig. 10) that lead the cell to a state of metabolic and genetic derangement that triggers apoptosis. In a situation, such as trauma or stroke, during which cell death may occur over a period of days to weeks and where some reversal of the processes associated with the insult will establish a more normal environment, antiapoptotic therapies may prove very useful. In effect, the antiapoptotic therapy keeps neurons alive for a time sufficient enough to establish a tissue milieu compatible with long-term survival and function of the neuron. In contrast, chronic neurodegenerative disease (e.g., ALS, Alzheimer's Disease, Parkinson's Disease) presents a different problem. In this case, the terminal apoptotic event is the result of the derangement of the neuron (again analogous to that of the activation, propagation, and commitment phase of Fig. 10) to a metabolically and morphologically altered, hypo- or nonfunctional state. In this case, antiapoptotic therapy alone may be of less use, as such therapies do not reverse the events leading to this hypo- or nonfunctional state (Sagot et al., 1995); and, in contrast to stroke or trauma, no healing processes are ongoing to reverse the underlying processes that are driving the neuron toward apoptosis. Future experiments studying antiapoptotic drugs or animals with genetic lesions in the apoptotic pathway (e.g., BAX knockouts), coupled with experimental or genetic models of stroke, trauma, or genetic disease, will assess the use of antiapoptotic therapies.

We thank D.A. Giegel and M. Santoro (Parke-Davis, Ann Arbor, MI) for preparing the recombinant enzymes and helping with the enzyme assays, and D. Creedon, R.M. Easton, T. Miller, and P.A. Osborne (Washington University, St. Louis, MO) for critical review of this manuscript.

This work was supported by National Institutes of Health grants NS 24679, AG 12947, and a Zenith award to E.M. Johnson, Jr. from the Alzheimer's Association.

Received for publication 19 August 1996 and in revised form 26 September 1996.

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