Involvement of caspases in sympathetic neuron apoptosis

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SUMMARY

In order to study the involvement of caspases in neuronal cell death, we have examined the effects of the viral caspase inhibitor p35 and peptide caspase inhibitors on sympathetic neurons isolated from the superior cervical ganglion (SCG). In these neurons, apoptosis can be induced by the withdrawal of nerve growth factor (NGF) and also by the addition of the kinase inhibitor staurosporine. p35 has been shown to be a broad spectrum inhibitor of the caspase family and promotes the survival of SCG neurons withdrawn from NGF. We show that p35 is also protective when apoptosis is induced by staurosporine. In addition, p35 inhibits a number of the morphological features associated with apoptosis, such as nuclear condensation, TUNEL labelling, and externalisation of phosphatidylserine. The tri-peptide caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp (O-methyl)-fluoromethylketone (zVAD-fmk) was effective at inhibiting NGF withdrawal-induced and staurosporine-induced apoptosis of SCG neurons. Two other peptide inhibitors, acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) and acetyl-Asp-Glu-Ala-Ala-aldehyde (Ac-DEVAD-CHO), also inhibited apoptosis induced by both means when microinjected into SCG neurons but peptides derived from the caspase cleavage site in p35 were not protective. We present data to suggest that apoptosis induced by separate death stimuli can result either in the activation of distinct caspases or in differences in the time of activation of the family members.

Key words: Apoptosis, SCG neuron, Caspase, Nerve growth factor, Staurosporine, p35.

INTRODUCTION

Apoptosis plays a key role in the shaping of the nervous system. Approximately 50% of all the neurons produced during development fail to make the appropriate connections and die by apoptosis (reviewed by Oppenheim, 1991). This is thought to be due to competition for limiting amounts of trophic factor, such as nerve growth factor (NGF). In addition, neurodegenerative diseases (Thompson, 1995; Nicholson, 1996) and neuronal death due to stroke are thought to have a significant apoptotic component (reviewed by Choi, 1996).

Members of the caspase family have been widely implicated in the control of apoptosis. The prototype caspase, interleukin-1β-converting enzyme (ICE), was initially identified as a homolog of the Caenorhabditis elegans gene, ced-3, which had been shown to be essential for programmed cell death in the nematode (Yuan et al., 1993). There are now approximately ten known members of the caspase/ICE-like protease family (reviewed by Alnemri et al., 1996; Fraser and Evan, 1996). Caspases are synthesised as pro-enzymes and are cleaved to release an amino-terminal pro-domain plus two subunits (20 kDa and 10 kDa for ICE), both of which are required in the active enzyme (Walker et al., 1994; Gu et al., 1995). The cleavage sites within the pro-enzyme occur after specific aspartic acid residues and, thus, conform to the preferred substrate cleavage sites for caspases (Lazebnik et al., 1994). There is some evidence that ICE itself can be activated by autocleavage (Thornberry et al., 1992) and that it is able to sequen-

tially cleave and activate pro-CPP32 (Tewari et al., 1995; Enari et al., 1996). This suggests that cascades of caspases are activated during apoptosis.

Evidence to support a role for caspases in apoptosis has come from experiments in which caspase inhibitors were shown to suppress apoptosis (Miura et al., 1993). These inhibitors are of two types: viral proteins, such as baculovirus p35 (Clem et al., 1991; Bump et al., 1995; Xue and Horvitz, 1995) and cowpox virus CrmA (Gagliardini et al., 1994), and derivatised synthetic peptides which bind within the active site (Milligan et al., 1995; Fearnhead et al., 1995).

Baculovirus p35 was first characterised as a viral protein which suppressed virus-induced apoptosis of Spodoptera frugiperda cells (Clem et al., 1991). Subsequently, p35 was demonstrated to inhibit apoptosis in a wide range of cells including neurons (Rabizadeh et al., 1993; Hay et al., 1994; Sugimoto et al., 1994; Beidler et al., 1995; Martinou et al., 1995). p35 has also been shown to inhibit a number of caspases in vitro, including ICE, ICH-1, ICH-2, CPP32 and Ced-3 (Bump et al., 1995; Xue and Horvitz, 1995). Although cowpox virus CrmA shares no structural homology with p35, it also is a potent inhibitor of the caspase family (Gagliardini et al., 1994; Komiyama et al., 1994; Tewari et al., 1995). However, it is unable to inhibit ICH-1 in vitro (Wang et al., 1994) or suppress developmental cell death in C. elegans (Xue and Horvitz, 1995), which suggests that p35 has a much broader spectrum of inhibition than CrmA.

Within the caspase substrate cleavage site, the sequence
immediately amino-terminal to the aspartic acid residue plays a key role in determining substrate specificity. This has allowed the synthesis of peptide inhibitors designed to inhibit one or more caspases. These include DEVD, derived from the cleavage site in poly(ADP-ribose) polymerase (PARP), a substrate of CPP32 (Nicholson et al., 1995), YVAD, derived from the cleavage site in interleukin-1β (Thornberry et al., 1992), and also smaller, potentially less specific, peptide based compounds such as benzyloxy carbonyl-Val-Ala-Asp (O-methyl)-fluoromethylketone (zVAD-fmk) (Boudreau et al., 1995; Fearnhead et al., 1995). These inhibitors have now been used in a number of systems to inhibit apoptosis (Milligan et al., 1995; Troy et al., 1996; Deshmukh et al., 1996).

While it is known that caspases are activated in many situations, what is less clear is which specific caspases are activated in given cell types induced to undergo apoptosis with particular death stimuli. This is of crucial importance if we are to design ways of targeting specific cell types and underlines the requirement to study these pathways in more detail. In this study, we have examined the role of caspases in apoptosis in rat superior cervical ganglion neurons (SCGs), NGF-dependant sympathetic neurons of the peripheral nervous system. Two different death stimuli were used, NGF withdrawal, a transcription-dependent death, and staurosporine addition, a transcription-independent death. Both stimuli result in the characteristic morphological changes associated with apoptosis (Martin et al., 1988; Bertrand et al., 1994; Philpott et al., 1996). We have investigated the effects of viral and peptide caspase inhibitors on the death of SCG neurons undergoing apoptosis induced by either means. We show that p35 can prevent apoptosis in SCG neurons induced by either stimuli and from the peptide studies we can infer that the caspases induced are either non-identical or that their time or sequence of activation is different. We further show that it is effective in blocking several of the individual nuclear and plasma membrane modifications associated with apoptosis.

MATERIALS AND METHODS

Neuronal cell culture

Superior cervical ganglia were removed from newborn rat pups and dissociated in 0.025% trypsin for 30 minutes followed by 30 minutes in 0.2% collagenase (Worthington). After pre-plating for 2 hours, to allow non-neuronal cells to attach (Deckworth and Johnson, 1993), the neurons were gently rinsed off, centrifuged and resuspended in culture medium. Cells were plated on poly-L-lysine/laminin-coated, 13 mm glass coverslips at 8×10³ cells per coverslip. The cell culture medium was Dulbecco’s modified essential medium (DMEM; Gibco BRL Laboratories) containing 10% fetal calf serum (Gibepharm Limited), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 100 ng/ml NGF (Promega). To limit the growth of non-neuronal cells, 20 μM fluorodeoxyuridine (Sigma) and 20 μM uridine (Sigma) were added. Cells were cultured for 5-7 days prior to use. For NGF withdrawal experiments, cells were washed once with DMEM, and fresh medium lacking NGF and containing 100 ng/ml of anti-NGF antibody (Boehringer Mannheim) added. When staurosporine was used to induce death, cells were refed with medium containing 100 ng/ml NGF and 1 μM staurosporine (Calbiochem). To prevent cells mechanically detaching from the coverslips, 0.6% methyl cellulose was added into the culture medium (Hawrot and Patterson, 1979).

Peptides

The peptide ICE inhibitors, acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), benzyloxy carbonyl-Asp-Gln-Met-Asp-aldehyde (zDQMD-CHO) and benzyloxy carbonyl-Asp-Gln-Met-Asp-Gl-Y-Phe-His-Asp-aldehyde (zDQMDGHFHDC-CHO) were synthesised at Eisai London Research Laboratories and shown to be greater than 98% pure. Since some of these peptides were not expected to be highly cell permeable, they were microinjected into neurons. Benzyloxy carbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (zVAD-fmk) and the control peptide benzyloxy carbonyl-Phe-Asp-fluoromethylketone (zFA-fmk) were purchased from Enzyme Systems Products Inc. and, as they are cell permeable were added directly into the culture medium. The control peptide acetyl-Leu-Leu-Arg-aldehyde (Ac-LLR-CHO; leupeptin), was purchased from Bachem and used in microinjection experiments.

Plasmid constructs

The cDNA for baculovirus p35 was cloned into the expression vector pcDNA1 (Invitrogen). To monitor the expression of p35, the 8 amino acid FLAG epitope, which can be detected with the monoclonal antibody M2 (UBI), was added to the amino terminus. Murine bcl-2, also cloned into pcDNA1, was a gift from J. Ham, and its construction has been previously described (Ham et al., 1995).

Microinjection

Microinjection was carried out using a Zeiss Axiovert 135M microscope with an Eppendorf injector and micromanipulator. Microinjection needles were pulled from glass capillaries using a horizontal electrode puller (Camden Instruments). Expression vectors were injected into the nucleus in 0.5×PBS at a DNA concentration of 0.05 mg/ml. Peptide ICE inhibitors were also injected in 0.5×PBS to give an intracellular concentration of approximately 1-10 μM (assuming an injection volume of 1-10% of the estimated cell volume).

Immunofluorescence analysis was carried out to verify that bcl-2 and p35 were expressed in injected neurons. Purified guinea pig IgG (Sigma) was added to the injection mix at a concentration of 5 mg/ml. After injection, the neurons were left 16 hours for the protein to be expressed. The cells were then fixed and stained with a rhodamine-conjugated donkey anti-guinea pig IgG antibody (Jackson ImmunoResearch Laboratories) at 1:100, to identify injected cells, and either an anti-bcl-2 antibody (Dako) or the anti-FLAG antibody.

To analyse the effects of p35 or the peptide ICE inhibitors on survival, neurons were injected 5-7 days after plating with DNA or peptides at the concentrations indicated. Neutral 70 kDa Texas Red dextran (Molecular Probes) at a final concentration of 5 mg/ml was used to mark the injected cells. After injection, the neurons were left for 3 hours and the number of Texas Red dextran positive cells that had survived injection counted. Apoptosis was then induced either by the withdrawal of NGF or by the addition of 1 μM staurosporine. After 48 or 72 hours survival was assessed as described below.

Live/dead assay

To assess cell viability, the Live/Dead assay (Molecular Probes) was used. Cells were incubated with 1 μM calcein AM, which is converted to a green fluorescent derivative by the activity of esterases in viable cells. For microinjection experiments, viability was assessed by counting the total number of injected cells (Texas Red dextran positive) which were also stained with calcein. For treatment with compounds added directly into the culture medium, viability was assessed by counting the number of calcein-stained cells in 10 random fields around the perimeter of the coverslip. All experiments were counted blind.
**TUNEL labelling**

To determine the effect of p35 on TUNEL labelling of SCG neurons, pcDFLAGp35 or the empty pcDNA1 vector were microinjected into neurons at a concentration of 0.05 mg/ml. Purified guinea pig IgG was included to mark injected cells. The neurons were left for 16 hours to allow the protein to be expressed and were then withdrawn from NGF for 48 or 72 hours, as previously described. They were fixed in 3% paraformaldehyde, permeabilised in 0.5% Triton X-100, and then labelled with the Fluorescein In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer’s instructions. Guinea pig IgG was detected using a rhodamine-conjugated donkey anti-guinea pig secondary antibody (Jackson ImmunoResearch Laboratories). Nuclei were stained with 1 μg/ml Hoechst 33342 (Sigma) for 5 minutes after which coverslips were mounted in Citifluor (Citifluor Ltd).

**Annexin V binding**

pcDFLAGp35 or the empty pcDNA1 vector and Texas Red dextran were microinjected into SCG neurons. The neurons were left for 3 hours to allow the protein to be expressed and then withdrawn from NGF for 48 or 72 hours. To detect externalised phosphatidylserine, cells were then stained with 1 μg/ml fluorescein-conjugated annexin V (R&D Systems) and fixed in 3% paraformaldehyde in the presence of 2 mM calcium chloride. Nuclei were stained with 1 μg/ml Hoechst 33342 for 5 minutes and the cells mounted in Citifluor.

**Photography**

Photographs were taken on a Nikon Microphot-FXA fluorescence microscope with Kodak 400 ASA film. Confocal images were taken on a Leica TCS4D confocal microscope.

**RESULTS**

**p35 prevents NGF withdrawal and staurosporine-induced apoptosis**

Baculovirus p35, a caspase inhibitor, has been shown to protect SCG neurons from apoptosis induced by NGF withdrawal (Martinou et al., 1995). To investigate the role of caspases in multiple death pathways we wished to determine if p35 overexpression would also block staurosporine-induced apoptosis of SCG neurons, which we have previously shown to be similar to NGF withdrawal-induced death (Philpott et al., 1996). This was achieved by microinjection of a p35 expression vector directly into SCG neurons.

To ascertain expression of p35 and bcl-2, and to determine where the proteins were localised, we microinjected pcDFLAGp35 or pcDbcl-2 plasmids into SCG neurons. p35 was expressed in over 90% of the cells which survived injection and was localised throughout the cytoplasm and nucleus (Fig. 1A), consistent with previous results (Rabizadeh et al., 1993; Hershberger et al., 1994). Bcl-2 was localised to membranous structures within the cytoplasm, again consistent with published data (Hockenbery et al., 1990; Jacobson et al., 1993) (Fig. 1B).

To determine whether p35 could prevent staurosporine induced death, pcDFLAGp35 was microinjected into SCG neurons. The results are shown in Fig. 1C and D. A control vector was injected since there is some protective effect reported by injection of DNA, possibly due to sequestering of transcription factors important in cell death. As a positive control, we used pcDbcl-2 which has previously been shown
to protect SCG neurons from apoptosis induced by NGF withdrawal (Garcia et al., 1992). After NGF withdrawal, neurons injected with pcDFLAGp35 showed increased survival compared with those injected with the empty vector pcDNA1, consistent with the findings of Martinou et al., (1995) (Fig. 1C). Importantly, in neurons treated with staurosporine, injection of either pcDFLAGp35 or pcDbcl-2 were effective in protecting these cells (Fig. 1D).

**p35 decreases the number of TUNEL and annexin V positive cells**

Apoptotic SCG neurons undergo a number of characteristic morphological and biochemical changes, including nuclear condensation, cell shrinkage and loss of mitochondrial function. Evidence in other cell systems, as well as in SCG neurons, suggests that the caspases are responsible for a number of these changes by cleaving specific substrate proteins responsible for maintaining cellular integrity. To further investigate p35 inhibition of apoptosis in SCG neurons, we examined two additional indicators of apoptosis, namely TUNEL labelling and binding of annexin V to externalised plasma membrane phosphatidylserine.

One key event associated with apoptosis is the activation of a nuclease which cleaves DNA into oligonucleosome-sized pieces. A method which has been developed to observe this fragmentation process is the TUNEL (TdT-mediated dUTP nick end labelling) method (Gavrieli et al., 1992). We injected neurons with p35 and then withdrew them from NGF. We observed a decrease in the number of TUNEL positive cells when compared to neurons injected with the pcDNA1 vector control, from 50% seen with control DNA to less than 10% when p35 was expressed (Fig. 2). The 50% labelling value obtained for control DNA microinjection corresponds to cells still remaining on the coverslip, but in fact many of these cells were already dead and had detached, so that the percentage of TUNEL-positive cells should be much greater. In addition, we have noted in our experiments that once the DNA is highly condensed it is no longer labelled using the TUNEL technique (data not shown).

During cell death, phosphatidylserine, which is normally concentrated within the inner leaflet of the plasma membrane, becomes exposed at the external surface of the cell (Fadok et al., 1992). Phosphatidylinerine exposed on the outer membrane can be detected by incubating unfixed cells with fluorescein-labelled annexin V and visualised by fluorescence microscopy (Koopman et al., 1994; Vermes et al., 1995; Rimon et al., 1997). After 48 or 72 hours of NGF deprivation, cells injected with the empty vector DNA showed the expected increase in the number of annexin V positive neurons (Fig. 3). In contrast, cells microinjected with p35 did not exhibit significant annexin V staining.
Inhibition of CED-3/ICE by p35 has been found to involve cleavage of the p35 protein at Asp-87-Gly-88, with the formation of a stable p35-ICE complex (Bump et al., 1995). In addition, although CrmA is normally unable to inhibit developmental cell death in *C. elegans*, it is possible to confer a protective effect by replacing the ICE cleavage site of CrmA with the eight amino acid cleavage sequence from p35 (Xue and Horvitz, 1995). We synthesised two peptides corresponding to the sequence of the cleavage site, Ac-DQMD-CHO and Ac-DQMDGFHD-CHO, and microinjected them into SCG neurons at a concentration of 100 μM. However, we found that after 72 hours, neither peptide prevented death induced by NGF withdrawal (Fig. 4).

**Peptide inhibitors of caspases prevent apoptosis in SCG neurons**

Investigation into the role of specific caspases is facilitated by use of peptide inhibitors derived from sequences of specific substrate cleavage sites. Ac-YVAD-CHO has been shown to be a potent ICE inhibitor (Milligan et al., 1995), and Ac-DEVD-CHO inhibits CPP32 (Nicholson et al., 1995). Both of these peptide inhibitors are thought to act as reversible competitive inhibitors due to the presence of an aldehyde leaving group. We found that microinjection of either of these peptides was

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**Fig. 3.** p35 decreases the externalisation of phosphatidylserine that normally occurs following NGF withdrawal. SCG neurons injected with pcDflagp35 or pcDNA1 were withdrawn from NGF for 48 or 72 hours and then stained with FITC-annexin V. (A) Cells injected with pcDflagp35 and maintained in NGF (filled squares), cells injected with pcDflagp35 and withdrawn from NGF (open squares), cells injected with pcDNA1 and maintained in NGF (triangles), cells injected with pcDNA1 and withdrawn from NGF (circles). The results shown are the mean ± s.e.m. of three independent experiments with over 200 neurons injected per coverslip. (B) Cells were injected with pcDflagp35 and withdrawn from NGF (a, b, c), injected with pcDNA1 and maintained in NGF (d, e, f), or injected with pcDNA1 and withdrawn from NGF (g, h, i). Injected cells labelled with Texas Red dextran (a, d, g), were stained with Hoechst 33342 to show nuclei (b, e, h) and labelled with FITC-annexin V (c, f, i). Cells in B (c) that were annexin positive were not injected with pcDflagp35. Bar, 20 μM.

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**Fig. 4.** Microinjection of p35 cleavage site peptides do not protect SCG neurons from death induced by NGF withdrawal. SCG neurons were microinjected with peptides corresponding to the p35 cleavage site to give an intracellular concentration of approximately 1 μM or 10 μM and withdrawn from NGF for 72 hours. Results are expressed as the percentage of surviving (injected) neurons at 72 hours relative to time 0 and are the mean ± s.e.m. of three separate experiments. In each experiment, approximately 200 neurons were injected per coverslip. (A) pcDflagp35 (solid bar), zDQMD-CHO, 10 μM (diagonal stripes), zDQMD-CHO, 100 μM (horizontal stripes), leupeptin 100 μM (clear bar). (B) pcDflagp35 (solid bar), zDQMDGFHD-CHO, 10 μM (diagonal stripes), zDQMDGFHD-CHO, 100 μM (horizontal stripes), leupeptin 100 μM (open bar).
neurons were microinjected with pcDL-AGp35 (solid bars) or 100 μM, to give an approximate intracellular concentration of 10 μM, of the peptides Ac-DEVD-CHO (diagonal stripes), Ac-YVAD-CHO (horizontal stripes), or leupeptin (open bars). Cells were either withdrawn from NGF (A) or treated with 1 μM staurosporine (B) for 72 hours. Results are expressed as the percentage of surviving (injected) neurons at 72 hours relative to time 0 and are the mean ± s.e.m. of more than three separate experiments. In each experiment 200 neurons were injected per coverslip.

able to protect SCG neurons induced to die by NGF withdrawal (Fig. 5A). We also injected these inhibitors and induced apoptosis by the addition of staurosporine. In this case the level of protection afforded by each inhibitor was somewhat less, but still significant (Fig. 5B).

zVAD-fmk is an irreversible, cell permeable inhibitor of caspases (Fearnhead et al., 1995; Pronk et al., 1996; Troy et al., 1996), and when added to the culture medium, was a potent inhibitor of NGF-withdrawal-induced death (Fig. 6A and C). After 72 hours, over 80% of neurons treated with 100 μM zVAD-fmk were viable by the Live/Dead Assay. One clear feature of zVAD-fmk treatment was that the neurites were well protected from the degeneration associated with apoptosis (Fig. 6C). The neurites were, however, thinner than those maintained in NGF and the cell bodies, although viable by criteria of the Live/Dead assay, were atrophic. We also observed a decrease in the number of annexin V-positive zVAD-fmk treated neurons after NGF withdrawal (data not shown). zVAD-fmk was also tested for its ability to block staurosporine-induced death. We found that a 4 hour pre-treatment with zVAD-fmk offered significant protection when neurons were treated with staurosporine for 72 hours (Fig. 6B and C). However, zVAD-fmk was not protective when added at the same time as staurosporine (Fig. 6B), and zVAD-fmk did not prevent staurosporine-induced neurite degeneration (Fig. 6C).

DISCUSSION

Many recent studies have provided information concerning intracellular regulators of neuronal apoptosis. Still, a clear understanding of the ways in which apoptosis is initiated in different neuronal types in response to various perturbations, such as, survival factor withdrawal and hypoxia, is lacking. Moreover, while it is clear that members of the caspase family of proteases are important, details surrounding their activation is also lacking.

One of the clearest demonstrations of the functional importance of caspases in apoptosis was the inhibition of NGF withdrawal-induced death of SCG neurons by the baculovirus caspase inhibitor p35 (Martinou et al., 1995). In this paper, we show that p35 is also able to block death caused by staurosporine addition to these neurons and that it is as effective as Bcl-2 (Fig. 1D). This demonstrates that both transcription-dependent and transcription-independent forms of neuronal death involve caspases.

Our preliminary analysis of injected cells was carried out using the Live/Dead assay which gives a measure of membrane integrity and cellular function, but is a somewhat late measure of apoptosis. We also looked in detail at the effects of caspase inhibitors on two additional morphological indicators of apoptosis in neurons. The first is the fragmentation of DNA, detected by TUNEL analysis. The second is the apoptosis-associated externalisation of phosphatidylinerine. Our experiments demonstrated that p35 can inhibit both of these changes.

To examine the mechanism of action of p35 more closely, we designed novel peptide inhibitors based on the known cleavage site in the p35 protein (Bump et al., 1995; Xue and Horvitz, 1995). Microinjection of the specific cleavage site peptide, zDQMD-CHO, or the larger peptide, zDQMDGFHD-CHO, into SCG neurons did not, surprisingly, protect the cells against NGF withdrawal induced death, suggesting that sequences distant from the actual cleavage site are important for p35 inhibition of caspases. This is supported by experiments with p35 in insect cells which demonstrated that both N- and C terminus mutants lost the ability to protect cells from death (Bertin et al., 1996). The residues involved in these mutations may have a role in the association of p35 with caspases during, or after, cleavage. The involvement of regions outside the cleavage site may explain to some extent why p35 is such an excellent survival mediator in a wide variety of death paradigms.

Next, in order to determine which types of caspases were being activated during the death of SCG neurons, we examined a number of additional peptide caspase inhibitors. Ac-YVAD-CHO and Ac-DEVD-CHO both inhibited NGF withdrawal-induced death of SCG neurons to a similar degree to that of p35 when they were microinjected into cells. This is in contrast to the results described by Deshmukh et al. (1996) in which Ac-YVAD-CHO was not found to protect SCG neurons when added into the media, perhaps due to low peptide permeability in these cells. In addition, while ICE knockout mice do not have any reported defects in neuronal apoptosis (Kuida et al., 1995; Li et al., 1995), treatment with YVAD reduced the number of pyknotic neurons present in the developing chick embryo (Milligan et al., 1995). There does, however, appear to be a clear role for CPP32 in some types of neurons since there is a substantial increase in the number of neurons in the brains of CPP32 deficient mice (Kuida et al., 1996). We see a decrease, by western blotting (data not shown), in the level of the pro-form of CPP32 in SCG neurons withdrawn from NGF for 24 hours, which is prevented by co-treatment with zVAD-fmk (see below). This is in contrast to published data where no decrease in pro-CPP32 levels was seen (Deshmukh et al., 1996) and it is unclear as to why there is a discrepancy. We therefore suggest that inhibiting either ICE-like or CPP32-like proteases
is sufficient to prevent/delay apoptosis in these cells, but that it is possible that these peptides are able to interact with multiple caspases when injected at high concentrations.

In addition to microinjecting inhibitors, we have also used the cell permeable inhibitor zVAD-fmk. This peptide has been shown previously to inhibit the processing of CPP32 in lymphoid cell lines (Slee et al., 1996), but did not prevent PARP cleavage in vitro, suggesting that it blocked the activation of CPP32 rather than CPP32 itself. More recently, zVAD-fmk has been used to inhibit programmed cell death in the interdigital webs in developing mouse paws in an organ culture system (Jacobson et al., 1996) and reaper induced death in Schneider cells (Pronk et al., 1996). In SCG neurons withdrawn from NGF, zVAD-fmk prevented the loss of neurites, although the cell bodies did appear smaller (approximately 50%). The removal of NGF from neurons induces a rapid decrease in protein synthesis (Greenlund et al., 1995), and peptide caspase inhibitors may not prevent this (Deshmukh et al., 1996). We found that if NGF is re-added to cells which have been maintained in zVAD-fmk for three days, a proportion of the cells do appear to recover completely, with the cells bodies becoming rounder and the neurites thicker and more extensive. This recovery upon re-addition of NGF indicates that inhibition of caspases by zVAD-fmk is sufficient to halt the progression of apoptosis at a point which is still reversible. This is an important observation, since in fibroblasts it has been

![Fig. 6](image_url). Effect of the zVAD-fmk on apoptosis induced by NGF withdrawal or staurosporine treatment. SCG neurons were treated with 0, 10 and 100 μM zVAD-fmk or 100 μM of the control peptide zFA-fmk and withdrawn from NGF (A) or treated with zVAD at the time of staurosporine addition (B). Cells were assayed 72 hours later and the results expressed as a percentage relative to cells maintained in NGF and are the mean ± s.e.m. for three separate experiments. (C). Cells maintained in NGF (a,f), withdrawn from NGF for 72 hours either without (b,g) or with (d,i) 100 μM zVAD-fmk, or treated with staurosporine for 72 hours either without (c,h) or with (e,j) 100 μM zVAD-fmk. Phase (a-e), Hoechst (f-j). Bar, 20 μM.

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**Peptide Concentration (μM)**

A. +NGF  -NGF

B. +NGF  -NGF  +STS

C. (a-f)
suggested that caspases act after the point of commitment and that cells maintained in zVAD-fmk are in a state of ‘living death’, unable to resume normal cellular activity following growth factor re-addition (McCarthy et al., 1997).

In the case of staurosporine-induced death, peptide inhibitors of caspasess were effective but not as effective as for NGF withdrawal-induced death. In addition, zVAD-fmk only protected SCG neurons when added 4 hours prior to staurosporine treatment. Further, zVAD-fmk did not prevent the rapid loss of neurites which occurred upon staurosporine addition. Degeneration of neurites and loss of cell body integrity can, however, be separated genetically. In C57BL/OLA mice, homologous for the ‘slow Wallerian degeneration’ allele (WldS), SCG cell bodies die after NGF withdrawal but the neurites remain intact (Deckworth and Johnson, 1994). Perhaps staurosporine is able to target additional enzymatic pathways involved in maintaining neurite integrity. One explanation for the decreased ability of peptide inhibitors to block staurosporine-induced death is that staurosporine may activate the caspase cascade at multiple or different points compared to that of NGF withdrawal. Evidence for multiple death cascades for different death paradigms has come from a number of experimental systems. In PC12 cells induced to die by NGF withdrawal or by the down regulation of superoxide dismutase (SOD1), there were differences in the amounts of caspase peptide inhibition required to rescue cells (Troy et al., 1996). In addition, in Jurkat cells CrmA expression prevented anti-APO-1-induced but not staurosporine-induced apoptosis (Chinnaiyan et al., 1996).

An additional possibility is that staurosporine activates the same caspasess, but with different kinetics. That zVAD-fmk was protective against staurosporine-induced death only when the cells were pre-treated suggests that staurosporine addition activates caspasess much faster than NGF withdrawal. However, we know from previous studies (Philpott et al., 1996) that the overall rates of death due to staurosporine addition or NGF depletion are similar. It may be that staurosporine addition results in the activation of potentially low levels of pre-existing caspasess, and that the death occurs slowly due to the small amount of active caspasess. On the other hand, NGF withdrawal has a lag phase in which transcription and new protein synthesis occur and hence may cause a significant increase in levels of pro-caspases. This could eventually amplify the late phase of apoptosis. There is some evidence for the induction of new ICE production in apoptotic mammary epithelial cells (Boudreau et al., 1995) and the requirement for new protein synthesis in neuronal cell death supports this hypothesis.

Our results in SCG neurons, in which zVAD-fmk could protect from both NGF-withdrawal induced death and staurosporine-induced death, contrast with experiments carried out in cerebellar granule neurons (Taylor et al., 1997). In these neurons zVAD-fmk protected cells from staurosporine-induced death but not from potassium withdrawal-induced death. They are also different from results with fibroblasts. In this case, zVAD-fmk could be added up to 2 hours after staurosporine treatment and still protect the majority of cells, but Ac-YVAD-CHO could not (Jacobson et al., 1996). These observations point to a somewhat surprising conclusion. All types of neuronal apoptosis known currently depend on activation of one or more caspasess. However, there seems to be extensive variability in the ways in which different kinds of neurites die in response to different death inducers. This suggests that not all forms of neuronal apoptosis are equivalent and that it may be possible to block some forms of apoptosis without affecting others. This information could be significant in designing therapies for various neurodegenerative disorders and highlights the need to study these proteases in specific neuronal types.

In summary, we have compared the survival promoting abilities of p35 with a range of peptide inhibitors in two different death paradigms and have suggested that an ICE-like and CPP32-like protease may be important in the death of SCG neurons. A detailed investigation comparing different mechanisms of inducing apoptosis with the protective effects afforded by p35 or peptide inhibitors has been undertaken in neurons and has highlighted some interesting and potentially important features. Dissecting out the molecular mechanism of neuronal cell death promises to enhance progress in the understanding and treatment of neurodegenerative disorders.

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