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JCB Article

Cyclosporin A inhibits caspase-independent death of NGF-deprived sympathetic neurons: a potential role for mitochondrial permeability transition

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Opening of the permeability transition pore (PTP) has been implicated as an important mitochondrial event that occurs during apoptosis. We examined the role of the PTP in the well-characterized cell death of rat sympathetic neurons deprived of nerve growth factor (NGF) in vitro. Removal of NGF causes these neurons to undergo either a classic apoptotic cell death or, when treated with a broad-spectrum caspase inhibitor such as boc-aspartyl(OMe)-fluoromethylketone (BAF), a delayed, nonapoptotic cell death. The PTP inhibitor, cyclosporin A (CsA), blocked commitment-to-die in the presence of BAF, as defined by the ability of NGF readdition to rescue cells, but had little effect on commitment-to-die in the absence of BAF. CsA did not have trophic effects on BAF-saved cells, but did block the decrease in mitochondrial membrane potential. These data suggest that PTP opening is a critical event in caspase-independent, nonapoptotic (but not caspase-dependent, apoptotic) death of NGF-deprived rat sympathetic neurons.

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Introduction

Programmed cell death (PCD)* is an orderly, cell-autonomous process by which excess cells are eliminated during development. Cells undergoing PCD display certain morphological features, such as cytoplasmic shrinkage, plasma membrane blebbing, chromatin condensation, and nuclear fragmentation. These features describe a form of cell death termed apoptosis (Kerr et al., 1972). Although the occurrence of PCD during development has long been known, its apoptotic features have been recognized more recently in pathological conditions of many tissues.

Apoptosis is executed by caspases, a family of cysteine proteases which are synthesized as relatively inactive zymogens, or procaspases (for review see Cryns and Yuan, 1998). In mammalian cells undergoing apoptosis, caspase activation is regulated principally by two mechanisms. In the intrinsic pathway, which is triggered by apoptotic stimuli such as trophic factor withdrawal and DNA damage, the activation of caspases is regulated by the convergence of signals at the mitochondrion, such as those mediated by the BCL-2 family of proteins. These signals lead to the release of cytochrome c from the intermembrane space of the mitochondria to the cytosol, where it interacts with the apoptosome, a large complex containing procaspase-9, APAF-1, and dATP, activating caspase-9 (Li et al., 1997). In contrast, in the extrinsic pathway, cell surface receptors, such as TNFR1 and Fas/CD95/APO-1, can directly activate caspase-8 through a signaling complex including the cytoplasmic tail of these receptors and procaspase-8 (Muzio et al., 1996). The separation between these pathways is not absolute, as cleavage of the proapoptotic BCL-2 family member, BID, by caspase-8 can lead to mitochondrial release of cytochrome c (Li et al., 1998; Luo et al., 1998).

The importance of the mitochondrion in apoptosis has lead to the examination of mitochondrial physiology for mechanistic roles in cell death. One such event is the opening of the permeability transition pore (PTP), a transient channel 2–3 nm in diameter connecting the cytosol and the mitochondrial matrix (for review see Crompton, 1999). The PTP is composed of at least three proteins in different compartments of the mitochondria: the voltage-dependent anion channel in the outer mitochondrial membrane (OMM), the adenine nucleotide translocase in the inner mitochondrial membrane, and cyclophilin D in the mitochondrial matrix. The PTP forms transiently under normal physiologic conditions, but under certain conditions, such as high calcium or

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*Abbreviations used in this paper: $\Delta\Psi$m, mitochondrial membrane potential; BAF, boc-aspartyl(OMe)-fluoromethylketone; CsA, cyclosporin A; NFAT, nuclear factor of activated T-cells; NGF, nerve growth factor; OMM, outer mitochondrial membrane; PCD, programmed cell death; PTP, permeability transition pore.

Key words: apoptosis; cytochrome c; mitochondria; permeability transition pore; programmed cell death

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low ATP levels, prolonged opening of the PTP leads to mitochondrial matrix swelling and rupture of the OMM. Two predicted consequences of this gross disruption of the OMM are the release of cytochrome c from the intermembrane space and the loss of the mitochondrial membrane potential ($\Delta\Psi_m$), leading to speculation that PTP opening may be the mechanism by which these events occur during apoptosis. Consistent with this, loss of $\Delta\Psi_m$ and cytochrome c release occur simultaneously in several models of cell death (Bradham et al., 1998; Heiskanen et al., 1999). Furthermore, pharmacologic inhibition of PTP opening by cyclosporin A (CsA) or bongkrekic acid inhibits cell death in a number of model systems (for review see Vieira et al., 2000). However, in some experimental paradigms, $\Delta\Psi_m$ is maintained, or even elevated, during and after cytochrome c release, arguing that the mechanism of cytochrome c release does not involve PTP opening (Vander Heiden et al., 1997; Bossy-Wetzel et al., 1998; Goldstein et al., 2000).

In this study, we examined the role of the PTP in NGF deprivation–induced death of sympathetic neurons from the superior cervical ganglion in vitro, a model of naturally occurring cell death in the developing nervous system. This cell death shows many morphological characteristics of apoptosis and requires protein synthesis (Martin et al., 1988), the proapoptotic Bcl-2 family member BAX (Deckwerth et al., 1996), and caspase activity (Deshmukh et al., 1996; Troy et al., 1996). Many of the early events in this cell death pathway are reversible, such that sympathetic neurons that have been deprived of NGF for a short time can fully recover after trophic factor readdition (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). In sympathetic neurons, the time course with which cells can no longer be rescued by NGF, or become committed-to-die, is virtually identical to the time course of cytochrome c release and rapidly ensuing caspase activation (Putcha et al., 1999). If caspase activity is inhibited by the broad-spectrum caspase inhibitor, boc-aspartyl(OMe)-fluoromethylketone (BAF), NGF-deprived sympathetic neurons can be rescued by NGF after they have released cytochrome c (Martinou et al., 1999). This protection is finite in duration, because BAF-saved cells eventually die by a nonapoptotic, caspase-independent mechanism (Deshmukh et al., 2000). This stands in contrast to the protection afforded by BAX deletion, which permanently prevents neuronal death after NGF deprivation (Deckwerth et al., 1996). Before caspase inhibitor–saved cells die, they lose the ability to be rescued by NGF and become committed-to-die. The commitment-to-die in the presence of a caspase inhibitor is termed Commitment 2, to distinguish it from the commitment-to-die in the absence of a caspase inhibitor, termed Commitment 1. The mitochondrial hit or the perturbation of the mitochondria that results from BAX translocation and cytochrome c release, is critical to Commitment 2, as BAX deletion prevents both these mitochondrial changes and the onset of Commitment 2.

Here, we report that CsA inhibited Commitment 2, but not Commitment 1, in rat sympathetic neurons, suggesting that PTP opening was a critical event in the commitment-to-die in the presence, but not the absence of, a caspase inhibitor. CsA acted downstream of cytochrome c release to block the loss of mitochondrial membrane potential, arguing that opening of the PTP was responsible for the loss of $\Delta\Psi_m$ associated with Commitment 2. Remarkably, CsA had no effect on Commitment 2 in mouse sympathetic neurons, suggesting important species differences exist in this cell death pathway. These results have important implications regarding the use of caspase inhibitors as neuroprotectants.

Results

CsA delays commitment-to-die in rat sympathetic neurons in the presence of a caspase inhibitor

To investigate the role of PTP opening in cell death in the presence of a caspase inhibitor, we examined the effects of the PTP inhibitor, CsA, on Commitment 2. Rat sympathetic neurons were deprived of NGF in the presence of the broad-spectrum caspase inhibitor BAF with or without 10 μM CsA for various times, after which rescue by NGF readdition was attempted. After 7 d of NGF rescue, the number of viable cells was counted to determine the percentage of cells that had not committed-to-die. Fig. 1 A shows examples of cells after 10 d of treatment. NGF-deprived cells treated with BAF or BAF and CsA appeared atrophic but maintained phase-bright soma and intact neurites. After 7 d of rescue with NGF, cells that were not committed-to-die in both treatment groups displayed an increase in cell size in response to 7 d of NGF rescue (Fig. 1 A).

As shown in Fig. 1 B, the proportion of NGF-deprived, BAF-saved cells that was rescued by NGF decreased with increasing time of NGF deprivation. About 60% of cells treated with BAF alone became committed-to-die within the first 12 d of NGF deprivation. After this, the rate of commitment-to-die decreased greatly, such that ~25% of BAF-saved cells were rescued by NGF readdition after 35 d of NGF deprivation. Concurrent treatment with 10 μM CsA dramatically inhibited the onset of Commitment 2, increasing the number of cells that was able to respond to readdition of NGF at all time points between 4 and 35 d of NGF deprivation. Most notably, CsA nearly completely blocked the initial, rapid onset of Commitment 2, such that after 10 d of NGF deprivation, ~90% of cells treated with BAF and CsA were rescued, versus ~40% of cells treated with BAF alone. Even after 35 d of NGF deprivation in the presence of BAF and CsA, >60% of neurons were rescued by NGF readdition. Thus, CsA conferred robust and sustained inhibition of Commitment 2 in rat sympathetic neurons, suggesting that PTP opening is a critical event in Commitment 2.

PTP opening is not required for commitment-to-die in rat sympathetic neurons in the absence of a caspase inhibitor

All known NGF deprivation–induced events that occur proximal to caspase activation are common to the pathways of both Commitment 1 and Commitment 2. Therefore, CsA could delay Commitment 2 indirectly by inhibiting signal transduction events upstream of cytochrome c release, such as activation of the JNK pathway, that are common to both Commitment 1 and Commitment 2. Alternatively, CsA could directly affect Commitment 2 by inhibiting events downstream of cytochrome c release that are specific

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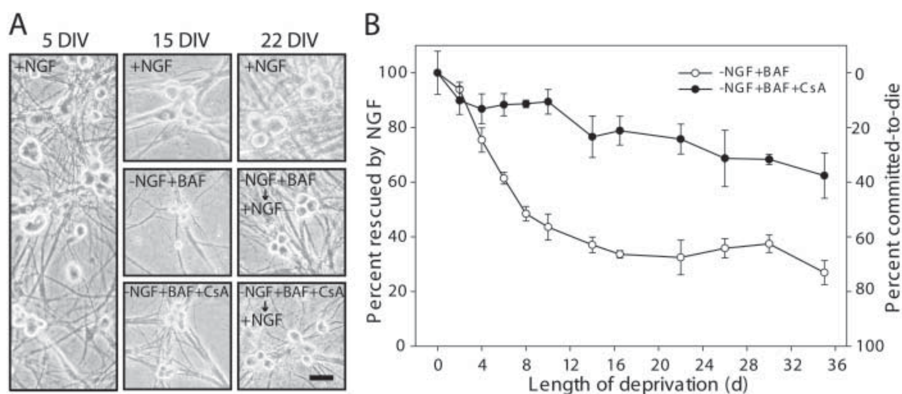

Figure 1. **CsA inhibits commitment-to-die in the presence of a caspase inhibitor.** Sympathetic neurons were deprived of NGF in the presence of BAF or BAF plus 10 μM CsA for various periods before being rescued with NGF. (A) Phase-contrast micrographs of cells at the time of treatment (5 d in vitro), after 10 d of treatment (15 d in vitro), and 7 d after NGF rescue (22 d in vitro) are shown. Bar, 40 μm. (B) The proportion of BAF-saved cells that were rescued by NGF is plotted as the percentage of cells rescued (left ordinate), or the percentage committed-to-die (right ordinate, calculated as 100% minus the percentage of cells rescued), versus length of NGF deprivation. Differences between BAF versus BAF plus CsA at all time points except 0 and 2 d were statistically significant ($P < 0.05$ by t test). The data represent mean ± SD of one experiment ($n \geq 4$) that was representative of three incomplete but overlapping independent time-course experiments.

to Commitment 2. To determine whether CsA affected proximal events in the cell death pathway, the effect of CsA on Commitment 1 was examined. Cells were deprived of NGF in the presence or absence of 10 μM CsA for various times before being rescued with NGF. In the absence of CsA, ~75% of neurons became committed-to-die by 24 h of NGF deprivation, with nearly all cells becoming committed-to-die after 4 d (Fig. 2 A). CsA had little effect on Commitment 1, although the proportion of CsA-treated cells that could be rescued by NGF was slightly increased. Although this effect was seen in multiple independent experiments, the magnitude of the inhibition was small and transient. Similarly, CsA slightly delayed, but did not prevent, the death of NGF-deprived cells, as determined by loss of toluidine blue staining (unpublished data). Thus, whereas CsA greatly inhibited Commitment 2, it had little effect on Commitment 1.

Next, we examined by immunocytochemistry whether CsA affects the loss of mitochondrial cytochrome c. NGF-maintained neurons exhibited a bright, punctate staining pattern (see Fig. 5 A), indicative of mitochondrial localization of cytochrome c, which is lost when cytochrome c is released into the cytosol and subsequently degraded (Deshmukh and Johnson, 1998; Neame et al., 1998). When deprived of NGF, ~50% of cells retained mitochondrial cytochrome c after 24 h of NGF deprivation, with this proportion decreasing to ~10% after 72 h. Exposure to 10 μM CsA had no effect on cytochrome c status after 24 h, and only slightly increased the number of cells with intact cytochrome c after 72 h of NGF deprivation. Similar to the effect of CsA on Commitment 1, its effect on the loss of mitochondrial cytochrome c was of small magnitude and transient, as all NGF-deprived cells treated with BAF and CsA eventually lost cytochrome c (see Fig. 5 A). Thus, CsA had only a minor effect on cytochrome c release as determined by both NGF rescue and immunocytochemistry.

To further exclude an action of CsA on events proximal to the mitochondria, we examined the effect of CsA on the rate of protein synthesis. Protein synthesis rates, as determined by measuring the incorporation of radiolabeled methionine and cysteine, decrease upon NGF deprivation (Deckwerth and Johnson, 1993) but are preserved by neuroprotectants that act proximal to the mitochondria, such as depolarizing levels of potassium, cyclic AMP analogues, and inhibition of the JNK pathway (Rydel and Greene, 1988; Harris et al., 2002). As shown in Fig. 2 C, CsA had no effect on this decrease in protein synthesis. Thus, CsA did not block early metabolic changes caused by NGF deprivation, further demonstrating that CsA does not inhibit Commitment 2 by acting proximal to the mitochondrial events of the cell death pathway. Notably, CsA itself did not inhibit protein synthesis, as treatment with 10 μM CsA for 24 h did not significantly decrease metabolic labeling of NGF-maintained cells. Because significant inhibition of protein synthesis is sufficient to block Commitment 1 and cytochrome c release, this finding was consistent with the inability of CsA to block these events (Fig. 2, A and B). Thus, these findings show that CsA does not act upstream of cytochrome c release and that CsA inhibits caspase-independent, but not caspase-dependent cell death.

To further examine the effect of CsA on the trophic status of the cells, the effect of CsA on protein synthesis rates of cells deprived of NGF in the presence of BAF for 10 d were examined. As seen in Fig. 2 D, CsA had no effect on the rate of protein synthesis of cells deprived of NGF in the presence of BAF for 10 d. A similar lack of effect was observed using somal diameter (Fig. 1 A; unpublished data) or MTT reduction (unpublished data) as measures of the trophic status of NGF-deprived, BAF-saved cells. Thus, CsA had no effect on the metabolic status of NGF-deprived, BAF-saved cells, further arguing that inhibition of upstream signal transduction events does not underlie the effect of CsA on Commitment 2.

Effect on Commitment 2 is not mediated by inhibition of calcineurin

CsA blocks PTP opening by binding to the mitochondrial matrix protein cyclophilin D (Halestrap et al., 1997). CsA also exerts immunosuppressive effects by inhibiting calcineurin/nuclear factor of activated T-cells (NFAT), JNK, and p38 pathways. Another immunosuppressant, FK-506, also inhibits these signal transduction pathways, but does not affect PTP opening (Matsuda et al., 2000). Therefore, we examined whether FK-506 affected Commitment 2. Cells treated with CsA showed a dose-dependent delay in Commitment 2, with the maximum effect reached at 10 μM (Fig. 3).

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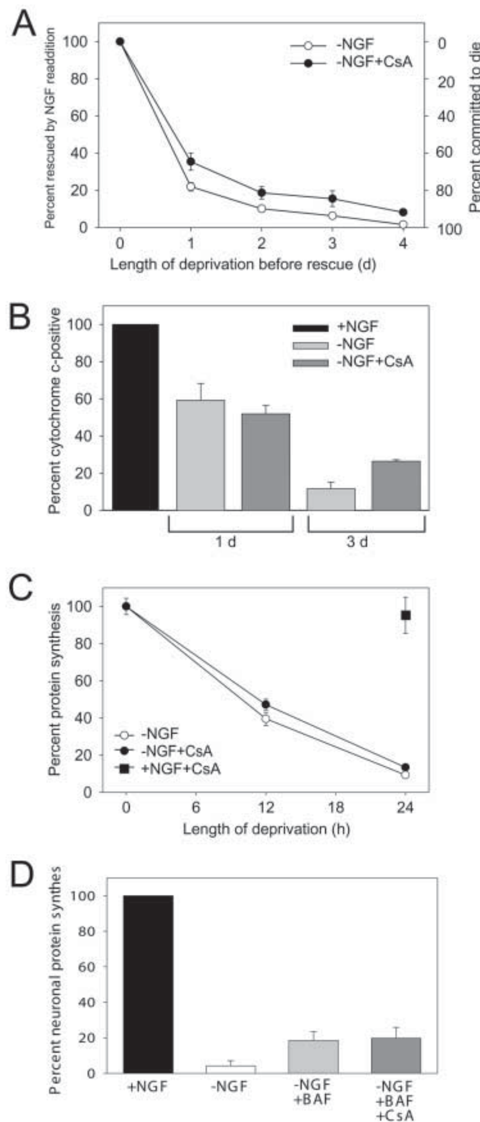

Figure 2. **CsA does not affect proximal events in the cell death pathway.** Rat sympathetic neurons were deprived of NGF in the presence or absence of 10 μM CsA. Cells were either rescued with NGF readdition (A) or processed for cytochrome c immunocytochemistry (B) after various times of NGF deprivation, as denoted on the x-axis of each graph. (A) Numbers of viable cells that remained after 7 d of trophic factor readdition and are plotted as a time course of commitment-to-die in the absence of a caspase inhibitor, or Commitment 1. The asterisks (*) at 1, 2, and 3 d denote statistical significance ($P < 0.05$). (B) Proportions of cells with intact mitochondrial cytochrome c after 1 and 3 d of NGF deprivation as determined by cytochrome c immunocytochemistry. (C) CsA does not affect the early NGF deprivation–induced drop in protein synthesis in NGF-deprived cells in the absence of or presence of 10 μM CsA. CsA did not inhibit protein synthesis in the presence of NGF ($P > 0.6$ vs. NGF alone) after 24 h of exposure. (D) CsA had no effect on the rate of protein synthesis in the presence of BAF after 10 d of NGF deprivation. Data in each panel represent mean ± SEM of three experiments performed in quadruplicate.

At this dose, 90% of cells could be rescued by NGF after 10 d of NGF deprivation, versus ~40% in BAF alone, consistent with the data shown in Fig. 1 B. In contrast, at doses ranging from 0.03 to 3 μM, FK-506 had no effect on the percentage of cells that had committed-to-die after 10 d of NGF deprivation. 3 μM FK-506 was not toxic to NGF-maintained neurons (Fig. 3). Because the IC_{50} for T-cell activation by FK-506 in intact cells is ~10–100-fold less than that of CsA (Kino et al., 1987), these doses of FK-506 should be sufficient to inhibit these signaling pathways as effectively as doses of CsA that alter Commitment 2. This is consistent with the lack of effect of CsA on early events of the cell death pathway (Fig. 2), which are blocked by inhibition of the JNK pathway (Harris et al., 2002). These findings argue that the effect of CsA on Commitment 2 is not due to inhibition of calcineurin/NFAT, JNK, or p38 pathways.

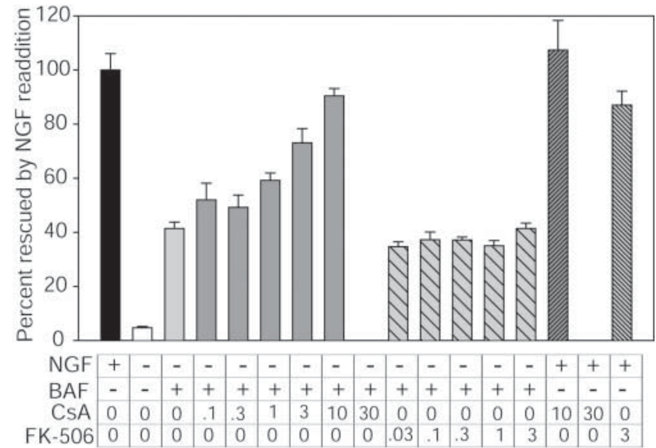

Figure 3. **CsA, but not FK-506, inhibits Commitment 2 in a dose-dependent fashion.** Cells were deprived of NGF in the presence of BAF alone or with different concentrations of CsA (0.3–30 μM) or FK-506 (0.03–3 μM) for 10 d and then rescued with NGF for 7 d. CsA conferred a dose-dependent effect on Commitment 2, whereas FK-506 had no effect on Commitment 2. Data are mean ± SD ($n = 4$) of one experiment that was representative of two independent experiments.

CsA blocks NGF deprivation–induced decrease in $\Delta\psi_m$

The loss of $\Delta\psi_m$, as measured by the $\Delta\psi_m$-sensitive dye, Mitotracker Orange CM-H_2TMRos, is a nearly perfect predictive marker for the onset of Commitment 2 in mouse sympathetic neurons (Deshmukh et al., 2000). To investigate the effect of CsA on $\Delta\psi_m$ in rat sympathetic neurons, cells were deprived of NGF in the presence of BAF or BAF plus 10 μM CsA for 10 d. The status of cytochrome c and $\Delta\psi_m$ in individual cells was determined by labeling cells with Mitotracker Orange, followed by cytochrome c immunocytochemistry. As seen in Fig. 4 A, NGF-maintained rat sympathetic neurons had intact cytochrome c staining and displayed bright Mitotracker staining. After 10 d of NGF deprivation, BAF-treated neurons lacked both cytochrome c staining and distinct Mitotracker staining. Rat sympathetic neurons deprived of NGF in the presence of BAF and CsA lacked cytochrome c staining but maintained distinct Mitotracker staining. This finding argues that CsA blocks NGF deprivation– induced decrease in $\Delta\Psi_m$.

To verify this finding by another method, sympathetic neurons were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracetylbenzimidazolylcarbocyanine iodide (JC-1), a $\Delta\psi_m$-sensitive dye that aggregates and changes spectral properties within polarized mitochondria. The intensity of JC-1 fluorescence in cells deprived of NGF in the presence of BAF for

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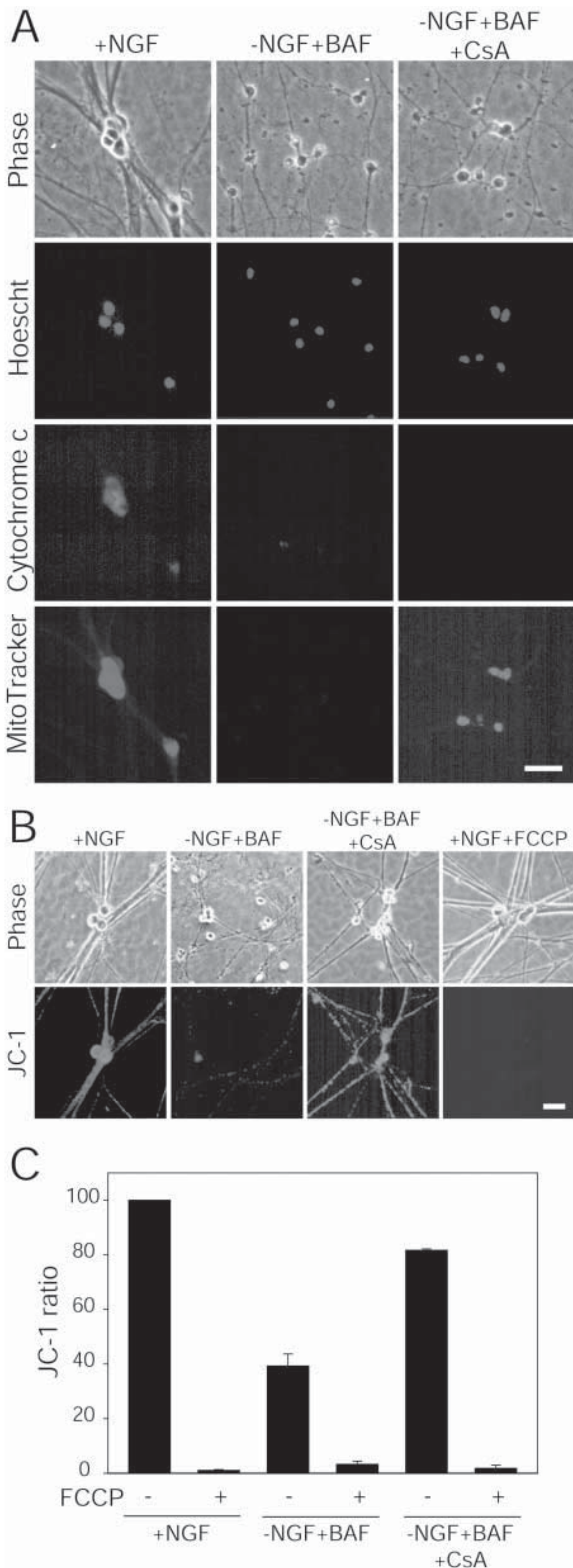

Figure 4. **CsA inhibits NGF deprivation-induced decrease of $\Delta\psi_m$.** $\Delta\Psi_m$ in rat sympathetic neurons deprived of NGF for 10 d in the presence of BAF or BAF plus CsA was determined by using Mitotracker Orange (A) or JC-1 (B, C). (A) Cells maintained in NGF had mitochondrial cytochrome c immunostaining and were Mitotracker positive. NGF-deprived, BAF-saved cells lacked cytochrome c staining and Mitotracker staining. In contrast, NGF-deprived cells treated with BAF plus CsA lacked cytochrome c staining but stained with Mitotracker. Nuclei of NGF-deprived cells treated with BAF or BAF plus CsA appeared atrophic but otherwise normal when compared with NGF-maintained cells. (B) Similar results were found when monitoring the aggregated form of JC-1 as an indicator of $\Delta\Psi_m$. JC-1 staining was absent in NGF-maintained cells pretreated with 10 μM FCCP. Quantification of the staining intensity is shown in C. Data shown represent mean ± SEM of three independent experiments. Differences between all groups in the absence of FCCP are statistically significant ($P < 0.01$). Bars, 50 μm.

10 d was greatly diminished compared to NGF-maintained cultures (Fig. 4 B). In contrast, JC-1 staining was largely maintained in NGF-deprived cells treated with BAF and CsA, verifying that CsA blocks the decrease in $\Delta\psi_m$. A 10-min pretreatment with the uncoupling agent, FCCP, completely abolished visible JC-1 aggregate staining, verifying the $\Delta\psi_m$-dependence of this signal.

Because the ratio of JC-1 aggregate to monomeric staining is independent of cell number or mitochondrial density, it can be used as a quantitative measure of $\Delta\psi_m$. The JC-1 ratio of NGF-deprived, BAF-saved neurons was <40% of that of NGF-maintained neurons when quantified with a fluorescent plate reader (Fig. 4 C). The combination of BAF and CsA increased the intensity of fluorescence to 81% of control. The dependence of the JC-1 ratio on $\Delta\psi_m$ was verified by exposing all treatment groups to FCCP, which decreased the signal to 3% or less of control. Thus, mitochondria in rat sympathetic neurons deprived of NGF in the presence of BAF were partially depolarized after 10 d of NGF deprivation. CsA significantly attenuated this NGF deprivation–induced decrease in $\Delta\psi_m$ in BAF-saved neurons without affecting the status of cytochrome c within the cell. Because we do not know the linearity of this assay, these results may not be rigorously quantitative. However, taken together, these data suggest that PTP opening is responsible for NGF deprivation–induced loss of $\Delta\psi_m$, and that inhibition of PTP opening accounts for the ability of CsA to block the onset of Commitment 2.

CsA is required during a distinct critical period after NGF deprivation

A striking feature of the time course of Commitment 2 in rat sympathetic neurons is that nearly all cells that commit-to-die over 35 d of NGF deprivation reach the Commitment 2 checkpoint within the first 12 d (Fig. 1 B). One possible explanation is that the trigger for CsA-inhibitable commitment-to-die is not sustained throughout the entire period of NGF deprivation, such that the 30% of cells that survive the first 12 d of NGF deprivation never undergo Commitment 2 because a drive for PTP opening no longer exists. To determine whether PTP opening is a transient or sustained event, we sought to identify the period after NGF deprivation during which CsA exposure was required to inhibit Commitment 2. We reasoned that if PTP opening were caused by a transient event or process, then after some critical period the continued presence of CsA would not be necessary to inhibit Commitment 2. Alternatively, if the driving force for PTP opening were a sustained event, continued exposure to CsA would be necessary to prevent Commitment 2.

To determine whether the continued presence of CsA was necessary for its effects on Commitment 2, CsA was

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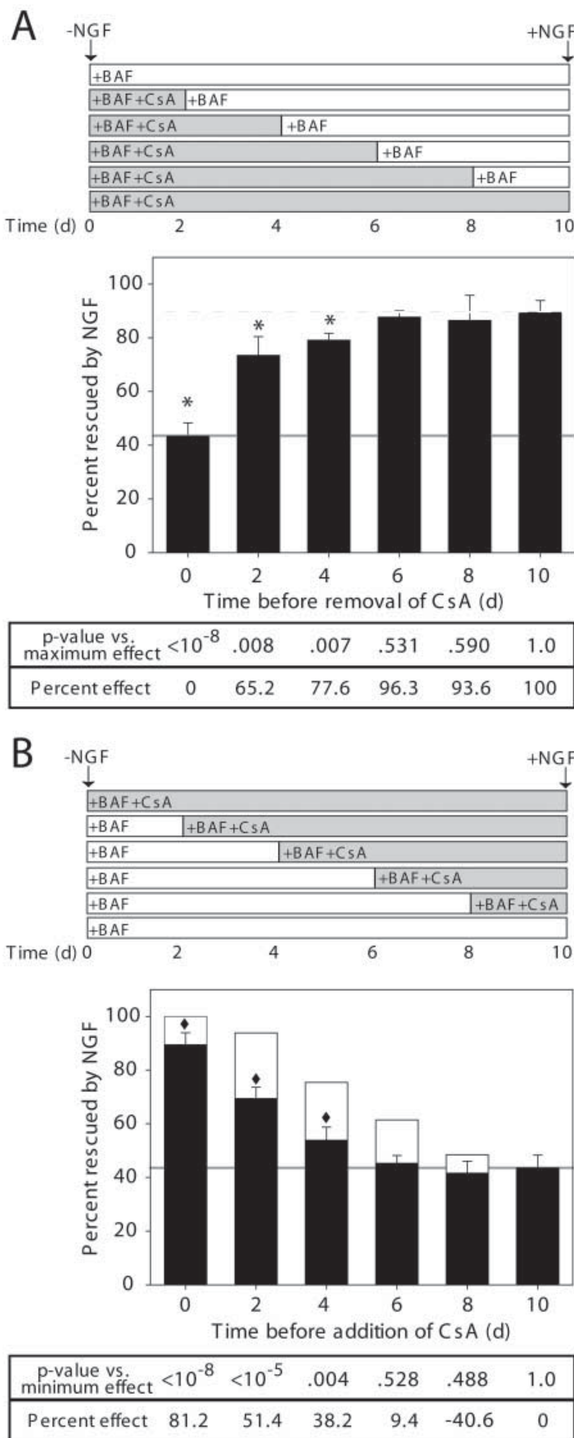

p-value vs. maximum effect	$<10^{-8}$	.008	.007	.531	.590	1.0
Percent effect	0	65.2	77.6	96.3	93.6	100

p-value vs. minimum effect	$<10^{-8}$	$<10^{-5}$	.004	.528	.488	1.0
Percent effect	81.2	51.4	38.2	9.4	-40.6	0

Figure 5. **CsA is required for distinct period during NGF-deprivation to affect Commitment 2.** The effect of early removal of A or delayed addition of B CsA to NGF-deprived, BAF-treated cells on Commitment 2 was determined. (A) Rat sympathetic neurons were deprived of NGF in the presence of BAF plus 10 μM CsA. As shown in the schematic, CsA was removed from cultures at various times after NGF deprivation, maintained in BAF for the duration of the 10 d, and then rescued with NGF. The proportion of cells that were rescued by NGF after this period of NGF deprivation is plotted as a function of the length of time cultures were exposed to CsA. The lower reference line (gray line) represents the percentage of cells treated with BAF alone that were rescued after 10 d of NGF deprivation, designated as zero percent effect. The upper reference line (dotted line) represents the maximal effect of BAF plus CsA for 10 d, or 100 percent effect. Removal of CsA after 6 or 8 d still provided maximal inhibition of Commitment 2. Asterisks (*) indicate that the proportion of cells rescued in a treatment group was statistically different from the maximal effect. (B) To determine the effect of delayed administration of CsA, it was added to NGF-deprived, BAF-treated cultures at various times, as schematized. The greater the delay before CsA addition, the closer the proportion of cells that could be rescued after 10 d of NGF-deprivation (solid bars) was to treatment with BAF alone (gray line). The number of cells treated with BAF alone that could be rescued (gray line) represents the minimum effect (0 percent effect). The proportion of cells that became committed-to-die after 10 d of NGF deprivation is statistically different (◆) than the minimum effect only when CsA was added after 0, 2, or 4 d of NGF deprivation. Note that the maximum possible saving effect of CsA addition (empty bars) decreased during the course of the experiment because NGF-deprived, BAF-saved cells became committed-to-die. *P* values from Student's unpaired *t* tests are shown. Data are mean ± SD of one experiment, performed in quadruplicate, that was representative of two independent experiments.

removed from cultures deprived of NGF in the presence of BAF and CsA at different times over a 10-d interval, after which all cells were rescued with NGF for 7 d (schematized in Fig. 5 A). The number of cells that could be rescued with NGF at the end of the 10-d period increased with increasing length of exposure to CsA (Fig. 5 A). When compared with cultures treated with CsA for the entire 10-d period, removal of CsA after 0, 2, or 4 d of NGF deprivation decreased the proportion of cells that were rescued by NGF. In contrast, removal of CsA after 6 or 8 d did not decrease the number of cells that were rescued, arguing that exposure to CsA after 6 d of NGF deprivation was not necessary for maximal inhibition of Commitment 2. These data argue that this CsA-sensitive event does not contribute to Commitment 2 after the first six d of NGF deprivation.

To determine whether delayed administration of CsA could inhibit Commitment 2, CsA was added to NGF-deprived, BAF-saved neurons at 2-d intervals over a 10-d period, after which all cells were rescued with NGF (Fig. 5 B). When added at d 0, 2, or 4, CsA increased the proportion of cells rescued by NGF over cultures treated with BAF alone. However, addition of CsA after 6 d of NGF deprivation did not increase the number of rescued cells, suggesting that the critical period for CsA-sensitive commitment event is complete after 6 d of NGF deprivation. To quantify the effects of delayed administration, the percent-maximum effect was calculated. Because BAF-saved cells become committed-to-die over this period, the maximum number of cells that can be saved by any intervention, including NGF readdition, decreases with time. This value, set as 100% effect, represents the time course of Commitment 2 (Fig. 1 B). This value decreased to close to zero when CsA was added after 6 d of NGF deprivation. Thus, CsA can modulate Commitment 2 if present during the first 6 d of NGF deprivation. These findings are consistent with the conclusion from the time course of removal of CsA (Fig. 5 A) that PTP opening must be inhibited during the first 6 d of NGF deprivation to alter Commitment 2 in rat sympathetic neurons. Taken together, these findings argue that the drive for PTP opening is not sustained throughout

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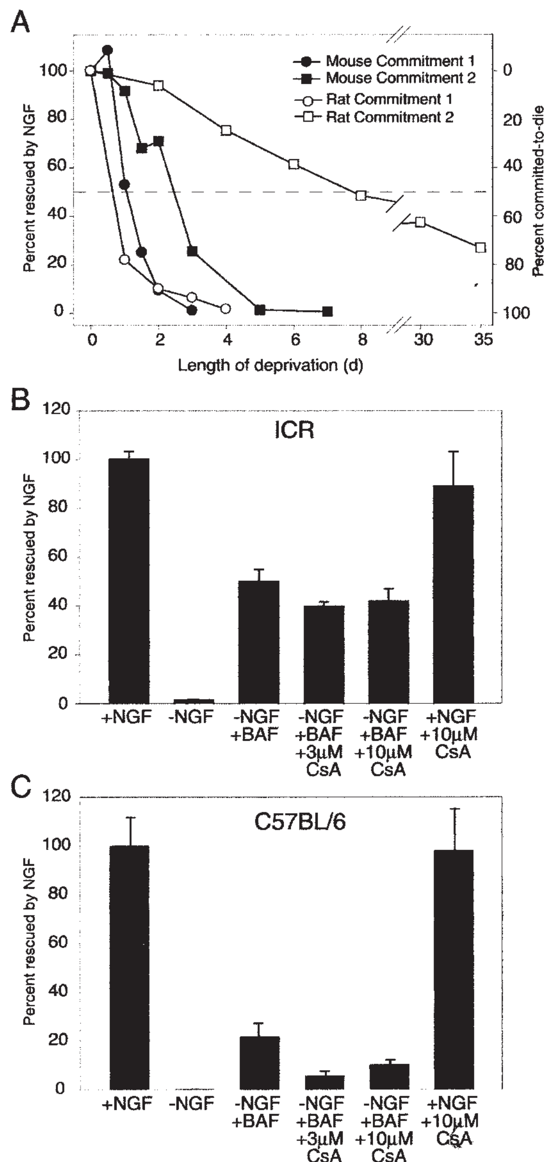

Figure 6. **CsA does not alter Commitment 2 in mouse sympathetic neurons.** (A) Comparison of time courses of Commitment 1 and Commitment 2 in rat and ICR mouse sympathetic neurons on a single graph. Data for rat time courses are taken from Figs. 1 B and 2 A, whereas those for mouse cells have been previously published (Deshmukh et al., 2000). The effects of CsA on Commitment 2 in sympathetic neurons from ICR (B) and C57BL/6J (C) mice after 72 h of NGF deprivation are shown. CsA, at 3 or 10 μM, did not significantly increase the proportion of cells that were rescued by NGF in either strain of mouse. Values represent mean ± SD of one experiment (performed in quadruplicate) that was representative of three independent experiments performed at slightly different time points.

the entire duration of NGF deprivation, but rather occurs during a distinct critical period.

CsA does not delay Commitment 2 in mouse sympathetic neurons

Although the time courses of Commitment 1 are very similar in rat and mouse sympathetic neurons, the time courses of Commitment 2 are very different. To illustrate this difference, time courses of Commitment 1 and Commitment 2 of sympathetic neurons from ICR mice (Deshmukh et al., 2000) and Sprague Dawley rat (Figs. 1 B and 2 A) were plotted on a single graph (Fig. 6 A). About 50% of mouse sympathetic neurons reached Commitment 2 after 60 h of NGF deprivation, versus 8 d in rat sympathetic neurons. By 5 d of NGF deprivation in the presence of BAF, all mouse neurons are committed-to-die, whereas nearly 30% of rat neurons can still be rescued after 35 d. To determine whether CsA had the same effect on Commitment 2 in these two species, sympathetic neurons from mice were deprived of NGF in the presence of BAF or BAF plus CsA for 72 h before rescue with NGF (Fig. 6 B). Surprisingly, CsA at doses up to 10 μM had no effect on the proportion of cells that were rescued by NGF. Pretreatment of cells with CsA for 2 h did not increase its effect on Commitment 2 (unpublished data). As seen with rat neurons, 10 μM CsA was not toxic to NGF-maintained neurons, whereas both NGF-maintained and NGF-deprived, BAF-saved mouse neurons treated died in the presence of 30 μM CsA (Fig. 3; unpublished data). A similar lack of effect on Commitment 2 was found in sympathetic neurons from different strains of mice, including C57BL/6J (Fig. 6 B), Swiss-Webster, and BALB/C (unpublished data). These data demonstrate that Commitment 2 in mouse and rat sympathetic neurons had striking phenomenological and mechanistic differences.

Discussion

In this study, we examined the role of PTP opening in NGF deprivation–induced death of sympathetic neurons. We asked whether CsA altered cell death in the presence or absence of a caspase inhibitor. From this study, we have three main conclusions. First, CsA blocks Commitment 2, the commitment-to-die in the presence of a caspase inhibitor, in NGF-deprived rat sympathetic neurons. Second, CsA does not prevent cytochrome c release or Commitment 1, the commitment-to-die in the absence of a caspase inhibitor, and thus does not act at the premitochondrial phase of the intrinsic pathway in these neurons. Third, the events that regulate Commitment 2 in mouse and rat sympathetic neurons are remarkably different.

CsA inhibits Commitment 2 in rat sympathetic neurons

In many models of neuronal cell death, caspase inhibition prevents apoptosis but not the ultimate death of cells, as they eventually undergo caspase-independent, nonapoptotic cell death (Miller et al., 1997; Stefanis et al., 1999; Deshmukh et al., 2000; Oppenheim et al., 2001). The events responsible for this nonapoptotic cell death are largely unknown. Here, we report that in NGF-deprived rat sympathetic neurons, the PTP inhibitor, CsA, provided robust and sustained inhibition of Commitment 2, suggesting that PTP opening may be a critical event in this nonapoptotic cell death (Fig. 1 B). CsA significantly increased the percentage of cells that could be rescued by NGF at all time points between 4 and 35 d of NGF deprivation. The most dramatic effect was after 10 d of NGF deprivation, when ~90% of neurons treated with BAF plus CsA were rescued by NGF readdition versus only 45% of cells treated with BAF alone. The protective effect of CsA on BAF-saved neurons was long term, as even after 35 d of NGF deprivation, the latest time point examined in these studies,

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>60% of CsA-treated, BAF-saved cells were able to respond to trophic factor readdition. These data suggest that PTP opening is a critical event in the commitment-to-die in the presence of a caspase inhibitor in rat sympathetic neurons.

CsA is widely used as an inhibitor of PTP opening, but its additional activities, such as inhibition of calcineurin/NFAT, JNK, and p38 pathways (Matsuda and Koyasu, 2000). This raises the possibility that the observed effect of CsA in these studies is not due to inhibition of PTP opening. However, the lack of effect of FK-506, which also inhibits these signaling pathways but does not block PTP opening, argues against this. In addition, the inability of CsA to block early cell death events (Fig. 2) argues that CsA is not inhibiting signal transduction events. Interventions that block these signaling pathways, such as depolarizing levels of potassium, cyclic AMP analogues, and inhibition of the JNK pathway, prevent cytochrome c release, Commitment 1, and the decrease in protein synthesis rates in NGF-deprived sympathetic neurons (Rydel and Greene, 1988; Franklin et al., 1995; Harris et al., 2002), none of which are seen with CsA treatment. Whereas we cannot rule out the possibility that CsA has unidentified activities, we favor the conclusion that CsA blocks Commitment 2 by inhibiting PTP opening.

CsA does not affect Commitment 1

Prolonged opening of the PTP mediates cytochrome c release in some models of cell death (Marchetti et al., 1996). Our data indicate that cytochrome c release in sympathetic neurons does not require PTP opening, as CsA had little effect on this process, as measured by the ability of NGF-deprived cells to be rescued by NGF (Fig. 2 A) and by cytochrome c immunocytochemistry (Fig. 2 B). This is consistent with the finding that $\Delta\Psi_m$, which would be dissipated upon prolonged PTP opening, remains intact after cytochrome c release in mouse sympathetic neurons (Deshmukh et al., 2000). In addition, for opening of the PTP, which connects the cytosol to the mitochondrial matrix, to cause release of cytochrome c, located in the intermembrane space, to the cytosol, secondary mitochondrial swelling and rupture of the OMM must occur. Ultrastructural analysis of NGF-deprived, rat sympathetic neurons reveals no such swelling or obvious rupture of the OMM after 2 d of NGF deprivation, either in the absence (Martin et al., 1988) or presence of a caspase inhibitor (Martinou et al., 1999). At this time point, virtually all cells released cytochrome c, but no cells have reached Commitment 2 (Fig. 1 B). Taken together, these findings provide strong evidence that PTP opening and rupture of the OMM are not required for the release of cytochrome c from mitochondria of NGF-deprived sympathetic neurons.

How does PTP opening cause Commitment 2?

If PTP opening causes commitment-to-die, it could do so by a gain-of-function mechanism. Loss of OMM integrity by PTP opening might release death-promoting factors that are normally sequestered in the mitochondria. As Commitment 2, by definition, occurs only in the absence of caspase activity, such molecules would have to act in a caspase-independent manner. Three candidates that warrant further investigation are AIF (Susin et al., 1999; Joza et al., 2001), HtrA2 (Suzuki et al., 2001), and endonuclease G (Li et al., 2001).

Alternatively, PTP opening could cause Commitment 2 by a loss-of-function mechanism. Mitochondrial disruption resulting from PTP opening might interfere with normal mitochondrial functions that are required for continued viability of the cell, such as ATP generation and calcium homeostasis. Interestingly, ATP inhibits PTP opening (Duchen et al., 1993), whereas elevated extramitochondrial calcium promotes PTP opening (Hunter and Haworth, 1979). Therefore, initial PTP opening could trigger a feed-forward cascade in which impairment of ATP production or calcium buffering promotes additional PTP opening, further compromising mitochondrial function. Generation of ATP by oxidative phosphorylation continues after cytochrome c release in actinomycin D–treated HeLa cells in the presence of the caspase inhibitor ZVAD-fmk, suggesting that mitochondrial ATP production after cytochrome c release may be important for continued cell viability (Waterhouse et al., 2001). The degree to which NGF-deprived, BAF-saved cells rely on oxidative phosphorylation as a source of ATP is not known. However, BAF-saved sympathetic neurons are less sensitive to anoxia than NGF-maintained cells, suggesting that oxidative phosphorylation is not required to maintain viability by morphological criteria (Xue et al., 2001).

What triggers the initial opening of the PTP?

If PTP underlies Commitment 2, what causes its initial opening? As noted above, mitochondria have a prominent role in regulating the cellular homeostasis of two known modulators of PTP opening: adenine nucleotides and cytosolic Ca^{2+}. By disrupting the ability of mitochondria to regulate these factors, the mitochondrial hit could serve as the initial trigger for catastrophic PTP opening. For example, the translocation of BAX to the mitochondria, an event necessary for Commitment 1 (Putcha et al., 2000), could be such a trigger. Once at the mitochondrion, BAX could modulate properties of the PTP, as ectopically overexpressed BAX does so by interacting with voltage-dependent anion channel (Shimizu et al., 1999, 2000).

Another way in which mitochondria could trigger this initial event is through increased reactive oxygen species production, another positive regulator of PTP opening (Crompton et al., 1987; Nieminen et al., 1995). One way in which $\Delta\psi_m$ could be maintained after loss of mitochondrial cytochrome c release is by continued electron transport with autooxidation of complexes I–III and generation of superoxide anions (Cai and Jones, 1998). The contributions of these events to PTP opening and Commitment 2 are a topic of current investigation.

The mitochondrial origin of all of these potential triggers for PTP opening strongly implicates the mitochondrial hit as an important event in the Commitment 2 pathway. Thus, mitochondria have a central role in the pathways to both apoptotic cell death and nonapoptotic cell death that occurs in the presence of a caspase inhibitor.

Comparison of Commitment 2 in rat and mouse sympathetic neurons reveals key species differences

Sympathetic neurons from mice undergo Commitment 1 at a very similar rate as those from rats. However, the time courses of Commitment 2 are remarkably different be-

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tween these species. Previously published time course studies show that in sympathetic neurons from ICR and C57BL/6J mice, 50% reach Commitment 2 by 60 h of NGF deprivation, and by 96 h, nearly all cells become committed-to-die (Deshmukh et al., 2000). In contrast, 50% of rat cells reach Commitment 2 after 8 d of NGF deprivation, demonstrating that Commitment 2 in rat and mouse sympathetic neurons occurs at drastically different rates (Figs. 1 B and 6 A).

An even more striking species difference is the complete lack of effect of CsA on Commitment 2 in mouse sympathetic neurons (Fig. 6, B and C). Nontoxic doses of CsA did not inhibit Commitment 2 in sympathetic neurons from four strains of mice, representing both inbred and outbred strains. One possible explanation is that CsA does not inhibit PTP opening in mouse sympathetic neurons because either these cells do not express cyclophilin D or the murine orthologue does not interact with CsA. We did not directly examine the expression of cyclophilin D, the target of CsA inhibition in the PTP complex, in either rat or mouse sympathetic neurons. However, these explanations seem unlikely as cyclophilin D is expressed and can bind CsA in murine hippocampal neurons (Khaspekov et al., 1999).

A more likely explanation is that the mechanism by which mouse sympathetic neurons commit-to-die in the presence of a caspase inhibitor does not require PTP opening. The event that causes the loss of $\Delta\Psi_m$ associated with Commitment 2 in mouse sympathetic neurons may not have the same consequences in rat sympathetic neurons. Consistent with an absence of, or resistance to, this event in rat cells, Commitment 2 in rat sympathetic neurons occurs with a much slower time course than in mouse cells. Although purely speculative, this could be related to differences in the intensity of the mitochondrial hit in these species or the ability of neurons from different species to generate sufficient ATP subsequent to the loss of mitochondrial cytochrome c.

Implications for therapeutic use of caspase inhibitors

Caspase inhibition has a limited ability to decrease cell death in some animal models of pathological cell death, including cerebral (Hara et al., 1997a, 1997b) and cardiac (Yaoita et al., 1998) ischemia. Because caspase inhibitor-saved cells have already undergone the mitochondrial hit, and the upstream events that cause it, they remain hypofunctional until the death-inducing stimulus is removed and a normal cellular environment is restored (e.g., reperfusion after an ischemic insult). In the interim, these cells are vulnerable to secondary caspase-independent, nonapoptotic death, which limits the potential for functional recovery. Our results suggest that inhibition of both caspases and PTP opening may provide synergistic protective effects in models of pathological cell death, even in paradigms where PTP inhibition alone is not beneficial. In addition, our results highlight the existence of species-specific pathways of cell death, which must be considered when applying neuroprotective strategies to both experimental and pathological cell death.

Materials and methods

Materials

Timed-pregnant Sprague Dawley rats and ICR, C57BL/6, ND4 Swiss Webster, and BALB/c mice were obtained from Harlan. Unless otherwise noted, all reagents were obtained from Sigma-Aldrich.

Cell culture

Primary cultures of sympathetic neurons from superior cervical ganglia of postnatal day 0–1 animals were prepared as previously described (Deshmukh et al., 1996). After 5 d in culture in medium containing 50 ng/ml NGF (AM50), cells were deprived of NGF by washing three times with AM0 (AM50 without NGF) before adding AM0 with goat anti-NGF neutralizing antibody. In experiments requiring inhibition of caspase activity, 50 μM BAF (Enzyme Systems) was included in the medium at the time of deprivation. When used, CsA was also added at the time of NGF deprivation, unless otherwise noted. During the period of NGF deprivation, the medium was replaced every 3–4 d. The same number of cells could be rescued after 6 d in BAF or in BAF plus CsA if fresh medium was provided every 2 d or if the medium was not changed, suggesting that CsA did not degrade and lose effectiveness during this period (unpublished data).

Rescue experiments

NGF-deprived cultures were rescued by washing cultures four times with AM50 to remove residual anti-NGF before final addition of AM50. After 7 d in AM50, cells were fixed with 4% paraformaldehyde for at least 8 h at 4°C and stained with 0.05% toluidine blue in TBS for at least 6 h. Intensely stained cells were counted by using an inverted bright-field microscope without knowledge of treatment group and expressed as a percentage of the average number of cells in NGF-maintained sister cultures.

For time-course experiments of the removal of CsA (Fig. 6 A), cultures were deprived of NGF in the presence of BAF plus CsA. To remove CsA, cultures were washed twice with AM0 and fed with AM0 containing anti-NGF and BAF. Fresh medium was given to all cultures every 2 d during the 10 d of NGF deprivation. To quantify the magnitude of the effect of early removal or delayed addition of CsA, the percent effect was calculated as:

$$\frac{\text{Percentage of cells rescued} - \text{mimimum possible effect}}{\text{maximum possible effect}} \times 100.$$

In both paradigms, the minimum possible effect was 43.6%, or the proportion of NGF-deprived, BAF-saved cells that were rescued after 10 d. In the experiments shown in Fig. 6 A, the maximum possible effect was 89.6%, the proportion of cells treated with BAF plus CsA that could be rescued after 10 d of NGF deprivation. In the experiments represented in Fig. 6 B, the maximum possible effect decreased over time because NGF-deprived, BAF-saved cells became committed-to-die.

Determination of protein synthesis

To determine rates of protein synthesis cells were labeled for 2 h in 500 μl of MEM lacking cysteine and methionine and supplemented with 10 μCi/ml ^{35}S-labeled cysteine and methionine (ICN Biomedical). After 2 h, cells were washed twice with PBS and lysed in lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% SDS, pH 7.5). Proteins were precipitated by addition ice-cold tri-chloroacetic acid (TCA) to a final concentration of 8% and incubating for at least 2 h on ice. TCA-precipitated protein was then retained on a 0.45-μm Protran nitrocellulose filter (Schleicher & Schuell), which was washed twice with 10% TCA and counted in a liquid scintillation counter.

In experiments where protein synthesis was determined after 10 d of NGF deprivation, cells were incubated in labeling medium for 4 h to increase the sensitivity of the assay. The small nonneuronal contribution to total protein synthesis was determined by measuring the protein synthesis rate of cultures that were maintained in AM0 containing anti-NGF from the time of plating. The mean nonneuronal value was subtracted from the values of other samples to calculate the rate of neuronal protein synthesis.

Determination of mitochondrial membrane potential

Mitotracker staining and cytochrome c immunocytochemistry were performed as described (Deshmukh et al., 2000). Briefly, cells were labeled for 1 h with 1 μM Mitotracker Orange CM-H$_2$TMRos (Molecular Probes) in the appropriate medium and washed extensively with PBS before fixing and processing for cytochrome c immunocytochemistry.

For JC-1 staining, cells were plated in 96-well white plates with clear bottoms (Costar). Cultures were washed once with PBS with 1 g/L glucose and labeled with 3 μM JC-1 (Molecular Probes) in PBS with glucose for 20

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min. After washing once in PBS, cells were either photographed with an inverted microscope or subjected to quantification by using a Titertek Fluoroskan II fluorescent plate reader. Excitation/emission filter sets of 544nm/590nm, corresponding to the J-aggregate form of JC-1, and 485nm/538nm, measuring its monomeric form. The JC-1 ratio was calculated as

$$\frac{(EM_{590} - EM_{590}^{NN})}{(EM_{538} - EM_{538}^{NN})},$$

where EM is the emission of the sample at the given wavelength and EM^{NN} is the average emission at that wavelength of cultures plated in AM0 containing anti-NGF. To completely depolarize mitochondria, cells were preincubated in 10 μM FCCP for 10 min before loading with the JC-1 solution, which also contained 10 μM FCCP.

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