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Activation of a PP2A-like phosphatase and dephosphorylation of τ protein characterize onset of the execution phase of apoptosis

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SUMMARY

The execution phase is an evolutionarily conserved stage of apoptosis that occurs with remarkable temporal and morphological uniformity in most if not all cell types regardless of the condition used to induce death. Characteristic features of apoptosis such as membrane blebbing, DNA fragmentation, chromatin condensation, and cell shrinkage occur during the execution phase; therefore, there is considerable interest in defining biochemical changes and signaling events early in the execution phase. Since onset of the execution phase is asynchronous across a population with only a small fraction of cells in this stage at any given time, characterizing underlying biochemical changes is difficult. An additional complication is recent evidence suggesting that the execution phase occurs after cells commit to die; thus, agents that modulate events in the execution phase may alter the morphological progression of apoptosis but will not affect the time-course of death. In the present study, we use a single cell approach to study and temporally order biochemical and cytoskeletal events that occur specifically in the execution phase. Microtubules de-acetylate and disassemble as terminally differentiated PC12 cells enter the execution phase following removal of nerve growth factor. Using phosphorylation sensitive antibodies to τ, we show that this microtubule-stabilizing protein becomes dephosphorylated near the onset of the execution phase. Low concentrations of okadaic acid inhibit dephosphorylation suggesting a PP2A-like phosphatase is responsible. Transfecting τ into CHO cells to act as a 'reporter' protein shows a similar dephosphorylation of τ by a PP2A-like phosphatase during the execution phase following induction of apoptosis with UV irradiation. Therefore, activation of PP2A phosphatase occurs at the onset of the execution phase in two very different cell types following different initiators of apoptosis which is consistent with activation of PP2A phosphatase being a common feature of the execution phase of apoptosis. Experiments using either taxol to inhibit microtubule disassembly or okadaic acid to inhibit τ dephosphorylation suggest that microtubule disassembly is necessary for τ dephosphorylation to occur. Thus, we propose that an early step in the execution phase (soon after a cell commits to die) is microtubule disassembly which frees or activates PP2A to dephosphorylate τ as well as other substrates.

Key words: Apoptosis, Programmed cell death, tau1, PHF1, Neuron, PC12, NGF, Protein phosphatase 2A, Okadaic acid, Microtubule, Acetylated tubulin, Alzheimer's disease

INTRODUCTION

Apoptosis can be divided into three sequential phases (Jacobson et al., 1994; Greenlund et al., 1995). The first (the induction or lag phase) depends on resident signal transduction machinery and is, by nature, cell-type specific. The next phase (the commitment phase) appears to be evolutionarily conserved and is characterized by activation of factors that eventually commit a cell to death. The final stage (the execution phase) is when all known morphological hallmarks of apoptosis occur, and the cell loses viability. Once this phase begins, death is inevitable (McCarthy et al., 1997; Messam and Pittman, 1998). It is becoming clear that the execution stage is evolutionarily conserved, lasts about an hour, regardless of cell type or species (Ellis et al., 1991; Raff, 1992; Barres et al., 1992; Earnshaw, 1995; Pittman et al., 1998), and all apoptotic cells pass through it before they die (Evan et al., 1992; Mills et al., 1997).

Proteins that transduce signals during the induction phase of apoptosis have been well studied, and considerable recent work has elucidated biochemical events that appear to occur during the commitment phase, such as delineating roles for the various bcl-2 and caspase family members (Steller, 1995; Chinnaiyan and Dixit, 1996; Reed, 1997). Although cellular events occurring during the execution phase such as DNA fragmentation, chromatin condensation, membrane blebbing, and formation of apoptotic bodies have been defined, little is

known about the biochemical pathways underlying these events.

Biochemical characterization of the execution phase is hindered by the asynchronous nature of apoptotic death (at any given time only 5-10% of cells in a population exhibit execution phase morphology). Investigators have attempted to overcome this asynchrony by isolating the small subset of execution phase cells (Casciola-Rosen et al., 1994) or by targeting experiments specifically to this population (Lazebnik et al., 1993; Newmeyer et al., 1994). Alternatively, an endogenous or exogenous 'reporter' molecule could be used to signal biochemical changes occurring only in execution phase cells. This was the approach used in the present study of apoptosis in PC12 cells.

The execution phase of apoptosis in nerve growth factor (NGF) differentiated PC12 cells is characterized by chromatin condensation, neurite beading, and extensive cellular blebbing (Pittman et al., 1993). The beading and blebbing suggest that the microtubule (MT) network might be altered in actively dying cells. A prominent regulator of MT dynamics in PC12 cells is τ protein. τ appears essential for developing and maintaining mature neurites (Hanemaaijer and Ginzburg, 1991; Teng et al., 1993; Esmaeli-Azad et al., 1994). MT stabilization by τ is regulated by protein kinases and phosphatases (Goedert et al., 1992; Biernat et al., 1993; Bramblett et al., 1993; Vincent et al., 1994). Protein phosphatase 2A (PP2A) has been shown to be the principal enzyme mediating τ dephosphorylation in vivo (Sontag et al., 1996).

The present study was designed to investigate cytoskeletal and biochemical changes occurring specifically during the execution phase of apoptosis. To overcome the inherent technical problem of asynchrony, a single cell approach was employed that coupled immunochemical staining using phosphorylation-sensitive τ antibodies with a dye that identified the pattern of chromatin condensation characteristic of the execution phase. Endogenous τ in PC12 cells following removal of NGF and transfected τ acting as a 'reporter' protein in CHO cells following UV irradiation was dephosphorylated early in the execution phase. Dephosphorylation of τ was inhibited by low concentrations of okadaic acid consistent with activation of a PP2A-like phosphatase being a common feature of the execution phase.

MATERIALS AND METHODS

Materials

NGF and rat-tail collagen were purchased from Collaborative Biomedical (Bedford, MA). RPMI, α-MEM, G418, and streptavidin/HRP were from Life Technologies (Grand Island, NY), fetal bovine serum from HyClone (Logan, UT), horse serum from ICN (Aurora, OH), ECL substrate from NEN/DuPont (Boston, MA), FITC- and TRITC-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA), Hoechst 33342 from Molecular Probes (Eugene, OR), Mowiol from Calbiochem (La Jolla, CA), okadaic acid (OKA) from RBI (Natick, MA), deltamethrin from Alomone Labs (Jerusalem, Israel). All other reagents were from Sigma (St Louis, MO).

Antibodies

Anti-tyrosinated (clone TUB-1A2) and anti-acetylated tubulin (clone 6-11B-1) monoclonal antibodies (mAbs) were from Sigma; anti-α-tubulin mAb (clone N 356) was from Amersham (Arlington Heights, IL). Tau1 mAb (from Dr L. Binder) recognizes residues 192-204 on τ protein (numbering based on full length human brain τ; Goedert et al., 1989) only when there are no phosphates present (Binder et al., 1985; Bramblett et al., 1993; Szendrei et al, 1993). PHF1 mAb (from Dr P. Davies) recognizes an epitope spanning residues 389-402 when S396 and S404 are phosphorylated (Greenberg and Davies, 1990; Otvos et al., 1994). The mAb T49 recognizes an epitope on the carboxyl terminus, and is independent of phosphorylation state (Mawal-Dewan et al., 1994). Polyclonal rabbit anti-NGF Ab was prepared as previously described (Pittman et al., 1993).

Cell culture

PC6-3 cells (Pittman et al., 1993) derived from the PC12 cell line (Greene and Tischler, 1976), were maintained as previously described in RPMI 1640 medium, supplemented with 5% fetal bovine serum, 10% horse serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Mills et al., 1995). For experiments, cells were sub-cultured onto collagen-coated plastic tissue culture dishes, and grown in the presence of 100 ng/ml NGF for 10 days (d10), at which point medium was replaced with either NGF-containing medium (+NGF) or medium lacking NGF but containing polyclonal anti-NGF antibodies (–NGF).

Wild-type CHO cells and CHO clone T40.19, stably expressing the longest human brain τ isoform (Bramblett et al., 1993), were maintained in α-MEM, 10% fetal bovine serum, and penicillin/streptomycin. CHO T40.19 cell medium was further supplemented with 0.2 mg/ml G418. Preliminary experiments using 20-1,000 J/M^2 light in a UV Stratalinker (Sratagene, La Jolla, CA) revealed that delayed apoptotic death in both T40.19 and wild-type CHO cells was maximally induced by 100 J/M^2.

Timelapse videomicroscopy

The procedure for long-term timelapse videomicroscopy has been detailed elsewhere (Pittman et al., 1993; Mills et al., 1995). Briefly, cells were grown in a tissue culture incubator in the presence of NGF for 9 days, then transferred to a specially designed video chamber that allowed for videorecording of one field of cells for up to 3 days under standard culture conditions (i.e. 37°C, 6% CO_2). Cells were observed for 24 hours in NGF-containing medium, and then death was induced by replacing +NGF medium with medium lacking NGF and containing anti-NGF IgG.

Immunocytochemistry

Ten-day NGF-differentiated cultures grown in 35 mm dishes were incubated in –NGF or +NGF medium for 24 hours, at which point media were removed (this and all the following steps were performed at room temperature unless otherwise specified). Cells were incubated in 0.06% Trypan Blue for 10 minutes, fixed in 4% paraformaldehyde for 8 minutes, and immediately permeabilized for 8 minutes in 0.1% Triton X-100. Cells were rinsed in PBS, incubated for 2.5 hours or overnight at 4°C in primary antibody in PBS, rinsed in PBS, then incubated in secondary antibody (either FITC- or TRITC-conjugated goat anti-mouse for 2 hours in PBS), and finally incubated in 5 μg/ml Hoechst 33342 in PBS for 30 minutes. After 2 rinses in PBS, and one in de-ionized water, a coverslip was mounted on a drop of Mowiol. Once the Mowiol had dried, dishes were suspended from the stage plate of an inverted Nikon Diaphot microscope by two strips of laboratory tape, and the microscope objective was raised to the coverslip. This technique was designed to allow the cells to be grown on tissue culture plastic, but still be observed through thin coverslip glass after fixation. Cells were treated with Trypan Blue prior to fixation. Preliminary experiments revealed that this pre-treatment permanently quenched subsequent Hoechst staining in Trypan Blue positive cells (i.e. those cells already dead at the time of fixation). Thus, Hoechst staining in the present report was also a marker for cellular viability. In some experiments, immunostaining for tau1 was also performed using glutaraldehyde/paraformaldehyde (see below) or methanol as a fixative, with no noticeable difference in the pattern of reactivity. More than 30 individual cell platings over the course of 10 months were immunostained with tau1 for various experiments; the

morphological pattern of increased tau1 in cells with execution phase nuclei was invariant.

To stain MTs, cultures were washed twice in 37°C PBS, once in 37°C PHEM MT stabilizing buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$) then fixed 10 minutes in 2% paraformaldehyde/0.05% glutaraldehyde in 50% PBS/50% PHEM and 25 μM taxol. This was followed by washing twice in PHEM and extraction for 10 minutes in 0.1% Triton X-100 in PBS at room temperature. To quench fluorescence arising from glutaraldehyde fixation, cultures were washed twice quickly in room temperature with 0.25 M Tris, pH 7.0, a third time overnight at 4°C, and then quickly again. The rest of the procedure was as described above. To test the sensitivity of τ and MTs to detergents, cultures were washed twice in 37°C PHEM, then pre-extracted in warm PHEM containing 0.2% Triton X-100 and 20 μM taxol, followed by fixation in glutaraldehyde/paraformaldehyde and post-extraction in 0.1% Triton X-100 as above. Preliminary timecourses of pre-extraction showed maximal extraction of α-tubulin immunoreactivity during execution occurred within 2 minutes, so this duration was chosen for all further experiments. It should be noted that 2 minutes of pre-extraction was long enough to produce filamentous MT staining in CHO cells (e.g. Fig. 9G), but 5-10 minutes was needed to extract all the tubulin dimer and produce filamentous staining in the smaller, spherical, pre-execution phase PC12 cells (not shown).

Correlating tau1 immunoreactivity with execution phase nuclear morphology

Cells immunostained as above for tau1 and Hoechst 33342 were scanned systematically for execution phase nuclei (defined as those with ≥4 or more ball-like chromatin aggregates) by an observer blinded to the experimental condition. All nuclei were counted, and, when an execution phase nucleus was encountered, the observer switched to the channel containing tau1 fluorescence to determine whether the immunoreactivity of that cell was substantially brighter than the surrounding ones. This process was reversed to determine the fraction of bright tau1-staining cells with execution phase nuclei. Counts were obtained in three independent experiments, counting approximately the same number of cells in each experiment, and the results summed.

Measurement of okadaic acid (OKA) effects

To determine whether τ dephosphorylation could be inhibited by OKA, NGF-deprived PC12 cells were treated with DMSO (0.1%), 20 nM, or 80 nM OKA for either 1.5 or 4.5 hours before standard fixation and immunostaining as described above. OKA was kept as a stock at 80 μM in DMSO at −80°C and diluted immediately before use. After labeling, an observer blinded to experimental condition, randomly selected fields containing a cell with an execution phase nucleus, surrounded by ≥3 cells with normal nuclei (fields usually contained only one execution phase cell but occasionally contained two). Next the fluorescent cube was changed so that the tau1 immunoreactivity of the field could be digitized (Image1 system from Universal Imaging, West Chester, PA). Camera gain and black level were adjusted in preliminary measurements for each new dish, so that fluorescence would be in the linear range across each field. Occasionally, the execution phase cell in a field was so bright (in non-OKA treated cultures) that camera levels could not be adjusted without making neighboring non-execution phase cells approach background levels. Such fields were excluded, and, thus, Fig. 5 represents a slight underestimate of the degree to which tau1 immunoreactivity increased in execution phase cells in vehicle-treated cultures. In other words, the effect of OKA is actually slightly stronger than is depicted.

Once the camera levels were set for a given dish, measurements were taken as follows: using the 'measure area brightness' feature of the software, a box was made, sized to about 1/3rd the area of an average cell body, and used to record intensity of staining in the brightest area in each cell. The average level of staining in the acellular (background) area immediately surrounding the cell was then subtracted from the peak value inside that cell. Using this technique, the background-subtracted intensity of all the morphologically normal cells was computed for each field and the average divided into the background-subtracted fluorescence of the execution phase cell to compute the relative execution phase fluorescence for each field. Between 5 and 15 fields were then averaged for each experiment, and four such experiments were performed. The same procedures were followed in CHO cell experiments except cells were treated with 100 nM OKA or 1 μM deltamethrin for 4 hours. Also, T49 was measured (in separate dishes) to control for variations in cell shape. Unlike CHO cells, execution phase PC12 cell bodies do not convert from a flat to rounded morphology, so it is unlikely that increase in tau1 reactivity is due merely to redistribution of epitope into a smaller area. Comparisons of multiple concentrations and times of OKA to DMSO were statistically assessed by ANOVA, followed by the test of Dunnet (1955) for comparison of control to experimental means. Statistical differences between CHO cell OKA and DMSO treatments were calculated using paired, one-tailed Student's *t*-test.

RESULTS

The execution phase of apoptosis is short and asynchronous

Most assays of cellular biochemistry such as western blots and enzymatic assays require large numbers of cells more or less homogeneously undergoing the biochemical events of interest. Thus, to study biochemical and cytoskeletal events during the execution phase of apoptosis, a substantial fraction of cells would need to be synchronously undergoing the characteristic active morphologic changes. However, apoptosis is known to be an asynchronous process and, indeed, previous work in this lab with apoptotic cultures of differentiated PC12 cells has shown that loss of cells begins at about 6 hours following induction of apoptosis by NGF removal and progresses steadily for up to 3 days until approximately 90% of the culture has died.

To demonstrate the degree of asynchrony in the onset of morphological changes, PC12 cells were differentiated in NGF for 9 days until they assumed a neuronal morphology with a complex neurite network. They were then transferred to a video chamber for long-term timelapse observation. For 24 hours cells were maintained in NGF. Then NGF was washed out, anti-NGF antibodies added, and the same field of cells observed for an additional 36 hours as they underwent apoptosis. As can be seen from Fig. 1, cells spent most of the time in a morphologically stable phase, indistinguishable from their morphology prior to NGF removal. The execution phase, characterized by dynamic cell blebbing, occurred suddenly in each cell and at variable times following NGF removal. But despite variability of onset, dying cells invariably spent about 45 minutes in this active phase. As is typical of apoptotic asynchrony, even though almost all the cells will eventually die, less than 15% of cells are in the execution phase at any given time. Thus, our early attempts to identify cytoskeletal changes in the execution phase using biochemical analysis of the population of cells (e.g. western blots) were only marginally informative. Consequently, a method for single cell analysis was devised and employed for the remainder of the experiments in the current study.

τ protein is dephosphorylated in differentiated PC12 cells during apoptosis

The phosphorylation state of τ in individual cells was analyzed

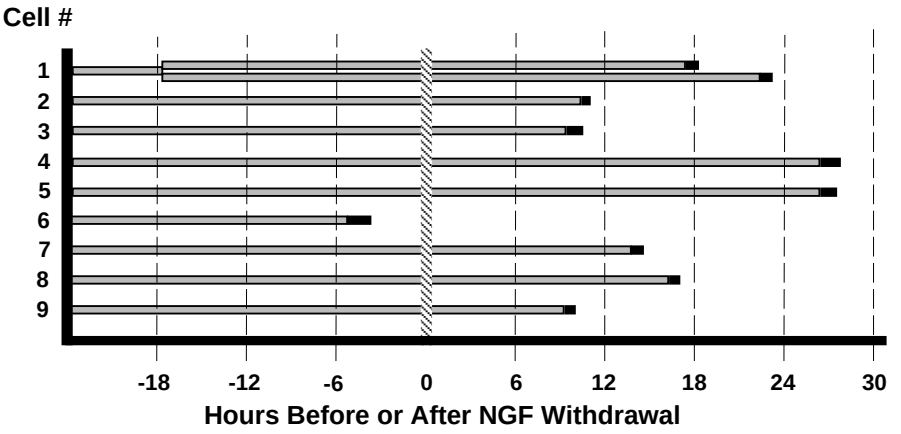

Fig. 1. Onset of the execution phase of apoptosis occurs at variable times following NGF withdrawal making it a markedly asynchronous process. PC12 cells were followed using long-term timelapse videomicroscopy for 24 hours in the presence of NGF, and then NGF was removed and the same field observed. The figure shows individual dying cells plotted in timeline format by their morphology. Apoptotic cells spend the vast majority of time in a morphologically normal state (gray bars). The execution phase, characterized by dynamic cell body blebbing and neurite beading and detachment (black bars), is very variable in time of onset but is quite invariant in duration.

by immunocytochemistry using phosphorylation sensitive monoclonal antibodies. Cultures of PC12 cells treated with NGF for 10 days followed by either withdrawal for 24 hours (–NGF, dying cells) or replacement of control medium (+NGF, control cells) were characterized using PHF1, tau1, and T49 mAbs. PHF1 is an antibody that binds τ when residues S396 and S404 are phosphorylated, whereas tau1 recognizes an epitope spanning amino acids 192-204 only when serine/threonine residues within this stretch are not phosphorylated (residues numbered using the system for full-length human τ; Goedert et al., 1989). T49 is an antibody that recognizes τ protein independent of phosphorylation state. With both T49 and PHF1 as primary antibodies, the general pattern of staining across populations of –NGF cells was similar to that seen in populations of +NGF cells (data not shown). Most cells in either dying or control cultures seemed to react with both antibodies, though there was some intercellular variability (see below for further characterization of T49 and PHF1 staining in individual cells). In contrast, tau1 immunostaining revealed a subset of very prominent immunoreactive cells present almost exclusively in –NGF cultures (Fig. 2).

To determine whether or not cells staining strongly with tau1 were in the execution phase of apoptosis, cultures of dying cells were treated with the vital dye Trypan Blue, fixed, permeabilized, indirectly immunostained and, before mounting, treated with the DNA-binding fluorescent probe Hoechst 33342 (see Materials and Methods). Those cells expressing significantly increased tau1 immunoreactivity usually also had execution phase nuclei with characteristic condensed chromatin (Fig. 3A,B; Table 1). In contrast, T49 immunoreactivity was independent of execution phase morphology (Fig. 3C,D), while PHF1 reactive cells showed a correlation inverse to that of tau1, with execution phase cells having almost undetectable immunofluorescence staining (arrowheads, Fig. 3E,F). Cell ghosts that had lost membrane integrity had no staining with any of the τ antibodies, indicating that τ is degraded and/or lost when cells become permeable to Trypan Blue. Immunocytochemistry, therefore, suggested that τ dephosphorylation is specific to execution phase cells.

Dephosphorylation of τ correlates temporally with formation of execution phase nuclei and neurite loss

The incidence of cells with strong tau1 immunoreactivity was assessed in dying cell cultures and compared to the incidence of cells containing execution phase nuclei. Neither phenotype was very common. Only 5.9% of the cells in the –NGF cultures had execution phase nuclei, and only 5.8% were strongly tau1 positive. There was strong correlation between the two phenotypes, however, as 90±6% of cells with execution phase nuclei had increased tau1 staining, and 75±4% of the strongly tau1 positive cells had execution phase nuclei (Table 1). Experiments were also conducted to determine whether T49 and PHF1 immunostaining were increased in cells with execution phase nuclear morphology. Cells with execution phase nuclei almost never had detectable PHF1 staining. T49 staining appeared slightly increased in about 25% of cells with

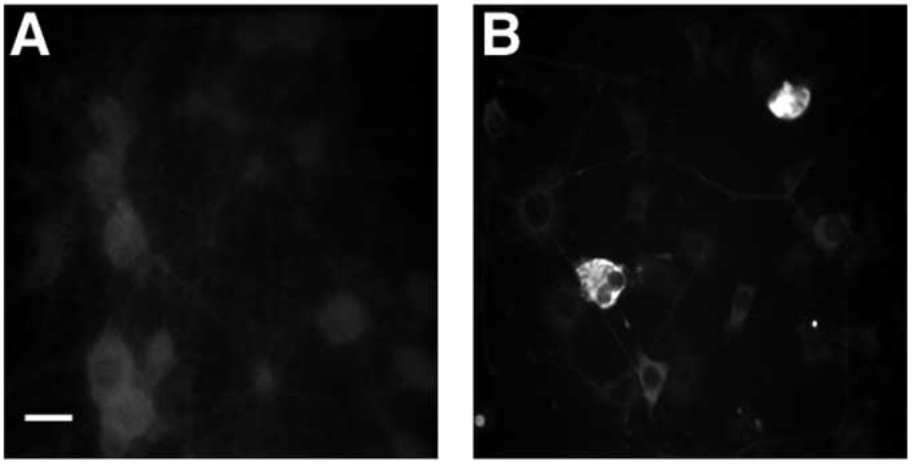

Fig. 2. τ protein is dephosphorylated at the tau1 site in a small subset of cells in cultures of apoptotic PC12 cells. Cultures were differentiated in NGF for 10 days and then treated for 24 hours with either +NGF medium (A) or –NGF medium (B). They were then fixed and processed for tau1 immunohistochemistry. Note the two very brightly stained cells in B. Bar, 25 μm.

Table 1. Correlation of τ dephosphorylation with execution phase nuclear morphology

Searching for:	Total cells counted	% Expression of searched for phenotype	% Correlation with other phenotype
Execution phase nuclei	4,650	5.90±0.52	90±6
Dephosphorylated τ	4,936	5.80±1.53	75±4

PC12 cells were fixed and double-labeled as for Fig. 3, using tau1 antibody to detect τ dephosphorylation. Cells with execution phase nuclei were located, and corresponding tau1 levels determined. Whenever an execution phase cell also had increased tau1, the two phenotypes were said to be correlated. Next, the converse was done: cultures were scanned for tau1 immunoreactivity, those cells with increased reactivity were assessed for execution phase nuclear state, and the percentage of cells that showed a correlation of the two phenotypes was again determined. Note that there are 20% more cells (75% vs 90%) with increased tau1 that do not have execution phase nuclear morphology than vice versa, consistent with dephosphorylation occurring slightly earlier than chromatin condensation.

execution phase nuclei, but only very rare cells were as bright relative to others in the field as was the norm for execution phase cells stained with tau1 (see, e.g. Figs 2B, 3B, 4B,D,F, and note in Fig. 5 the ~3-fold increase). Thus, although total τ protein might be slightly increased in a fraction of execution phase cells (as evidenced by occasional ones with increased T49), the increase is unlikely to account for the dramatic rise in tau1 staining seen in the vast majority of execution phase cells.

Because nearly all execution phase nuclei had increased tau1 immunoreactivity (90%, Table 1) but the converse correlation was not as strong (75% of strong tau1 positive cells had execution phase nuclei), the results are consistent with τ dephosphorylation occurring slightly before or during onset of chromatin condensation. Support for this temporal relationship came from studying the morphology of cells with execution phase nuclei and/or increased tau1 staining. As shown above, 20% of the cells with increased tau1 immunoreactivity did not have execution phase nuclei that met the rigorous criterion of ≥4 condensed chromatin aggregates. Such cells exhibited a variety of cellular morphologies, from rare cells having mostly normal neurites and normal nuclei (Fig. 4A,B) to more commonly observed cells with beaded, fragmenting neurites and nuclei exhibiting early changes (Fig. 4C,D). On the other hand, among the thousands of apoptotic cells examined for the present study, those with clearly execution phase nuclei were only very rarely observed to have normal neurites. Occasionally, however, evidence of retracted or disintegrating neurites could be observed (Fig. 4E,F). The above observations suggested that tau1 reactivity increased, on average, very soon before neurite breakdown, which, in turn, preceded full-fledged execution phase chromatin condensation. The presence of tau1

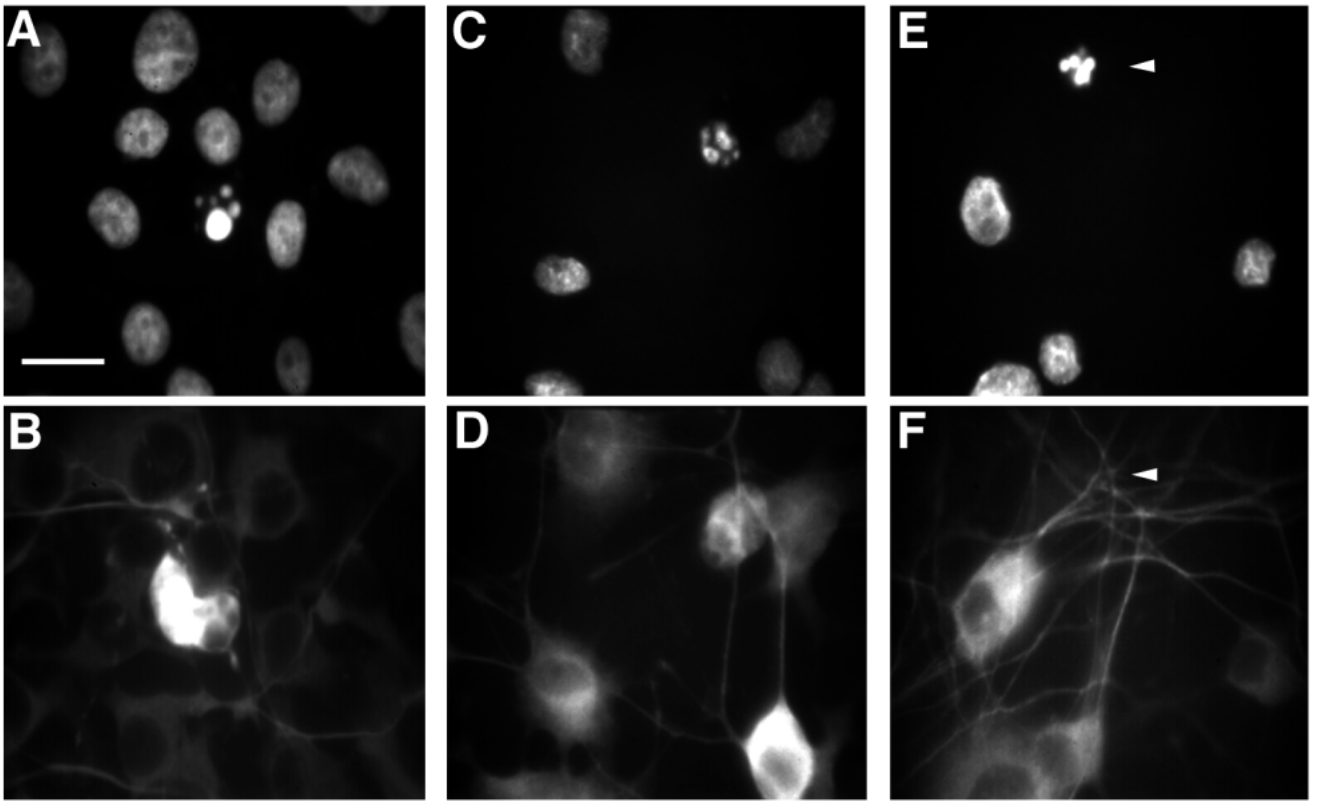

Fig. 3. Dephosphorylation of τ protein occurs in cells with apoptotic nuclei. Cultures were differentiated in NGF for 10 days and apoptosis was induced by 24 hour treatment in –NGF medium. Cells were then treated with Trypan Blue to quench fluorescence in dead cells, immunostained with tau1 (B), T49 (D), or PHF1 (F) antibodies, and double-labeled with Hoechst 33342 to visualize chromatin (A,C,E). Note that the cell with fragmented, condensed chromatin in A has greatly increased tau1 (i.e. dephosphorylated τ), the one in C has relatively similar T49 (total τ) levels to neighboring cells with normal nuclei, and the cell in E (demarcated by arrowheads) has almost no detectable PHF1 (phosphorylated τ). Note also that the morphology of the cell in 3B is typical of a blebbing cell in execution phase of apoptosis with its nucleus eccentrically positioned (previous experiments have shown that actively blebbing cells often extrude and retract nuclei into large membrane pouches). Note that panel B is slightly over-exposed for the execution phase cell, so that the cytoplasm in the morphologically normal cells could be visualized. Bar, 20 μm.

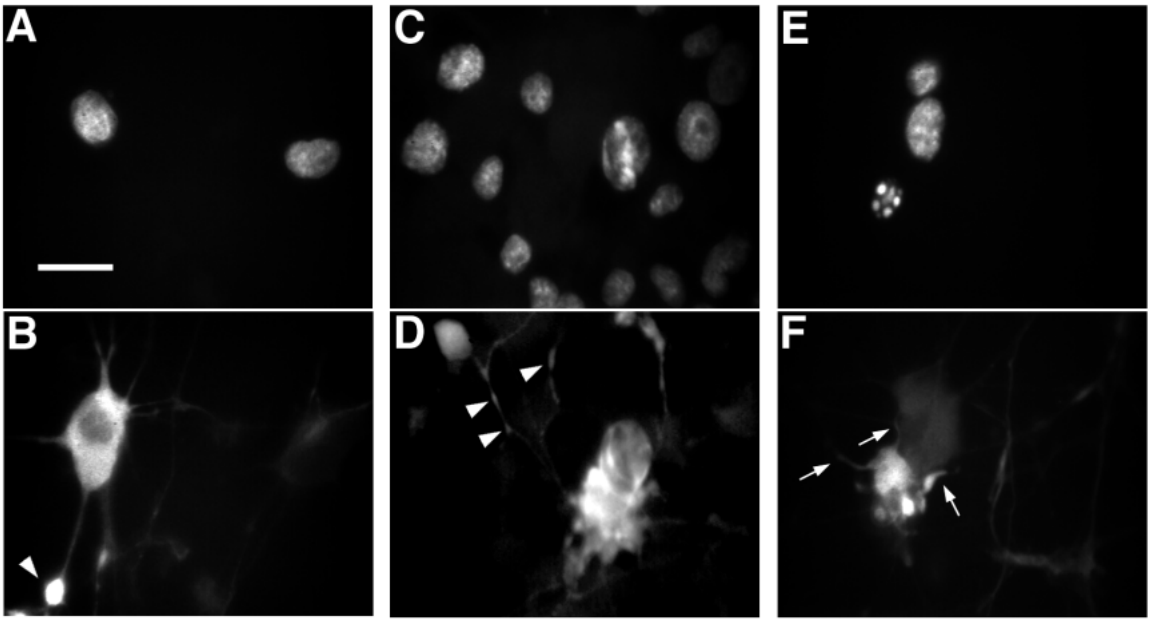

Fig. 4. τ protein dephosphorylation coincides with neurite beading and cell body morphological changes. Cultures were treated as described in Fig. 3. (A and B) Two cells with essentially normal nuclei, but the cell on the left shows considerable τ dephosphorylation. Its neurites are mostly intact, whereas D depicts a cell with detaching neurites and early cell body blebbing (nucleus shows early signs of apoptotic execution phase, C). (F) A cell with a fully execution phase nucleus (E) commencing cell body dynamic blebbing with neuritic remnants (arrows). Arrowheads in B and D mark examples of neuritic beading. Bar, 20 μm.

reactivity in degenerating neurites (as demonstrated below) further supports this temporal sequence.

It should be reiterated that asynchrony of the execution phase (Fig. 1) means that, at any one time, marked dephosphorylation of τ can be seen only in the small fraction of cells undergoing execution phase changes. Time-lapse studies such as that shown in Fig. 1, however, demonstrate that almost all cells go through the execution phase before they die, and the large majority of cells in these cultures eventually die within 2 to 3 days (Mills et al., 1997). Thus, τ dephosphorylation is a general feature of the end stage of apoptosis in this system and likely occurs in almost every cell in a culture.

τ Dephosphorylation during apoptosis is inhibited by okadaic acid (OKA)

The phosphorylation state of τ, and accordingly its MT binding affinity, is thought to be determined by the reciprocal actions of cellular kinases and phosphatases. Thus, it seemed probable that τ dephosphorylation in apoptotic PC12 cells was enzymatically mediated. To test this hypothesis, cultures of dying PC12 cells were treated with OKA, a serine/threonine phosphatase inhibitor which is most effective against PP2A, less so against PP1 and only marginally against PP2B (Bialojan and Takai, 1988).

NGF-deprived cultures were treated with 20 or 80 nM OKA for 1.5 or 4.5 hours immediately prior to fixation, double-labeling for tau1 immunoreactivity, and Hoechst nuclear staining. Using computerized fluorescent imaging (see Materials and Methods), it was determined that, on average, tau1 staining in cell bodies with execution phase nuclei was 3.10±0.52 times brighter than cells with normal nuclei (Fig. 5). OKA treatment at 20 nM for 4.5 hours or 80 nM for either 1.5 or 4.5 hours resulted in a statistically significant reduction in relative execution phase tau1 intensity to 2.19±0.11, 2.23±0.39, and 1.5±0.4, respectively (Fig. 5). If the baseline fluorescence (i.e. relative intensity of 1.0) is subtracted, OKA

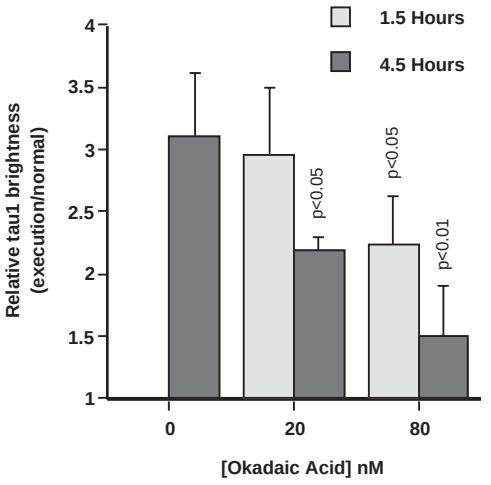

Fig. 5. Okadaic acid inhibits τ dephosphorylation. Cells were cultured in the absence of NGF for 24 hours to induce apoptosis, then fixed and immunostained. Either 4.5 (shaded bars) or 1.5 hours (stippled bars) prior to fixation, cells were treated with DMSO vehicle, 20 nM, or 80 nM OKA. Those cells containing execution phase nuclei were located, their tau1 fluorescent intensity digitized by computer and then ratioed to the intensities of neighboring cells with normal nuclei. Note that treatment with OKA at 20 nM for 4.5 hours or at 80 nM for 1.5 and 4.5 hours resulted in a statistically significant decrease in relative tau1 immunoreactivity in execution phase cells. Treatments showing a statistically significant difference relative to DMSO control are marked. Data represent the mean ± s.e.m. from 4 separate experiments.

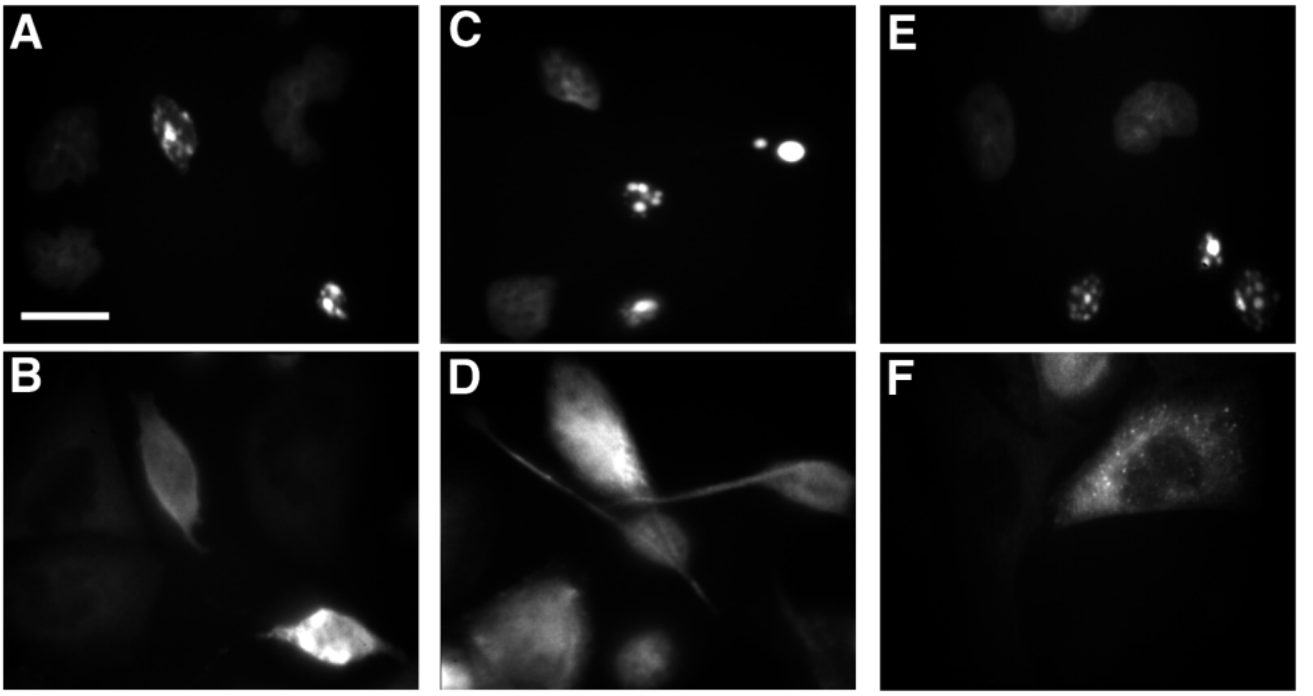

Fig. 6. τ is dephosphorylated in apoptotic, τ-expressing CHO cells. CHO cells stably expressing full length human τ were induced to undergo apoptosis by UV irradiation and stained as in Fig. 3. Just as for Fig. 3, A depicts the nuclei of the tau1-stained cells in B; C shows the nuclei for the T49-stained cells in D; and E shows the nuclei for the PHF1-stained cells in F. Note the increase in tau1 staining in both the cell with early execution phase nuclear changes (middle, B) and in the cell with typical execution phase nuclear morphology (lower right of B). T49 staining is approximately equal in all cells (D), while PHF1 staining is completely lost in the execution phase cells (E). Also note the long neurite-like extensions in two of the execution phase cells in D. Bar, 20 μm.

inhibited tau1 dephosphorylation in execution phase cells by 43%, 41%, and 76% (20 nM for 4.5 hours, or 80 nM for 1.5 or 4.5 hours, respectively). Another PP2A inhibitor which is equally potent against PP1 (calyculin A) was toxic above, and had no effect below, a concentration of 200 nM (data not shown). In other experiments, enzymatic activity of PP2A in the population was assessed by release of ^{32}P from labeled phosphorylase a. Only a slight, non-statistically significant difference was found in PP2A activity between dying and non-dying cultures, a result that re-enforced the need to characterize biochemical changes during the execution phase at the level of single cells rather than across an entire population.

τ is also dephosphorylated in cultures of apoptotic CHO cells stably transfected with τ

To determine whether protein phosphatase-mediated τ dephosphorylation was unique to neural apoptosis or also occurred in other apoptotic cells, experiments were performed with CHO cells stably transfected with τ. τ is a neural protein; therefore, its exogenous expression in CHO cells allows it to function as a 'reporter' protein providing information on changes in kinase/phosphatase activity during the execution phase in a non-neural cell. Preliminary experiments had shown that approximately one quarter of CHO cells develop execution phase nuclei 24 hours after treatment with 100 J/M^2 UV irradiation. Like PC12 cells, CHO cells with execution phase nuclei exhibited increased tau1 immunoreactivity (Fig. 6A,B). T49 immunostaining, on the other hand, was similar in execution phase and normal cells (Fig. 6C,D); although, it sometimes appeared slightly brighter in execution phase cells, probably due to concentration of cellular protein as the cells adopted a more rounded morphology. Staining with T46, another phosphorylation-independent antibody resembled that of T49 (not shown). As with PC12 cells, PHF1 staining was almost undetectable in CHO cells with execution phase nuclei (Fig. 6E,F). Expression of τ in CHO cells had no noticeable effect on rate or incidence of death, as wild-type CHO cells died similarly (unpublished observations).

To investigate the role of protein phosphatases in altered τ phosphorylation during apoptosis of τ-expressing CHO cells, cells were UV irradiated and, on the following day, treated with 100 nM OKA for 4 hours immediately prior to fixation. In vehicle-treated cultures, execution phase cells were, on average, a statistically significant 2.2±0.11 times brighter than cells with normal nuclei (Fig. 7A). On the other hand, in OKA-treated cultures, execution phase cells exhibited almost the same tau1 immunoreactivity as neighboring healthy cells (relative fluorescence of 1.07±0.09, Fig. 7A), suggesting that OKA significantly inhibited τ dephosphorylation.

Because CHO cells tended to change morphology in the execution phase (see text above and Fig. 6B,D), it was important to control for the possibility that increased tau1 immunofluorescence in execution phase cells was caused merely by concentrating existing τ in a smaller area. Thus, T49 relative immunofluorescence (i.e. T49 in execution phase vs T49 in normal cells) was calculated in sister cultures, using the same technique as for tau1 (T49 recognizes all τ and can serve as a control for any increase in immunofluorescence that results

from change in shape alone). Then the relative immunofluorescence of tau1 in each experiment was divided by that of T49, creating a ratio of the two ratios (Fig. 7B). Even when changes in morphology and total τ were accounted for, OKA still caused a statistically significant 66% inhibition of τ dephosphorylation in execution phase cells (Fig. 7B, with baseline fluorescence subtracted). To assess the role of the other principal serine/threonine phosphatase in τ dephosphorylation, cultures of UV irradiated CHO 40.19 cells were treated with deltamethrin, which inhibits cellular PP2B (Enan and Matsumura, 1992). A concentration of 1 μM deltamethrin did not differ significantly from vehicle in its effect on tau1 or T49 relative fluorescence (Fig. 7A,B). Interestingly, execution phase CHO cells transfected with τ often formed long, straight processes that were not seen in apoptotic wild-type cells (Fig. 6D). Also of note, mitotic CHO cells, almost without exception, had undetectable tau1 staining and very bright PHF1 staining (unpublished observations and Preuss et al., 1995). Thus, the increase in tau1 staining in execution phase CHO cells is likely the result of a biochemical event specific to the execution phase and not simply a general feature of morphological change.

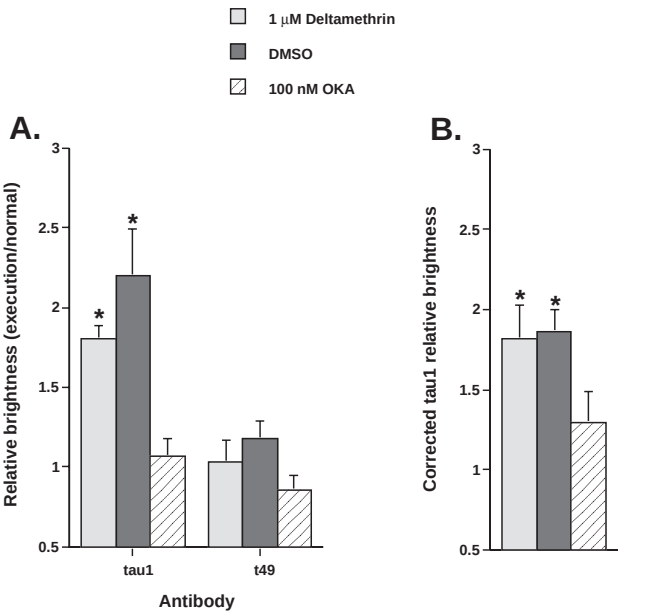

Fig. 7. τ dephosphorylation in dying CHO cells is inhibited by OKA. CHO cells were induced to undergo apoptosis by exposure to UV irradiation 24 hours before fixation; then, 4 hours prior to fixation, they were treated with either deltamethrin (1 μM), OKA (100 nM), or DMSO vehicle. (A) Relative fluorescence of execution phase cells to neighbors was calculated (as in Fig. 5) using tau1 and T49 as primary mAbs. Note that tau1 fluorescence is decreased significantly by OKA but not by deltamethrin. (B) The decrease is also statistically significant when changes in cell shape are controlled for by ratioing tau1 to T49. Asterisks denote a statistically significant difference relative to the corresponding OKA value. Data represent the mean ± s.e.m. from 4 separate experiments. Legend applies to both panels.

Dephosphorylation of τ protein correlates with MT disassembly

Fragmenting neurites have a characteristic 'beads on a string' morphology that can be found throughout cultures of apoptotic neurons and can reach hundreds of μms in length (Deckwerth and Johnson, 1993, 1994; Pittman et al., 1993). As might be expected, given the earlier findings that τ is dephosphorylated at the time of neurite collapse, these execution phase PC12 neurites showed strong immunoreactivity with tau1, even when no longer attached to a cell body (Fig. 8A; Table 2). Fragmented neurites were also stained by T49 (Fig. 8B; Table 2), though less intensely than tau1 relative to surrounding neurites. An antibody against α-tubulin showed a similar pattern (Fig. 8C; Table 2). Both τ and tubulin staining could be extracted from beaded neurites with short prior detergent treatment, a finding consistent with other studies showing that beads from fragmenting neurites are filled with disassembled MTs (Horie et al., 1983; Joshi et al., 1986; Baas and Ahmad, 1993). Cultures immunostained with antibodies against phosphorylated S396/S404 in τ (i.e. PHF1) and against acetylated tubulin contained no detectable beaded

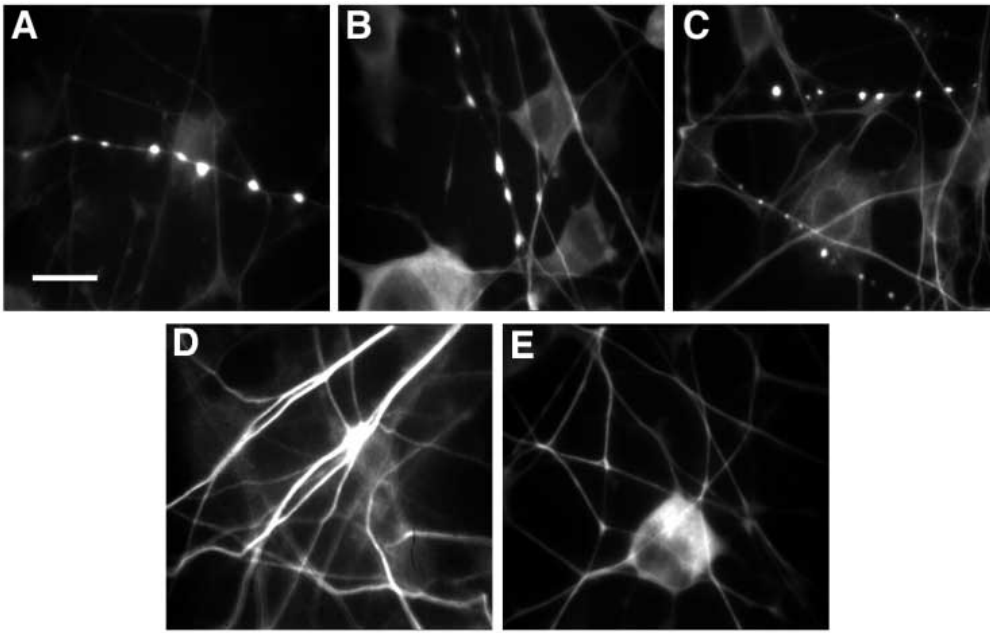

Fig. 8. Beaded PC12 cell neurites contain dephosphorylated τ and α-tubulin. Cultures were fixed and immunostained for tau1 (A), T49 (B), α-tubulin (C), acetylated tubulin (D), and PHF1 (E). Note that beaded neurites stain very brightly for dephosphorylated τ but also stain brightly for total τ and for α-tubulin (A-C). Neurites from cultures immunostained with acetylated tubulin and PHF1 antibodies never show beading (e.g. D,E). Note that detergent extraction removed immunoreactivity of all antibodies in beaded neurites, suggesting that the MTs are in a disassembled state (see Results and Table 2). Bar, 20 μm.

Table 2. Fragmenting, execution phase neurites lack phosphorylated τ and stable, acetylated tubulin

Antibody	Epitope	Strength of signal
tau1	Dephosphorylated τ	+++
tau49	Total τ	++
PHF1	Phosphorylated τ	–
N 356	α-tubulin	++
TUB-1A2	Tyrosinated tubulin	+
6-11B-1	Acetylated tubulin	–

PC12 cells were fixed and immunostained using the listed primary antibodies. Apoptotic cultures, which due to asynchrony always contain mostly healthy neurites, were scanned for the smaller subset of beaded, fragmenting, execution phase neurites. The intensity of staining in the beaded neurites was scored relative to the staining in surrounding healthy neurites.

+++, well above baseline intensity; ++, slightly above, +, similar to, –, less than baseline or none at all.

neurites, although the normal network of healthy neurites was strongly immunoreactive (Fig. 8D,E; Table 2). Acetylation is a post-translational tubulin modification that is a marker for more stable MTs (Piperno et al., 1987; Baas et al., 1991; Caceres et al., 1992; Lee and Rook, 1992). Antibodies against tyrosinated tubulin, a marker for less stable MTs (Brown et al., 1993) showed slight immunoreactivity against beaded neurites (Table 2). Thus, in execution phase neurites, τ is dephosphorylated, MTs are de-acetylated and disassembled, and both τ and MTs redistribute to form characteristic, periodic beads.

Similar results were obtained in execution phase cell bodies. As demonstrated earlier, tau1 staining is increased in actively dying cells, while PHF1 staining is decreased. Immunostaining revealed that α-tubulin was also present in execution phase cell bodies (Fig. 9A,B). An antibody against tyrosinated tubulin showed a similar pattern (not shown). α-Tubulin, as well as T49 and tau1, staining could be extracted from both execution phase cell bodies and beaded neurites within 2 minutes by 0.2% Triton X-100, a procedure that had no effect on the stable MT networks in normal neurites and healthy cell bodies (Fig. 9C,D). Execution phase CHO cells also had tubulin immunoreactivity that was sensitive to detergent pre-extraction (Fig. 9G,H).

τ is thought to be an important MT-stabilizing protein, and its phosphorylation state is likely to dictate MT-binding. To assess the effect of τ dephosphorylation on MT changes during the execution phase, dying PC12 cells were treated for 4.5 hours with 20 nM OKA and then immunostained for α-tubulin. OKA treatment did not result in a statistically significant change in MT susceptibility to detergent pre-extraction: 83±12% of OKA-treated execution phase cells had extractable α-tubulin staining (see, e.g. Fig. 9E,F) vs 71±6% of control.

As demonstrated earlier (Table 1, Fig. 4A-D), τ dephosphorylation probably occurs early in the execution phase. To determine whether MT disassembly is also an early event, PC12 cell cultures were scanned for nuclei with early morphological changes (i.e. aggregated or 'streaky' chromatin not yet clearly coalesced into distinct apoptotic 'balls'). A large fraction (56±9%) of cells with early nuclear changes had α-tubulin staining that was completely or nearly completely removed in a brief detergent pre-extraction (see, e.g. Fig. 10A,B). Treatment with OKA caused an even greater portion of cells with early execution phase nuclei to have extractable tubulin (from 56±9% to 73±4%, $P<0.05$, Fig. 10C,D). Thus, as inhibition of τ dephosphorylation seems to increase disassembly of early execution phase MT, it is possible that τ dephosphorylation normally plays a role in slowing MT collapse once it has been initiated (also see below). Note that double-labeling cells to correlate τ dephosphorylation with detergent-extractable α-tubulin in individual cells could not be performed because detergent pre-extraction also removes most τ staining.

PP2A is thought to be the principal τ phosphatase, and a significant fraction of this enzyme is bound to MTs (Sontag et

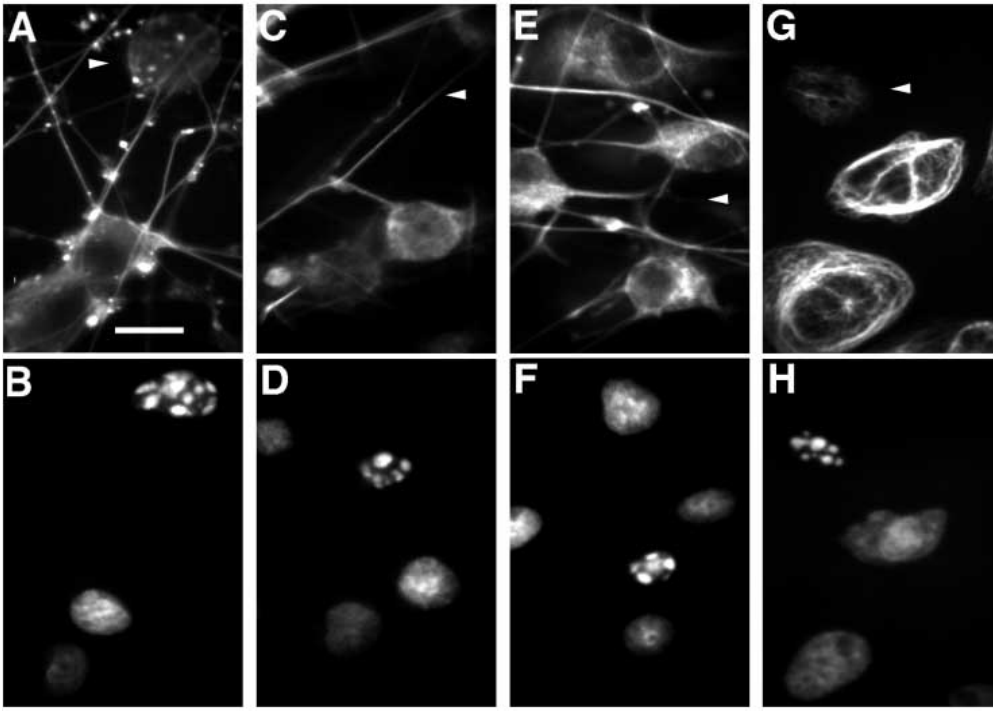

Fig. 9. Execution phase cell bodies contain detergent-extractable tubulin. Cultures were fixed and immunostained for α-tubulin (PC12 cells: A,C,E; CHO cells: G) and double-labeled with Hoechst (PC12 cells: B,D,F; CHO cells: H). Cells in C-H were pre-extracted briefly in detergent; A and B were fixed without pre-extraction. Execution phase cells often still contain tubulin immunoreactivity (A), but it is readily detergent-extractable (C), suggesting it is in a disassembled form. 20 nM OKA treatment for 4.5 hours did not prevent detergent sensitivity (E). Detergent pre-extraction also removes most tubulin immunoreactivity in execution phase CHO cells (G). Note that cellular debris and neurite remnants are present in small 'balls' of tubulin staining in non-extracted cultures (A). Arrowheads mark execution phase cells. Bar, 20 μm.

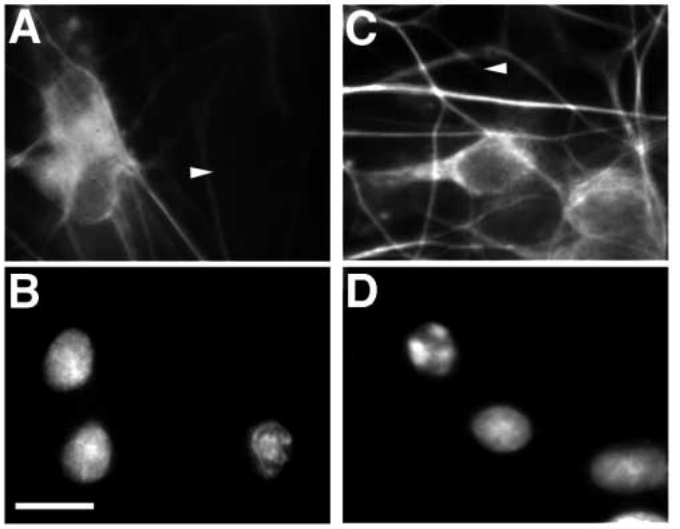

Fig. 10. Cells with nuclei exhibiting early execution phase changes also often have detergent-sensitive MTs. Cells were fixed and stained as for C-H in Fig. 9. Pre-extraction with detergent causes loss of tubulin immunoreactivity in vehicle (A and B) and OKA-treated (C and D) cells. Cells with early execution phase nuclei marked with arrowheads. Bar, 20 μm.

al., 1995, 1996). MT disassembly in execution phase cells may free PP2A to interact with a variety of substrates including phosphorylated, non-MT-bound τ. To test whether MT disassembly might lead to PP2A release and subsequent τ dephosphorylation, apoptotic PC12 cell cultures were treated with the MT-stabilizing agent taxol (20 nM) for 4.5 hours prior to fixation, a treatment which significantly decreased the fraction of execution phase cells with extractable tubulin (to 18±4% from 71±6%, $P<0.0005$, not shown). Relative tau1 fluorescence (as computed for Fig. 5 and Fig. 7) in taxol-treated execution phase cells was much less than relative tau1 fluorescence in vehicle-treated execution phase cells (1.60±0.15 vs 3.10±0.52, $P<0.005$, not shown). In other words, taxol inhibited τ dephosphorylation, suggesting that MT disassembly contributes to increased PP2A activity.

DISCUSSION

Apoptosis is a physiological process that is characterized by a conserved pattern of specific morphological events including dynamic blebbing, chromatin condensation, fragmentation of DNA, and formation of apoptotic bodies. These morphological changes appear to be initiated *after* cells commit to die (McCarthy et al., 1997; Messam and Pittman, 1998; J. C. Mills et al., 1998) and are thought to be involved in packaging the cell for phagocytosis. Cellular packaging during the execution phase is thought to be important for avoiding inflammation, a property of apoptosis that is probably its most critical feature in vivo. Thus far, study of apoptosis has focused almost exclusively on upstream events, on how apoptotic signals are translated into a commitment toward death. We present in this report for the first time evidence for several biochemical events that begin early in the execution phase of apoptosis in two unrelated cell types with two different apoptotic inducing agents. Using techniques devised to assess and temporally order biochemical changes occurring at a single cell level, it was found that τ protein is dephosphorylated, and MTs disassemble early in this stage. Dephosphorylation was inhibited by treatment with relatively low concentrations of the phosphatase inhibitor OKA, indicating that a PP2A-like phosphatase may be responsible. To ascertain whether PP2A activation occurred in apoptosis of other cell types, τ was used as a 'reporter' protein in CHO cells. τ that had been stably transfected into these non-neuronal cells was also dephosphorylated by an OKA-sensitive enzyme, suggesting

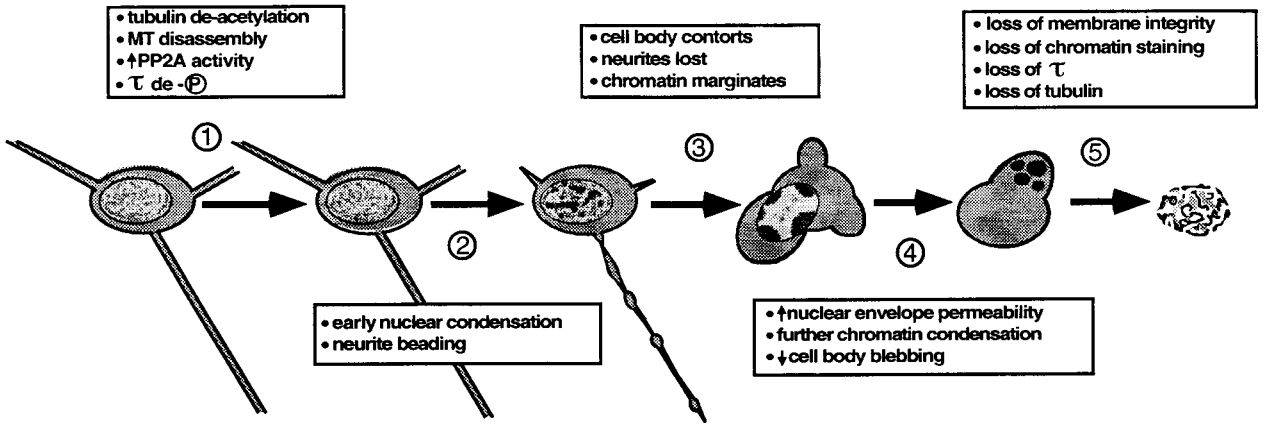

Fig. 11. Model of the progression of biochemical and morphological changes in the execution phase of neural apoptosis. Despite the asynchrony of death in cell culture, extensive timelapse video analysis has shown that each dying neuron eventually undergoes a stereotypical sequence of active morphological changes (Pittman et al., 1993; Mills et al., 1997). Before a cell shows any obvious morphological changes, several biochemical events occur. First, tubulin is de-acetylated. Because acetylated tubulin was only found in neurites with normal morphology, tubulin de-acetylation is probably complete before onset of the execution phase. MT disassembly also likely begins early in the execution phase, as MTs in beaded neurites and cells with early nuclear changes are already detergent-extractable (Figs 8, 9, Table 2). τ dephosphorylation (mediated by PP2A activation) begins early in the execution phase, after microtubule disassembly in most cells (Fig. 4, Table 1), but may continue for some time thereafter. The first signs of execution phase morphology are neurite beading and slight condensation and 'streaking' of chromatin, followed by cell membrane blebbing and loss of neurites. As the cell loses viability (assayed by loss of membrane integrity), chromatin, τ, and tubulin staining are lost, all motion stops, and often the cell debris detaches from the surface of the culture dish.

that PP2A activation may be a common feature of the evolutionarily conserved execution phase of apoptosis.

One hypothesis that would explain our findings is that changes occurring at the end of the commitment phase lead to modification and de-stabilization of MTs. Disassembly of MTs frees or activates previously bound and sequestered PP2A (Sontag et al., 1996) which should dephosphorylate numerous proteins, including (in neurons) τ (see Fig. 11). Evidence for this sequence of events is that changes in MT extractability and τ dephosphorylation both occur very early in the execution phase. MT disassembly is likely to be the catalytic event, because OKA treatment to inhibit τ dephosphorylation did not inhibit MT extractability, whereas slowing MT disassembly with taxol did inhibit τ dephosphorylation and, therefore, PP2A activation. MTs were always de-acetylated before disassembling in neurites, so one pre-disassembly biochemical modification of MTs is evident.

MT-τ interactions in the execution phase of apoptosis have not previously been studied, but our hypothesis seems plausible based on available data. It has been shown, for example, that cells treated with MT depolymerizing agents also dephosphorylate τ (Pope et al., 1994; Merrick et al., 1996; J. C. Mills and R. N. Pittman, unpublished observations), a process that is PP2A-mediated and may represent an attempt to re-stabilize disassembling MTs. PP2A has been shown to be MT-associated in many cell types, including neural cells (Sontag et al., 1995). Thus, it is not unreasonable to propose that MT disassembly in apoptosis would have the effect of activating or altering the cellular compartment of PP2A activity.

Several studies have shown protein phosphatase-mediated changes in phosphorylation in cultures of dying cells (Bøe et al., 1991; Baxter and Lavin, 1992; Song and Lavin, 1993; Ohoka et al., 1993; Gjertsen et al., 1994; Shibasaki and McKeon, 1995; Morana et al., 1996); these studies, however, have focused on upstream signaling (i.e. induction phase) events rather than on the execution phase. Thus, it is unlikely they are directly relevant to interpretation of execution phase processes.

Long-term treatment with taxol does not significantly inhibit death in differentiated PC12 cells (unpublished observations). Thus, our results suggest MT disassembly and PP2A activation are not directly responsible for causing loss of viability. However, they are very likely important in other aspects of 'normal' physiologically relevant apoptosis. The MT network is a fundamental regulator of cellular structural stability. It would be surprising if such a dramatic reorganization of MTs that occurs very early in a critical phase would not play a significant role in the progression of normal apoptosis. It is easy to see, for example, how disassembly of MTs might be critical to facilitate cross-linking and packaging of cellular constituents. Likewise, PP2A is one of the principal cellular phosphatases. PP2A that has been freed to interact with endogenous τ in PC12 cells and exogenous τ in CHO cells would undoubtedly interact with other substrates. Its role in τ dephosphorylation alone may be important, because phosphorylation inhibits normal calpain-mediated turnover of τ (Litersky and Johnson, 1995; Mercken et al., 1995). In Alzheimer's disease, where PP2A deficiency is a proposed underlying mechanism, hyperphosphorylated τ accumulates in insoluble aggregates that are an important pathological feature of the disease. Thus, the τ dephosphorylation that occurs in physiological death may be a mechanism to prevent τ aggregation and potential pathological consequences.

Using a single cell approach, the present report outlines a sequence of conserved biochemical changes that occur during initiation of the execution phase of apoptosis. MT disassembly, activation of a PP2A-like phosphatase, and dephosphorylation of tau occur in two unrelated cell types following two different inducers of apoptosis suggesting that the cellular changes may be conserved and fundamental features of the execution phase of apoptosis. Further characterization of the execution phase as a whole is clearly needed. The data presented in the current study suggest several lines for further investigation: (1) determining which event during the commitment stage causes MT de-acetylation and destabilization (e.g. are caspases involved); (2) determining other substrates for PP2A; and (3) developing assays for studying aspects of apoptosis other than loss of viability to determine the ramifications of MT disassembly and PP2A activation. As a first step in directing future studies, a model of our working hypothesis of events occurring in active apoptosis is presented (Fig. 11).

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