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Effects of Elevated Intracellular Calcium Levels on the Cytoskeleton and Tau in Cultured Human Cortical Neurons

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ABSTRACT

Considerable evidence suggests that altered neuronal calcium homeostasis plays a role in the neuronal degeneration that occurs in an array of neurological disorders. A reduction in microtubules, the accumulation of 8–15 nm straight filaments, and altered antigenicity toward antibodies to the microtubule-associated protein tau and ubiquitin, as well as granulovacuolar degeneration, are observed in many human neurodegenerative disorders. Progress toward understanding how and why human neurons degenerate has been hindered by the inability to examine living human neurons under controlled conditions. We used cultured human fetal cerebral cortical neurons to examine ultrastructural and antigenic changes resulting from elevations in intracellular calcium levels. Elevation of intracellular calcium by exposure to a calcium ionophore or a reduced level of extracellular Na^+ for periods of hours to days caused a loss of microtubules, an increase in 8–15 nm straight filaments, and increased immunostaining with Alz-50 and 5E2 (tau antibodies) and ubiquitin antibodies. Granulovacuolar degeneration was also observed. Antigenic changes in tau were sensitive to phosphatases, and the electrophoretic mobility of tau was altered in cells exposed to calcium ionophore, indicating that tau was excessively phosphorylated as the result of elevated intracellular calcium levels. Colchicine also caused an accumulation of

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straight filaments and altered tau immunoreactivity, suggesting that a disruption of microtubules secondary to altered calcium homeostasis may be a key event leading to altered tau disposition and neuronal degeneration. These data demonstrate that aberrant rises in intraneuronal calcium levels can result in changes in the neuronal cytoskeleton similar to those seen in neurodegenerative disorders, and suggest that this experimental system will be useful in furthering our understanding of the cellular and molecular mechanisms of human neurological disorders.

Index Entries: Alzheimer's disease; cell culture; cytoskeleton; human cerebral cortex; microtubules; neurodegeneration; phosphorylation; tau.

INTRODUCTION

The proper regulation of intracellular calcium levels is of critical importance for the maintenance and adaptive modification of neuronal structure (Kater et al., 1988; Mattson, 1990–1990a). Many environmental signals that influence neural plasticity and cell survival utilize the calcium signaling system to elicit their responses. For example, the neurotransmitter glutamate can selectively inhibit the elongation of dendritic growth cones in embryonic hippocampal neurons by a calcium-mediated mechanism (Mattson et al., 1988), and calcium influx is believed to play a key role in long-term potentiation of synaptic transmission (del Cerro et al., 1990). The calcium signaling pathway affects neuronal cytoarchitecture by influencing the cytoskeleton. Calcium can have direct influences on cytoskeletal elements (Weisenberg, 1972), or it can activate protein kinases that phosphorylate and thereby modify cytoskeletal proteins (Browning et al., 1985).

Activation of the calcium pathway is not always beneficial to a neuron. Indeed, it is now well established that an excessive and sustained rise in intracellular calcium levels can cause cell death. Such a loss of calcium homeostasis may result from the overactivation of excitatory receptors (Choi, 1988; Mattson et al., 1988; 1991a), growth factor deficiencies (Mattson et al., 1989a), or faulty calcium buffering mechanisms (Gibson and Peterson, 1987). This destructive potential of calcium has led to the hypothesis that abnormalities in calcium homeostasis underlie the neuronal degeneration in an array of disorders, including Huntington's disease, Alzheimer's disease, and stroke (Gibson and Peterson, 1987; Choi, 1988; Greenamyre and Young, 1989; Mattson, 1989). This hypothesis is particularly attractive since cytoskeletal proteins are quite sensitive to calcium, and alterations in the neuronal cytoskeleton are hallmarks of neurodegenerative disorders. Neurofibrillary tangles are abnormal accumulations of cytoskeletal proteins with unique chemical and antigenic properties that allow their selective staining (*see* Selkoe, 1989 for review). The detailed ultrastructure of tangles varies somewhat depending on the

disease, but in general, they are devoid of microtubules and possess accumulated straight or paired helical filaments (Terry, 1963; Wisniewski et al., 1979; Metuzals et al., 1988; Perry et al., 1987a). Granulovacuolar neuronal degeneration is also prominent in Alzheimer's disease (Woodard, 1962; Ball, 1978). The straight filaments and paired helical filaments contain the microtubule-associated protein tau (Delacourte and Defossez, 1986; Kosik et al., 1988; Love et al., 1988; Nukina et al., 1987), as well as ubiquitin (Mori et al., 1987; Perry et al., 1987b). The results of biochemical and immunological studies of tau suggest that it is excessively phosphorylated in Alzheimer's disease neurofibrillary tangles (Grundke-Iqbal et al., 1986, 1988; Ihara et al., 1986; Bancher et al., 1989; Ueda et al., 1990). Other cytoskeletal components including intermediate filament proteins may also be abnormally phosphorylated in Alzheimer's disease (Sternberger et al., 1985). These observations are consistent with the possibility that aberrant kinase activity plays a role in the pathophysiology of neurodegenerative disorders. Further support for the calcium hypothesis has come from recent data demonstrating that calcium calmodulin-dependent protein kinase II is concentrated in neurons that are selectively vulnerable in Alzheimer's disease (McKee et al., 1990), and that calcium-dependent kinase systems and/or protein kinase C may be altered in Alzheimer's disease (Saito and Iimoto, 1989). In addition, we recently found that antigenic changes similar to those seen in neurofibrillary tangles could be elicited by elevating intracellular calcium levels in cultured rat hippocampal neurons (Mattson, 1990b).

Previous approaches to understanding the dynamic aspects of human neurodegenerative processes have relied largely on postmortem analyses of human neural tissue or animal studies. Although these approaches have been useful, it has proven very difficult to retrace the events leading to neurodegeneration. Perhaps another valuable approach to understanding the mechanisms of human neurological disorders would be to study living human neurons in an experimental setting. We recently developed procedures for long-term cryopreservation and cell culture of human fetal cerebral cortical neurons (Mattson and Rychlik, 1990). In the present study, we have used this human cortical culture system to examine the process of neuronal degeneration induced by experimental elevation of intracellular calcium levels.

METHODS

Cell Cultures and Experimental Treatments

Dissociated cell cultures of human embryonic cerebral cortex were established from our cryopreserved cell stocks. Methods for tissue procurement, cell cryopreservation, and culture are detailed in our recent paper (Mattson and Rychlik, 1990). The protocol for obtaining postmortem embryonic neural tissue is reported in our initial study of cul-

tured human neurons (Mattson and Rychlik, 1990) and compiled with all federal guidelines for fetal research, with the Uniformed Anatomical Gift Act, and with guidelines established by the Medical Institutional Review Board of the University of Kentucky. Cerebral cortices were placed in a Ca^{2+} and Mg^{2+} -free Hanks Balanced Saline Solution (HBSS; Gibco). Tissue was cut into pieces of approx. 1–3 mm^3, and the tissue pieces were incubated in saline solution containing 2 mg/mL trypsin for 20 min, washed, suspended in cryopreservation medium (containing 8% dimethylsulfoxide (DMSO)), and then cells were dissociated mechanically by trituration through the narrowed bore of a fire-polished Pasteur pipet. Cells were frozen to –70°C at a rate of –1°C/min and were then transferred to liquid nitrogen for long-term storage. Cells were thawed rapidly to 37°C, and were seeded into polylysine-coated culture dishes containing bicarbonate-buffered Eagles MEM, supplemented 10% (v/v) with fetal bovine serum (Sigma). Cultures were maintained at 37°C in a 6% CO_2/94% room air humidified incubator. All experiments were done in cells that had been in culture for 7–22 d.

Calcium ionophore A23187 was prepared as a concentrated stock in DMSO and was diluted into the culture medium from the stock, such that the final concentration of DMSO never exceeded 1% (DMSO alone was added to control cultures). Calcium-deficient medium consisted of HBSS lacking calcium and containing 1m*M* EGTA, 1m*M* $MgCl_2$, and 20 m*M* glucose. The free calcium concentration in this solution was nominally zero. In some experiments, 90% of the NaCl in the culture medium was replaced with *N*-methyl-D-glucamine. This procedure reduces Na^+-dependent calcium extrusion and reduces the ability of the neurons to extrude calcium (Mattson et al., 1989b).

Light and Electron Microscopic Analyses

Cells were visualized and photographed with a Nikon Diaphot inverted microscope equipped with phase-contrast and bright-field optics. For transmission electron microscopy, cells were fixed in a solution of 3% glutaraldehyde in PBS, and postfixed in a solution of 1% osmium tetroxide in PBS. Cultures were stained en bloc with 1% uranyl acetate for 1 h, dehydrated in a graded ethanol series, and embedded in epon. Thin sections were stained with lead citrate and were examined and photographed in an Hitachi-H-7000 transmission electron microscope.

Measurements of Intracellular Calcium Levels

These procedures were similar to those of our past work (Mattson et al., 1991a). Briefly, cells were loaded at 37°C for 40 min with 4 μ*M* of the acetoxymethyl ester (AM) form of fura-2 (Molecular Probes). The loaded cells were then washed with fresh medium and incubated for at least 1 h prior to imaging. Prior to imaging, the normal culture medium was replaced with HBSS containing 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 1%

glucose. Images were obtained with a Dage MTI SIT videocamera and a Leitz Diaplan microscope using a 63X, N.A. 1.3 fluorescence objective. The imaging system consisted of an IRIS 3120 graphics work station (Silicon Graphics) and an AP-512 digital image processing system (Imaging Technology, Inc.). UV filters of 350 nm and 380 nm were housed in a filter wheel that was controlled manually. Intracellular calcium levels were determined based on the ratio of fluorescence emitted during 350 nm excitation to that emitted during 380 nm excitation following background fluorescence subtraction, using the method of Grynkiewicz et al. (1985). Statistical comparisons of intracellular calcium levels between treatment groups or cell types were made using Students *t*-test.

Antibodies

Four different antibodies that recognize tau were used in the present study. Tau-1 is a mouse monoclonal raised against purified tau from bovine brain (Wood et al., 1986), and was a generous gift from L. I. Binder. Alz-50 is a mouse monoclonal raised against Alzheimer's disease neurofibrillary tangle material (Wolozin et al., 1986) and was a kind gift from P. Davies.; Alz-50 reacts preferentially with phosphorylated tau (Ueda et al., 1990). 5E2 is a mouse monoclonal raised against fetal human tau that immunostains neurofibrillary tangles (Joachim et al., 1987; Kosik et al., 1988) and was kindly provided by K. S. Kosik. The polyclonal antisera to tau was from ICN; the recognition of tau by this antiserum is not phosphorylation dependent. Antibody AP14 is a mouse monoclonal that recognizes MAP2 and was a gift from L. I. Binder. Polyclonal rabbit antiserum against ubiquitin (Perry et al., 1987b) was a generous gift from G. Perry.

Immunocytochemistry

For all comparisons, cortical cell cultures were established, maintained, treated, and processed in parallel. Following exposure to treatments, cells were fixed for 20–30 min in either 4% paraformaldehyde in PBS (for subsequent staining with Alz-50, tau-1, MAP2, and ubiquitin antibodies) or PLP fixative that contained 2% paraformaldehyde, 100 m*M* DL-Lysine, and 10 m*M* sodium *m*-periodate (5E2 antibody). Fixed cultures were exposed for either 2 min (MAP2 antibody) or 5 min (other antibodies) to a 0.2% solution of Triton X-100 in PBS, and were processed using primary antibodies and appropriate Vectastain ABC avidin-biotin-peroxidase kits with diaminobenzidine tetrahydrochloride as a substrate. Antibody dilutions were: 5E2, 1:400; Alz-50, 1:10; tau-1, 1:500; anti-ubiquitin, 1:500; MAP2, 1:300. These antibody dilutions were chosen based on preliminary studies showing that these dilutions resulted in minimal staining in control cultures in the case of antibodies 5E2, Alz-50, ubiquitin, and MAP2; and relatively high level of staining in the case of tau-1. Negative controls consisted of substitution nonimmune serum for

the primary antibodies, or preadsorption of primary antibodies with excess antigen (ubiquitin). In addition, cell specificity of the staining provided an important criterion for judging the specificity of staining. (e.g., tau immunostaining was present in neurons but not in glia). The same exposure conditions were used to photograph all cells, and bright-field prints were exposed for the same time period. For semiquantitative comparisons, neuronal staining intensities were scored on a scale from 0 to 3 (0 = no staining, 1 = low, 2 = moderate, 3 = intense). A total of 300 neurons in three separate cultures (100 neurons/culture) were scored without knowledge of the treatment history of the cultures.

Phosphatase Treatments

Fixed and permeabilized cells were exposed to acid or alkaline phosphatases prior to immunocytochemical processing. These methods were similar to those recently reported by Ueda et al. (1990). Briefly, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5–10 min. Cells were then exposed to either 3 U/mL acid phosphatase in 0.1*M* acetate buffer (pH 4.9) or 3 U/mL alkaline phosphatase in 0.1*M* Tris buffer containing 0.01*M* PMSF (pH 8.0) for 20–30 min. Cells were then washed with PBS and immunostained. Parallel cultures not exposed to the phosphatases were used as reference controls.

Western Blots

Cells were cultured in 60-mm diameter plastic culture dishes at a cell density of approx. 10^6 cells/dish. Following experimental treatment, cells were rinsed once in PBS and then solubilized in sample buffer that contained 2 m*M* EDTA, 2.3% SDS, 10% glycerol, and 62.5 m*M* Tris (pH 6.9). Equivalent amounts of protein from each culture were separated by sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis (14% acrylamide) according to Laemmli (1970). Proteins from unstained polyacrylamide gels were transferred electrophoretically onto immobilon (Millipore) and immunoreacted with rabbit polyclonal antisera to tau (ICN) or tubulin (T_1T_2; from G. Perry). This tau antibody was chosen for Western blots in the present study because its binding to tau is not phosphorylation dependent. The antibodies were visualized using goat antirabbit IgG labeled with peroxidase, and diaminobenzidine as enzyme substrate. Molecular mass markers consisted of β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa).

RESULTS

Effects of Elevated Intracellular Calcium Levels on Neuronal Ultrastructure

We have previously employed the calcium indicator dye fura-2 to examine in detail the effects of calcium ionophore A23187 on intracellular

calcium levels in cultured central neurons (Mattson et al., 1990; 1991b). In the present study, we found that 2 μM A23187 caused a highly significant and sustained elevation in intracellular calcium levels in the cultured human cortical neurons. Intracellular calcium levels rose from a pretreatment level of 75 $\pm$ 1.8 nM to 555 $\pm$ 50.0 nM at 10 min posttreatment and 623 $\pm$ 46.4 nM at 1 h posttreatment ($n = 8$ neurons; $p < 0.001$). Calcium levels remain elevated and neurons degenerated during a period of several hours of exposure to A23187 (cf Mattson et al., 1988; 1991a).

In untreated human cortical cultures, the neuronal cytoskeleton consisted predominantly of 25 nm microtubules that, in axons, were oriented in parallel with the long axis (Fig. 1A). Intermediate filaments (10 nm) were very sparse in these embryonic neurons, and microfilaments (6 nm) were also rarely seen in the axons and somata but were present in growth cones (not shown). This cytoskeletal organization was seen in all 27 neurons examined. Cytoskeletal structure was dramatically altered in neurons exposed to 2 μM calcium ionophore A23187. Calcium influx caused a rapid and marked loss of microtubules, and the accumulation of 8–15 nm straight filaments in both the somata and axons (Fig. 1B and C). Within 2 h of exposure to A23187 microtubules were drastically reduced in numbers, and those still present were often curved along their length (Fig. 1B). Within 2 h of exposure to A23187, filaments ranging in diameter from 8–15 nm had accumulated (25 of 28 neurons examined). Paired helical filaments, such as those seen in Alzheimer's disease, were not observed, although structures that appeared to consist of twisted filaments were occasionally seen (Fig. 1C), but they were highly variable in their apparent twist periodicity and diameters. Such structures may be microtubules that were in the process of disassembly. Cultures that were exposed to A23187 and maintained beyond 2 h subsequently degenerated over a period of several hours as previously reported (Mattson et al., 1988; Mattson, 1990) and were generally not present beyond 12 h.

Since neuronal degeneration in neurodegenerative disorders probably occurs more slowly than was induced by A23187 in the present study, we exposed cultured cortical neurons to a condition that caused a more moderate elevation in intracellular calcium levels and slower neuronal degeneration. Cultures were incubated in medium in which 90% of the NaCl was replaced with *N*-methyl-D-glucamine. We have previously shown that this manipulation reduces the ability of neurons to reduce intracellular calcium levels (Mattson et al., 1989b). Preliminary experiments using the calcium indicator dye fura-2 showed that Na^+ replacement with *N*-methyl-D-glucamine resulted in a significant twofold elevation of intracellular calcium levels over a 24-h period. Intracellular calcium levels 24 h following exposure to reduced Na^+ medium averaged 155 $\pm$ 14.3 nM, whereas calcium levels in neurons maintained in medium containing the normal level of Na^+ averaged 75 $\pm$ 1.8 nM ($n = 8$ neurons; $p < 0.01$). Ultrastructural changes in neurons exposed to the

Fig. 1. Effects of calcium ionophore A23187 on the ultrastructure of cultured human cortical neurons. Transmission electron micrographs of human cortical neurons in a control culture (**A**) and a culture treated with 2 μ*M* A23187 for 2 h (**B and C**). In the control (untreated) culture, parallel arrays of microtubules (mt) are the predominant cytoskeletal component in the somata and axons; intermediate-sized filaments are rare in these embryonic neurons (**A**). Following exposure to A23187, microtubules are reduced in numbers and those present are curved and have lost their parallel orientation (mt). There is also a striking accumulation of intermediate-size filaments ranging in diameters from 8 to 15 nm (**B**). Occasional helical-shaped filamentous structures are also observed (e.g., arrowhead in **C**). N, nucleus. Scale bar, 150 nm.

reduced Na^+ medium were striking. Gravulovacuolar-like degeneration was observed in 17 of 27 neurons examined. The presence of vacuoles was pronounced and these neurons showed massive accumulations of filaments that were often so tightly packed in the axons that relationships between individual filaments often could not be discerned (Fig. 2).

Effects of Elevated Intracellular Calcium Levels on Neuronal Immunoreactivity with Tau and Ubiquitin Antibodies

In a number of neurodegenerative disorders, degenerating neurons are highly immunoreactive with antibodies to tau (Delacourte and Defossez, 1986; Wood et al., 1986; Kosik et al., 1988; Love et al., 1988) and ubiquitin (Mori et al., 1987; Perry et al., 1987b; Love et al., 1988). We therefore used two monoclonal antibodies, 5E2 (Joachim et al., 1987; Kosik et al., 1988) and Alz-50 (Wolozin et al., 1986; Ueda et al., 1990) that recognize tau epitopes in neurofibrillary tangles, as well as a ubiquitin antiserum, to test whether antigenic changes similar to those seen in neurofibrillary tangles could be induced by experimental elevation of intracellular calcium levels (Figs. 3–7, Table 1). In untreated cultures, 5E2 and Alz-50 immunoreactivities were present at relatively low levels in the neurons (Figs. 3A, 4). Immunoreactivity toward the 5E2 and Alz-50 antibodies increased greatly in neurons exposed to calcium ionphore A23187 (Figs. 3 and 4). Staining was very intense in both the somata and axons of neurons exposed to A23187 (Fig. 3B). Glial cells did not stain with the tau antibodies (Fig. 3), and no staining was observed in neurons when nonimmune serum was substituted for the primary antibodies (not shown), indicating that the staining was specific. No increase in immunoreactivity toward 5E2 and Alz-50 antibodies was seen in neurons exposed to A23187 in culture medium lacking calcium and containing 1 m*M* EGTA ($n = 3$ cultures), indicating that an influx of extracellular calcium was required for the altered tau immunoreactivity. In contrast to staining with 5E2 and Alz-50, neuronal staining with antibody tau-1 was moderately reduced in cultures exposed to A23187 (Fig. 4).

In order to determine whether the slower form of calcium-dependent neuronal degeneration induced by reduced Na^+ medium also caused altered tau immunoreactivity, we immunostained neurons that had been maintained for 24–48 h in medium in which 90% of the Na^+ was replaced with *N*-methyl-D-glucamine. Neurons maintained in the reduced Na^+ medium stained intensely with 5E2 and Alz-50 antibodies, but tau-1 immunoreactivity was decreased (Figs. 3C, 4). When cultures were exposed to either A23187 for 2–4 h (Figs. 4, 5) or medium with a reduced level of Na^+ for 24 h (Table 1), ubiquitin immunoreactivity was increased greatly in the degenerating neurons. Thus, altered calcium homeostasis in the cultured human cortical neurons resulted in both ultrastructural and antigenic changes similar to those seen in neurofibrillary tangles *in situ*.

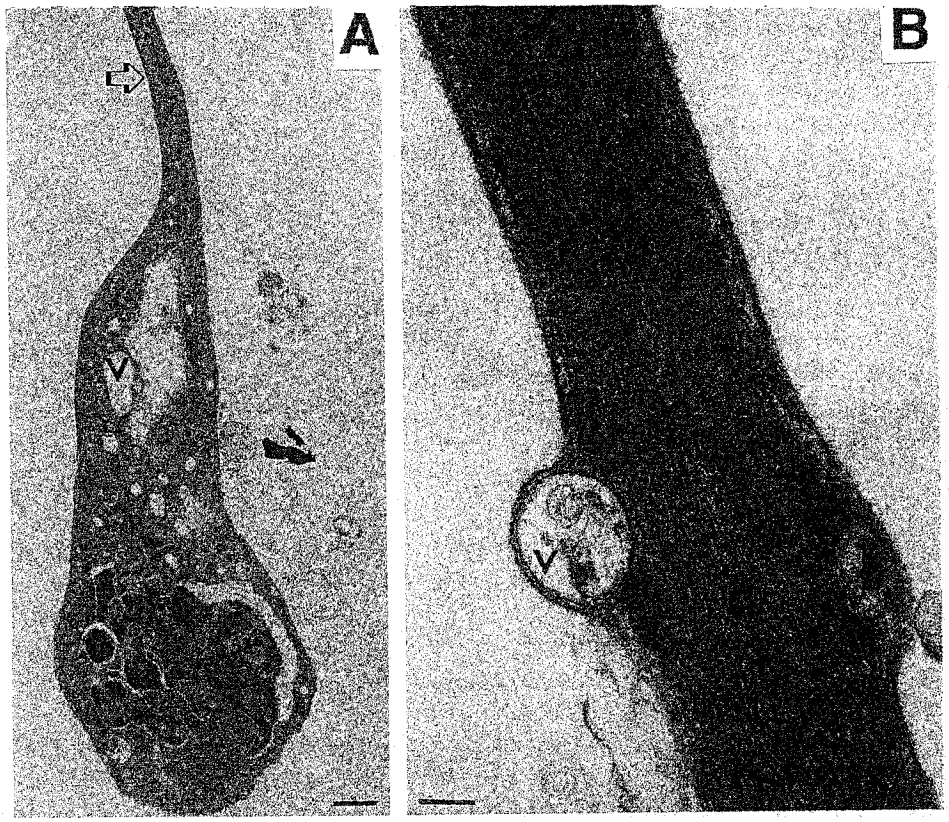

Fig. 2. Effects of reduced Na^+ medium on the ultrastructure of human cortical neurons. Shown are electron micrographs of a cortical neuron in culture, 48 h following exposure to medium in which 90% of the NaCl was replaced with *N*-methyl-D-glucamine (this manipulation impairs the plasma membrane Na^+/Ca^{2+} exchanger and results in an elevation in intracellular calcium levels). (**A**) The cell body is extensively vacuolated and there is a massive accumulation of filamentous material throughout the cytoplasm (open arrow points to the axon). (**B**) Higher power view of a region of the axon of the cell in A. the large accumulation of material in the cytoplasm obscures the identity of individual filaments. V, vacuole. Scale bars: **A**, 1 μm; **B**, 250 nm.

Several observations indicated that the altered immunoreactivity toward the tau and ubiquitin antibodies observed in degenerating neurons was not the result of some nonspecific change, such as an increased permeability of the cells to the antibodies. Thus, 5E2, Alz-50, and ubiquitin immunoreactivities were increased by elevated intracellular calcium levels, whereas tau-1 staining decreased (Fig. 4). In addition, staining with an antibody to MAP2 was not affected by A23187 or reduced Na^+ medium (Table 1).

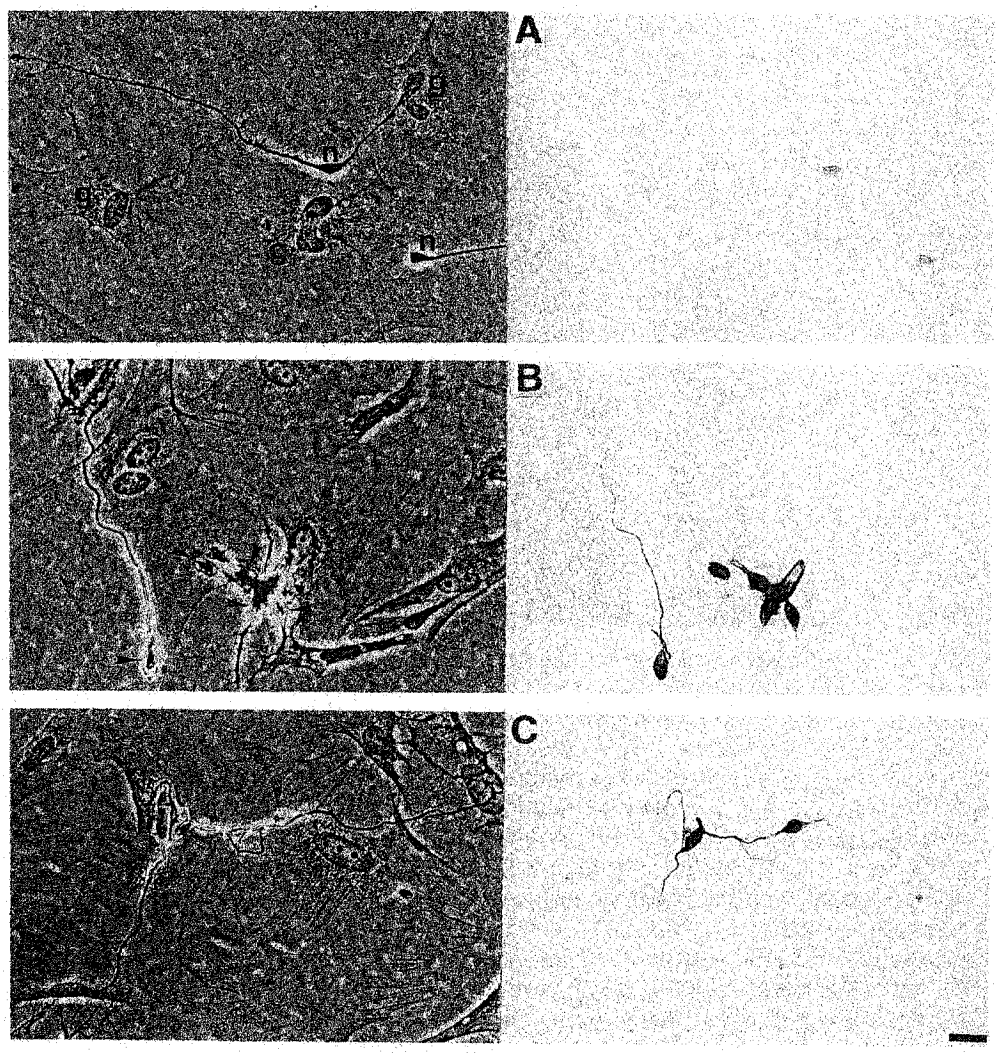

Fig. 3. Elevated intracellular calcium levels after tau immunoreactivity in cultured human cortical neurons. Cells were either left untreated (**A**), or were exposed to 2 μM A23187 for 2 h (**B**) or medium in which 90% of the NaCl was replaced with *N*-methyl-D-glucamine for 24 h (**C**) and were then processed in parallel by immunocytochemistry using tau monoclonal antibody (5E2). Each panel (**A–C**) contains a phase-contrast (left) and a bright-field (right) micrograph of a microscope field of cells. Note that 5E2 immunoreactivity was greatly increased in the neurons exposed to the calcium elevating conditions (**B** and **C**). n, neuron; g, glial cell; arrowheads, neuronal somata. Scale bar, 20 μm.

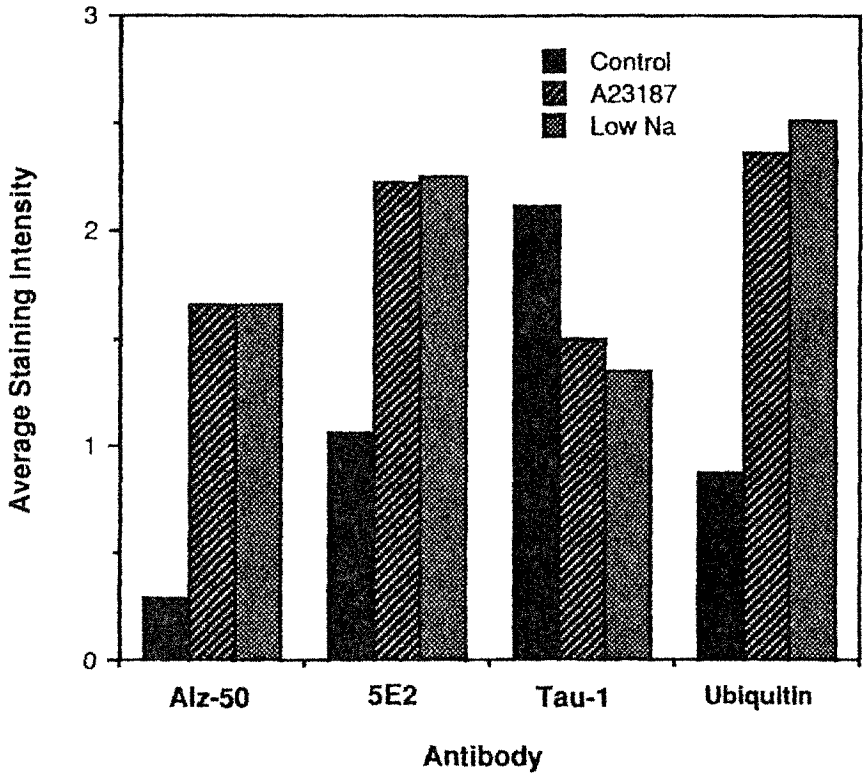

Fig. 4. Effects of calcium ionophore A23187 and low Na^+ medium on relative staining intensities of cultured cortical neurons with tau and ubiquitin antibodies. Cultures were left untreated (control) or were exposed to 2 μM A23187 for 2 h, or medium in which 90% of the Na^+ was replaced with *N*-methyl-D-glucamine for 24 h, and were then immunocytochemically processed using the indicated antibodies. Staining intensities of 300 neurons in each group were scored on a scale from 0 to 3 (0 = no staining, 1 = light, 2 = moderate, 3 = intense) and the values presented represent the average staining intensity for the 300 neurons.

Calcium-Induced Alterations in Tau Immunoreactivity and Phosphorylation

Considerable data indicate that tau is abnormally phosphorylated in Alzheimer's neurofibrillary tangles (Grundke-Iqbal et al., 1986; Ihara et al., 1986; Baudier and Cole, 1987; Bancher et al., 1989; Ueda et al., 1990). Previous studies indicated that Alz-50 recognizes a phosphorylated epitope on tau (Ueda et al., 1990) and that tau-1 preferentially binds to dephosphorylated tau (Wood et al., 1986). Phosphorylation of tau causes a shift in its electrophoretic mobility to a higher molmass form (Baudier and Cole, 1987). We therefore tested whether elevated intracellular calcium levels were causing tau phosporylation in the cultured human

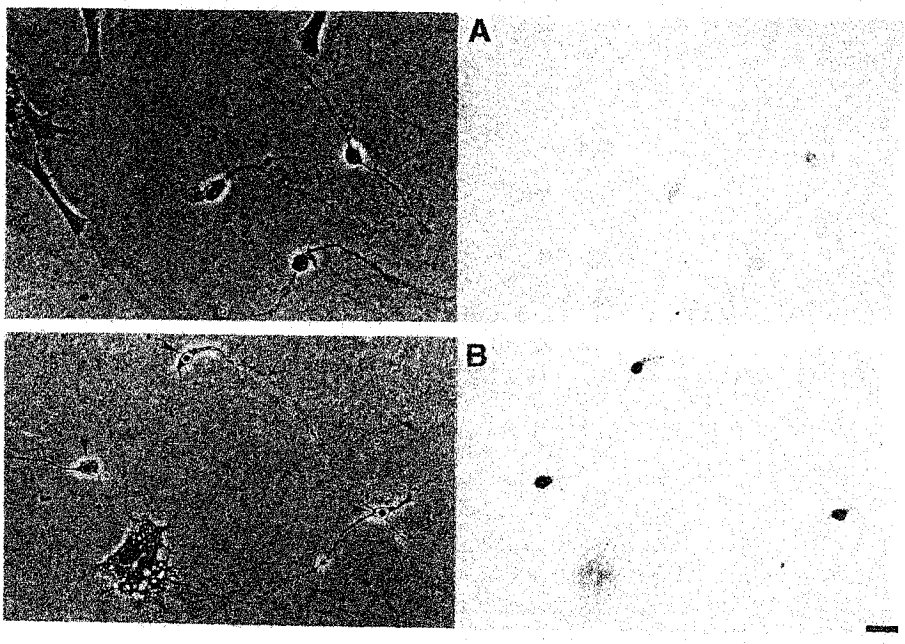

Fig. 5. Elevated intracellular calcium levels cause an increase in the immunoreactivity of cultured human cortical neurons toward a ubiquitin antibody. Phase-contrast (left) and bright-field (right) micrographs of cells in a control culture (**A**) and a culture treated 2 μM A23187 for 4 h (**B**). Note that ubiquitin immunoreactivity is greatly increased in the degenerating neurons (arrowheads point to degenerating somata and arrows point to fragmented degenerating neurites). Scale bar, 20 μm.

cortical neurons. Western blot analysis of tau in cell homogenates from control cultures revealed three immunoreactive bands of approx. 49, 52, and 61 kDa. Treatment with 2 μM A23187 caused a reduction in the staining intensities of all three bands and, noteably, a shift in electrophoretic mobility of the ~61 kDa band to a higher molecular weight of ~63 kDa (Fig. 6). This electrophoretic shift is similar to that previously shown to occur as the result of tau phosphorylation (Baudier and Cole, 1987). Western blot analysis of tubulin in control and ionophore-treated cells indicated that calcium influx caused a reduction in tubulin levels (Fig. 6).

In order to further test whether tau phosphorylation was responsible for the altered tau immunoreactivity, we examined the effects of phos-

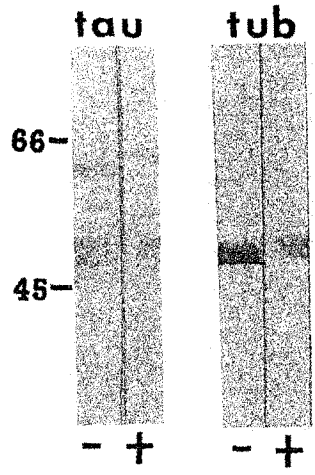

Fig. 6. Western blot analysis of the effects of calcium ionophore on tau and tubulin in cultured human cortical cells. Cultures were incubated in the absence (—) or presence (+) of calcium ionophore A23187 (1 μM for 4 h). Equivalent amounts of protein from each culture were then separated by polyacrylamide slab gel electrophoresis and transferred to immobilon. Transfers were reacted with antisera to tau (left) or tubulin (right). Note that A23187 caused decreases in both tau and tubulin, and also note the reduced migration of the upper tau immunoreactive band in the ionophore-treated culture (arrowhead). Molecular mass calibration markers were bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

phatases on the immunoreactivity of the human cortical neurons to the different tau antibodies. Cultures were exposed for 24 h to medium in which 90% of the Na^+ was replaced with *N*-methyl-D-glucamine, a condition that increased intracellular calcium levels and elicited ultrastructural and antigenic changes similar to those seen in neurofibrillary tangles (*see above*). Cells were fixed and then exposed to either acid or alkaline phosphatases prior to immunocytochemical staining. Staining in the phosphatase-treated neurons was then compared to that in cultures treated and processed in parallel, but without exposure to a phosphatase. Alz-50 immunoreactivity was markedly reduced in neurons treated

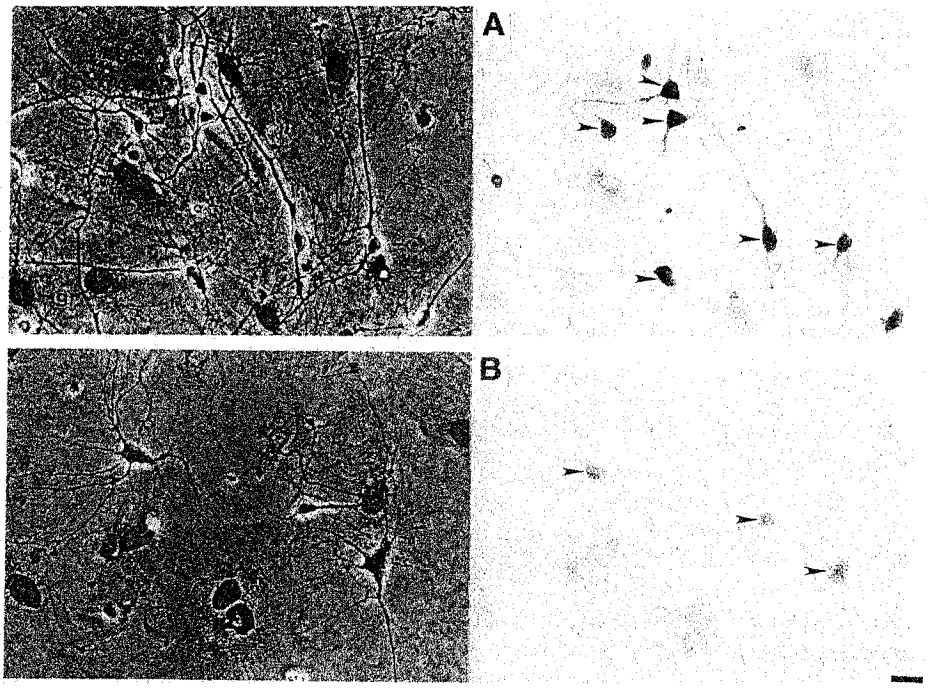

Fig. 7. Acid phosphatase treatment reduces neuronal immunoreactivity toward Alz-50. Phase-contrast (left) and bright-field (right) micrographs of cells from parallel cultures that had been exposed for 24 h to medium in which 90% of the NaCl was replaced with *N*-methyl-D-glucamine. Following membrane permeabilization, one culture (**A**) was left in buffer and the other was exposed for 20 min to 5 u/mL of acid phosphatase (**B**) (*see* Methods). The cultures were then immunocytochemically processed in parallel using Alz-50. Arrowheads point to neuronal somata. Also note that glia (g) did not stain with Alz-50. Scale bar, 10 μm.

with acid phosphatase and was also reduced in neurons treated with alkaline phosphatase (Table 1, Fig. 7). In contrast to Alz-50 staining, neuronal staining with tau-1 was increased by phosphatase (Table 1). Phosphatase treatment had little effect on immunostaining with antibody 5E2 although there was a trend toward reduced staining (Table 1). These data are consistent with previous studies, which indicated that Alz-50 recognizes phosphorylated tau (Ueda et al., 1990), tau-1 recognizes dephosphorylated tau (Wood et al., 1986), and 5E2 binding to tau is probably not affected by phosphorylation (Kosik et al., 1988). Neuronal staining with ubiquitin and MAP2 antibodies was similar in control

Table 1
Summary of the Effects of Calcium-Elevating Agents and Phosphatases[a] on Neuronal Immunoreactivities to Tau, Ubiquitin, and MAP2 Antibodies in Human Cortical Cell Cultures

	Antibody				
Treatment	Alz-50	Tau-1	5E2	Ubiquitin	MAP2
A23187 (1–2 μM)	+ + +	o,–	+ + +	+ +	o
Reduced $[Na^+]_o$	+ + +	o,–	+ + +	+ + +	o
Acid phosphatase	– – –	+,o	o,–	o	o
Alkaline phosphatase	–	+,o	o,–	o,–	o,–

+, increase; o, no change; –, decrease.
[a]Phosphatase treatments were done in neurons that had been exposed for 24 h to culture medium in which 90% of the sodium was replaced with *N*-methyl-D-glucamine to elevate intracellular calcium levels. At least 100 neurons were examined for each antibody and experimental treatment. *See* Methods and Results for details.

and phosphatase-treated cultures (Table 1). Taken together, these observations indicated that elevated intracellular calcium levels in human neurons can result in excess phosphorylation of tau and neuronal degeneration.

Effects of Microtubule Disruption on Neuronal Ultrastructure and Tau Immunoreactivity

Depolymerization of microtubules may be an early event in the process of neurofibrillary tangle formation (*see* Metuzals et al., 1988, and data above). It was therefore of interest to determine whether microtubule depolymerization alone (i.e., in the absence of calcium influx) might provoke some of the other alterations in the cytoskeleton that were observed to result from increased intracellular calcium levels. To this end, cultures were exposed to the microtubule-disrupting agent colchicine (10 μg/mL) for several hours and were then processed for either transmission electron microscopy or immunocytochemical staining with tau antibodies. Ultrastructural examination of colchicine-treated neurons revealed a complete absence of microtubules and the presence of bundles of straight filaments throughout the cytoplasm in the soma and axons (Fig. 8). The straight filaments had somewhat variable diameters ranging from approx. 8 to 15 nm (18 of 18 neurons examined). Colchicine also caused increased neuronal immunoreactivity toward 5E2 (Fig. 9A) and Alz-50 (not shown). The effects of colchicine on neuronal ultrastructure and immunoreactivity toward tau antibodies did not involve alterations in intracellular calcium levels, since colchicine did not affect intracellular calcium levels in the cultured neurons. Intracellular calcium levels in neurons treated for 2 h with 10 μg/mL colchicine were 78 ± 2.9 n*M*. In addition, colchicine caused increased neuronal immunoreactivity toward Alz-50 and 5E2 in neurons incubated in medium lacking calcium and containing 1 m*M* EGTA (a condition that prevents calcium influx; data

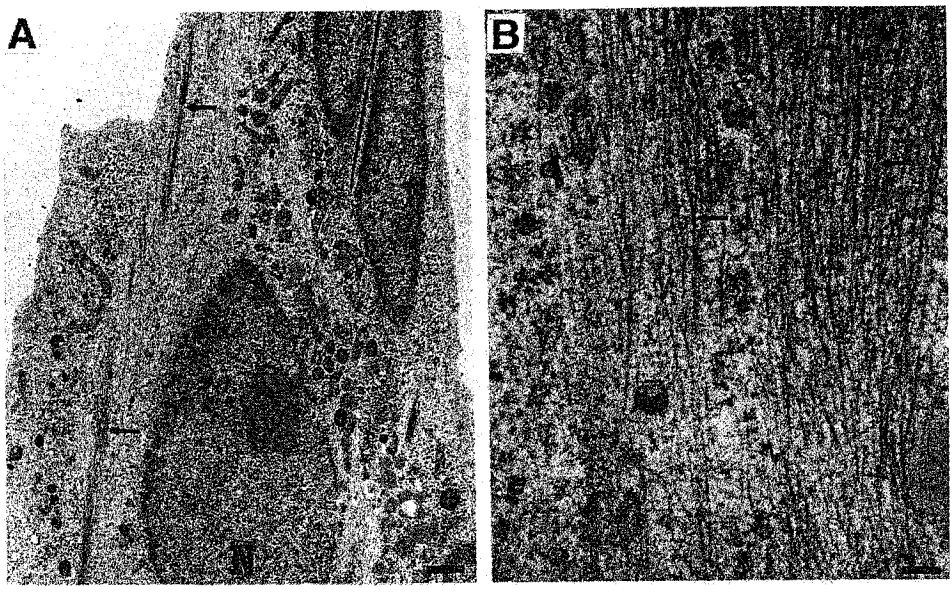

Fig. 8. Ultrastructural alterations resulting from selective microtubule disruption in cultured human cerebral cortical neurons. Transmission electron micrographs of a neuron that had been exposed to 10 μg/mL colchicine for 2 h. (A) Low-power micrograph shows a lack of microtubules and the accumulation of bundles of straight filaments (arrows). N, nucleus. Scale bar, 800 nm. (B) Higher magnification micrograph showing accumulated straight filaments (arrows). Scale bar, 100 nm.

not shown). The antigenic changes induced by colchicine were specific for microtubule disruption, since they were not observed in neurons exposed to the microfilament-disrupting agent cytochalasin D (Fig. 9B). Furthermore, microfilament disruption did not prevent altered tau immunoreactivity associated with calcium influx, since the antigenic changes were seen in neurons exposed to both cytochalasin D and A23187 (Fig. 9C). Apparently, disruption of microtubules in human cortical neurons is a sufficient stimulus to induce an accumulation of straight filaments and antigenic changes in tau similar to those seen in neurofibrillary tangles.

DISCUSSION

The present findings indicate that a loss of calcium homeostasis can result in neuronal degeneration in cultured human cortical neurons that shares several features with pathological neuronal degeneration *in situ*. We found that elevated intracellular calcium levels caused the following alterations in cultured human cortical neurons: A loss of microtubules and a decrease in tubulin levels; an accumulation of 8–15 nm straight filaments; granulovacuolar degeneration; altered electrophoretic mobility

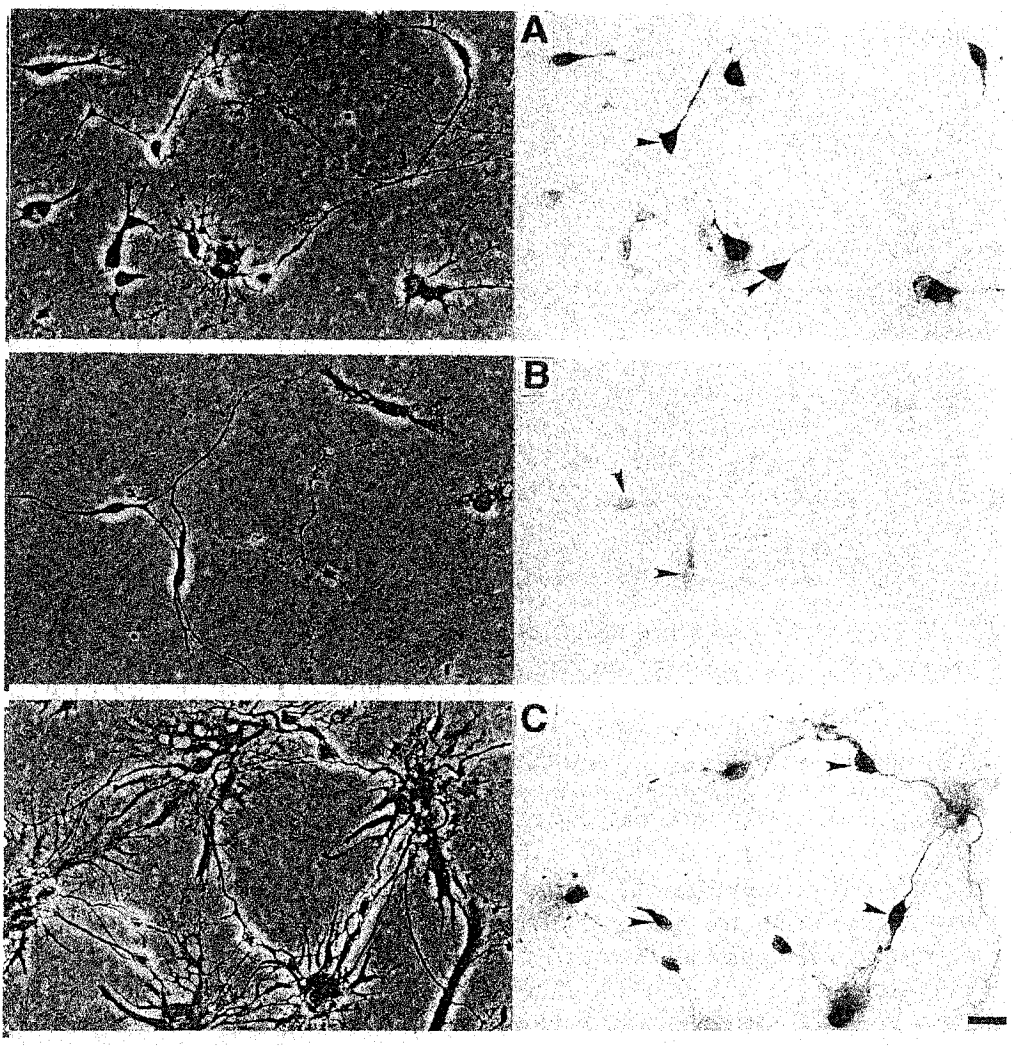

Fig. 9. Microtubule disruption in cultured human cortical neurons elicits an increase in immunoreactivity toward tau antibody 5E2. Phase-contrast (left) and bright-field (right) micrographs of cultured human cortical cells following immunocytochemical staining with antibody 5E2. (**A**) At 2 h following exposure to 10 μg/mL colchicine. (**B**) At 2 h following exposure to 1 μg/mL cytochalasin D. (**C**) At 2 h following exposure to both 1 μg/mL cytochalasin D and 2 μ*M* A23187. Scale bar, 20 μm.

of tau; antigenic changes in tau and ubiquitin. These alterations have been noted in a number of disorders, including Alzheimer's disease (Selkoe, 1989), Down syndrome and subacute sclerosing panencephalitis (Wisniewski et al., 1979), Pick's disease (Murayama et al., 1990), and anoxia/stroke (Kim, 1971; Kato et al., 1988) Taken together, these data indicate that many of the cytoskeletal alterations that are observed in neurodegenerative disorders are also observed in neurons degenerating

as the result of experimentally-induced elevation of the intracellular calcium level. Indeed, even nonneural cells exposed to adverse conditions exhibit an accumulation of straight filaments and altered immunoreactivity toward tau and ubiquitin antibodies (Blass et al., 1990; Ko et al., 1990).

Paired helical filaments are a prominent cytoskeletal aberration in Alzheimer's disease and related disorders (Terry, 1963; Selkoe, 1989) that were not observed in the human cortical neurons in the present study. This suggests that either aberrant calcium homeostasis is not sufficient to induce these structures or, alternatively, the status of the neurons or the experimental conditions used in the present study were not permissive for paired helical filament formation. For example, it is possible that paired helical filament formation is specific for mature neurons. The fetal neurons examined in the present study express the low molmass (68 kDa) neurofilament protein but not the higher molmass forms (Mattson and Rychlik, 1990; M. P. Mattson, unpublished). Such differences in the complement of cytoskeletal proteins present might play a role in the specific abnormal structures that form in response to altered calcium homeostasis. On the other hand, it might be the case that paired helical filaments would form in the cultured human fetal neurons under appropriate conditions of altered calcium homeostasis. Perhaps the short time-course of degeneration examined here (hours to days) was not sufficient for paired helical filament formation. In support of this possibility, De-Boni and Crapper-McLachlan (1985) reported the presence of paired helical filaments in cultured human spinal neurons exposed to glutamate for a period of several weeks. Since the degenerative actions of glutamate are calcium-dependent (Mattson et al., 1988; Mattson, 1990; Mattson et al., 1991a), it is likely that calcium played a role in paired helical filament formation in the human neurons exposed to glutamate. Other evidence also indicates that paired helical filaments form in the later stages of the process of neurofibrillary degeneration. For example, electron microscope studies of brain biopsy specimens from early and advanced Alzheimer's patients indicate that early events in the progression of cytoskeletal changes include a loss of microtubules and the accumulation of straight filaments, and that the formation of paired helical filaments occurs very late in the process (Metuzals et al., 1988). It should be noted in this regard that although paired helical filaments are a prominent feature of the end stage of Alzheimer's disease, the accumulation of 8–15 nm straight filaments is perhaps a more pervasive cytoskeletal alteration that may precede and accompany paired helical filament (Perry et al., 1987a).

It is not clear what proteins comprise the experimentally-induced straight filaments observed in the present study. However, the rapidity of their formation (within several hours) suggests that the proteins are most likely already present within the neurons at the time of calcium elevation. Western blot analysis of cells exposed to calcium ionophore for 4

h indicated that levels of tau and tubulin were actually reduced. Furthermore, we previously found that neurofibrillary-like antigenic changes in cultured rat hippocampal neurons exposed to glutamate or calcium ionophore were not prevented by the protein synthesis inhibitor cycloheximide (Mattson, 1990b). We are currently examining the ultrastructural localization of tau and neurofilament proteins in order to determine whether they are components of the straight filaments that are induced by calcium influx.

We found that overactivation of the calcium signaling system can lead to neuronal degeneration that displays many of the features characteristic of pathological neurodegeneration *in situ*. Associated with elevated intracellular calcium levels was an apparent overphosphorylation of tau. Thus, in neurons exposed to conditions that elevated intracellular calcium, we observed: Decreased neuronal immunoreactivity toward tau-1, an antibody that recognizes dephosphorylated tau (Wood et al., 1986); increased immunoreactivity toward Alz-50, an antibody that apparently recognizes a phosphorylated epitope of tau (Ueda et al., 1990); a reduction in Alz-50 staining and an increase in tau-1 staining in phosphatase-treated cells; a shift in electrophoretic mobility of tau taken from cells exposed to calcium ionophore (cf Baudier and Cole, 1987). In addition, we found that phospatase treatment had little effect on neuronal immunoreactivity toward tau antibody 5E2, which apparently recognizes a phosphate-independent epitope (Kosik et al., 1988). The kinases involved in the cytoskeletal alterations induced by calcium are not known but several have been implicated (Kennedy, 1989; Kishimoto et al., 1989; Saito and Iimoto, 1989). Cell-free studies of tau phosphorylation have shown that calcium/calmodulin-dependent kinases can phosphorylate tau in a way that causes a shift in electrophoretic mobility similar to that seen in Alzheimer's disease (Baudier and Cole, 1987). Interestingly, calcium/calmodulin-dependent protein kinase II is concentrated at high levels in hippocampal neurons predisposed to neurofibrillary tangle formation, suggesting a possible role for this kinase in Alzheimer's disease neuropathology (McKee et al., 1990). On the other hand, the phosphorylation site on tau within the region recognized by Alz-50 may not be phosphorylated by calcium/calmodulin-dependent protein kinase II (Ueda et al., 1990). Protein kinase C is another kinase implicated in the neuronal degeneration that occurs in neurodegenerative disorders (Saito and Iimoto, 1989). This kinase can phosphorylate tau (Hoshi et al., 1987) and we recently provided evidence that overactivation of protein kinase C can cause antigenic changes in tau in cultured human cortical neurons similar to those seen in neurofibrillary tangles (Mattson, 1991). Another kinase that has been implicated in neurofibrillary degeneration is casein kinase II (Iimoto et al., 1989). Taken together, these data indicate that different kinases may be involved in the overphosphorylation of tau that appears to be a common feature of degenerating neurons.

Although the accumulating data strongly suggest that cytoskeletal proteins in neurodegenerative disorders are abnormally phosphorylated, the extent to which the phosphorylation is involved in the cytoskeletal alterations and/or neuronal death are not clear. Is altered phosphorylation necessary for the changes in tau immunoreactivity in degenerating neurons? It is known that phosphorylation of tau reduces its ability to promote microtubule polymerization, resulting in a destabilization of microtubules (Lindwall and Cole, 1984). This property of tau is consistent with a sequence of events in which elevated intracellular calcium levels activate a protein kinase(s), which results in tau phosphorylation and microtubule depolymerization. Tau might then either self-aggregate or associate with other cytoskeletal proteins, resulting in the formation of straight filaments and paired helical filaments. On the other hand, it is also possible that the depolymerization of microtubules induced by calcium is not mediated by tau phosphorylation, since calcium can have direct depolymerizing actions on microtubules (Weisenberg, 1972). In the present study, we found that colchicine caused increased neuronal staining with tau antibodies Alz-50 and 5E2, and an accumulation of straight filaments. These effects of colchicine were independent of calcium influx and therefore suggest that depolymerization of microtubules may be a sufficient stimulus (in the absence of aberrant phosphorylation) to cause alterations in the disposition and antigenic properties of tau. However, since we did not directly examine the state of tau phosphorylation in colchicine-treated cells, we cannot rule out the possibility that tau phosphorylation did occur in response to colchicine. In recent studies, we have found that the microtubule-stabilizing agent taxol can prevent the increased Alz-50 and 5E2 immunoreactivities in cultured rat hippocampal neurons exposed to glutamate or calcium ionophore (M.P. Mattson, unpublished data). Thus, stabilization of microtubules under conditions expected to result in phosphorylation of tau (i.e., calcium influx) prevents at least some of the antigenic changes in tau that would otherwise occur as the result of calcium influx. We therefore propose that epitopes of tau that are not normally exposed in the presence of microtubules may become exposed and available to the 5E2 and Alz-50 antibodies when microtubules depolymerize. In addition, microtubule depolymerization seems to be a trigger for the accumulation of intermediate-size filaments in both neural and nonneural animal cells (Seil, 1968; Wisniewski and Terry, 1967). Microtubule depolymerization may therefore be a very important early event in the process of neurofibrillary tangle formation, as has been suggested in previous studies of Alzheimer's disease (Metuzals et al., 1988).

In addition to the alterations in tau induced by calcium influx, we observed increased immunoreactivity toward ubiquitin antibodies in neurons exposed to calcium ionophore or reduced Na^+ culture medium. Previous work demonstrated that ubiquitin associates with neurofibrill-

ary tangles (Mori et al., 1987; Perry et al., 1987b). It is not yet clear whether ubiquitination precedes or follows the phosphorylation and altered disposition of tau. In cultured human cortical neurons, we find that increased immunoreactivity toward Alz-50 and 5E2 occurs very rapidly following exposure to calcium ionophore (within 10–30 min; Mattson, 1990b). Increased ubiquitin immunoreactivity occurs more slowly following exposure to A23187 in cell culture and staining intensity progressively increases over a period of several hours (M.P. Mattson, unpublished data). Whether the increased ibiquitin immunoreactivity represents increased expression of the protein, altered immunoreactivity of extant protein, or both remains to be determined. In any case, the available data suggest that phosphorylation and altered disposition of tau may largely precede ubiquitination of the cytoskeletal proteins as neurofibrillary changes progress.

It is becoming increasingly clear that Alzheimer's disease is a disorder of heterogenous causes that may include both genetic and environmental components (St George-Hyslop et al., 1990). The calcium hypothesis is therefore particularly attractive since there are a number of potential sites where alterations would lead to excess intracellular calcium levels, aberrant phosphorylation, and cell death (e.g., glutamate levels or receptors; growth factor levels or receptors; and neuronal systems for calcium homeostasis). Systems for calcium homeostasis that are likely to influence neuronal cytoarchitecture and cell survival include plasma membrane calcium channels and extrusion systems, calcium binding proteins, and organellar sequestration/release sites (Carafoli, 1987). For example, the presence of high levels of a 28-kDa calcium-binding protein in hippocampal neurons apparently protects them against calcium influx and excitotoxicity (Mattson et al., 1991b). In any case, the results of the present study are consistent with the possible involvement of altered calcium homeostasis in pathological neurodegeneration and suggest potential regulatory targets (e.g., glutamate and growth factor receptors, systems for calcium homeostasis) for preventing or reducing pathological neuronal degeneration.

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