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tau protein kinase I is essential for amyloid β-protein-induced neurotoxicity

(programmed cell death/Alzheimer disease)

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ABSTRACT **Pathological changes of Alzheimer disease are characterized by cerebral cortical atrophy as a result of degeneration and loss of neurons. Typical histological lesions include numerous senile plaques composed of deposits of amyloid β-protein and neurofibrillary tangles consisting predominantly of ubiquitin and highly phosphorylated tau proteins. Previously, tau protein kinase I (TPK I) was purified and its cDNA was cloned. To examine the biological role of this enzyme in neurons, we have studied the induction of its kinase activity in primary cultures of embryonic rat hippocampal neurons. Treatment of cultures with amyloid β-protein significantly increased TPK I activity and induced the appearance of tau proteins recognized by the Alz-50 monoclonal antibody. In addition, though amyloid β-protein was neurotoxic, either cycloheximide or actinomycin D prevented neuronal death. Death was also prevented by TPK I antisense oligonucleotides but not by sense oligonucleotides. These observations suggest that rat hippocampal neurons undergo programmed cell death in response to amyloid β-protein and that TPK I is a key enzyme in this process.**

The histopathological lesions of Alzheimer disease (AD) are well known yet poorly understood. At autopsy, AD brains typically show diffuse cerebral cortical atrophy with varying distributions that often include the hippocampus, reflecting widespread granulovacular degeneration and loss of neurons. Microscopically, amyloid deposits are found in large numbers of senile plaques scattered throughout the extracellular matrix as well as in the walls of the cerebral blood vessels. Many neurons also contain intracytoplasmic neurofibrillary tangles (NFT) (1). Amyloid β-protein (Aβ), a polypeptide derived by proteolytic cleavage of amyloid precursor protein, is a major component of senile plaques (2). Ubiquitin (3, 4) and highly phosphorylated tau proteins, members of a family of microtubule-associated phosphoproteins, predominantly compose NFT. The tau proteins form paired helical filaments (PHF), which are found in NFT and degenerative neurites of senile plaques (5–7). The molecular mechanisms by which these lesions arise are unknown. They are thought to result from defects in proteolytic processing of the corresponding precursor polypeptides, but whether they are the primary causes of neurodegeneration or merely remnants of this process remains obscure.

Genetic studies of familial AD have demonstrated a variety of mutations in the gene coding for the amyloid precursor protein (8, 9). Middle-aged patients with Down syndrome (trisomy 21), in whom the amyloid precursor protein is overexpressed due to a gene dosage effect, also exhibit neuropathologic changes characteristic of AD (10). One longstanding hypothesis is that aberrant accumulation of Aβ occurs in the AD brain and is associated with NFT formation and neuronal death (1, 2, 11, 12). The specific details of this process are lacking, however, and whether neurons die by passive necrosis or by programmed cell death requiring protein synthesis is a matter of speculation. It is a reasonable assumption that the ability of NFT-containing neurons to function normally is severely damaged. Accordingly, the appearance of NFT may be an important indicator of the extent of brain damage. Despite intensive efforts, however, the sequence of events leading to the formation of NFT is unknown in AD, and for ethical reasons *in vitro* model systems are needed for analysis of these events at the molecular level.

In search of such a system, work in this laboratory initially focused on a protein kinase that phosphorylated tau proteins, converting them to PHF *in vitro*. When purified and characterized, this enzyme was named tau protein kinase I (TPK I), and it was found to modify normal tau proteins to a highly phosphorylated form bearing an epitope of PHF and exhibiting a large shift in electrophoretic mobility (13–16). In this study, we examined the role of TPK I in Aβ-induced neurotoxicity by using primary cultures of neurons from embryonic rat hippocampus. Treatment with Aβ caused an increase in the activity of TPK I that correlated with both neurotoxicity and the appearance of the epitope for the Alz-50 monoclonal antibody, characteristic of NFT. Neurons were protected from the toxic effects of Aβ by treatment of cultures with TPK I antisense oligonucleotides, and inhibitors of protein and RNA synthesis were also effective in preventing cell death. The results suggest that rat hippocampal neurons succumb to programmed cell death triggered by Aβ and that TPK I participates in this process by contributing to the formation of NFT. This system may offer an approach for studying the pathogenesis of AD.

MATERIALS AND METHODS

Cell Culture. Primary cultures of embryonic rat hippocampus were established by a modification of the protocol of Banker and Cowan (17). Briefly, the hippocampus was dissected from randomly bred Wistar rat embryos at 18 days post coitum. The tissue was digested with papain (10 units/ml) for 20 min at 37°C and was dissociated by gentle pipetting. Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (heat-inactivated, HyClone), 5% horse serum (heat-inactivated, Whittaker Bioproducts), insulin (10 μg/ml), transferrin (0.1 mg/ml), aprotinin (1 μg/ml), sodium pyruvate (1 mM), and gentamicin (84 μg/ml). Then they were seeded at a density of 2×10^5 cells per cm^2 into poly(L-lysine)-coated tissue culture wells. On day 3 of culture, cells were treated with 1 μM 1-β-D-arabinofuranosylcytosine for 24 hr to reduce the num-

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Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer disease; LDH, lactate dehydrogenase; NFT, neurofibrillary tangle(s); PHF, paired helical filament(s); TPK I, tau protein kinase I.

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ber of proliferating nonneuronal cells. On day 5, cultured hippocampal cells were used for various experiments. When the protein-synthesis inhibitor cycloheximide (0.1 μg/ml) or the RNA-synthesis inhibitor actinomycin D (0.01 μg/ml) were used, cultures were pretreated with either reagent for 5 min at 37°C before addition of Aβ.

Peptides and Oligonucleotides. TPK I sense and antisense oligonucleotides were designed as 18-mers corresponding to the cloned rat TPK I sense and antisense sequences flanking the translation initiation region as follows: TPK I sense, 5′-ATGTCGGGGCGACCGAGA-3′; TPK I antisense, 5′-TCTCGGTCGCCCCGACAT-3′. The oligonucleotides were synthesized by an automated DNA synthesizer (MilliGen, Bedford, MA). Oligonucleotides recovered from a 20% acrylamide/urea gel were purified by ethanol precipitation and were suspended in water. The concentration of oligonucleotide was adjusted to 1 mM, and 1 μl of the solution was added to 1 ml of medium 5 min before addition of Aβ to each culture. Control cultures were unaffected by this concentration of either oligonucleotide, but at concentrations > 5 μM toxic effects were observed. Human Aβ peptides (aa 1–43 and 25–35) were synthesized and purified as described (18, 19). Aβ-(1–43)-peptide was dissolved in 35% acetonitrile to make a 1 mM stock solution, and Aβ (25–35)-peptide was dissolved in water to make a 2 mM stock solution. Both peptides showed neurotoxicity consistent with previous reports (18, 19).

Determination of Neurotoxicity. Normal "healthy" neurons were counted by phase-contrast microscopy as an index of neuron survival after various treatments. A healthy neuron was defined morphologically as having a cell body with a smooth contour, round to oval shape, and multiple neuritic processes. A degenerating neuron was identified by an irregularly shaped cell body, fragmented neurites, cell lysis, or detachment from the substrate. Cell counts of surviving neurons were performed in triplicate per well. More than 400 cells per well were counted in control cultures. Morphological identification of neurons was confirmed by immunocytochemical staining with a neuron-specific antibody, anti-microtubule-associated protein 2. The protective effect of TPK I antisense oligonucleotide against Aβ-induced cell death was biochemically confirmed by assay of lactate dehydrogenase activity released into the medium (20). Medium supplemented with 0.25% bovine serum albumin was used as non-Aβ control. Experiments were performed at least three times on three independent cultures. The data presented are from one of those experiments.

tau Phosphorylation Assay. After various treatments, cells were washed three times with ice-cold phosphate-buffered saline and harvested from the culture plate with a rubber policeman. Cells were suspended and homogenized in buffer A (20 mM) (4-morpholine ethanesulfonate/0.5 mM magnesium acetate/1 mM EGTA, pH 6.8) containing phosphatase inhibitor (1 μM okadaic acid; Seikagaku Kogyo, Tokyo) and protease inhibitors (phenylmethylsulfonyl fluoride at 1 mM and leupeptin, pepstatin, and aprotinin each at 1 μg/ml). Homogenates were centrifuged at 14,000 × *g* for 1 hr. The supernatant was used for the phosphorylation assay. Rat tau protein expressed in *Escherichia coli* BL21 was purified as described (15). One microliter of cell extract was added to aliquots of a solution of recombinant rat tau (400 μg/ml) in buffer A containing 1 mM [γ-^{32}P]ATP (10–20 Ci/mmol; 1 Ci = 37 GBq) and 10 μM okadaic acid, to a final volume of 10 μl. After incubation at 37°C for 3 hr, the reaction was stopped by adding SDS sample buffer. After SDS/10% PAGE, the ^{32}P incorporated into tau proteins was visualized by laser image analysis (Fuji BAS 2000).

Analysis of TPK I Transcripts by Polymerase Chain Reaction (PCR). TPK I mRNA transcripts in cells treated with either sense or antisense TPK I oligonucleotide were measured by a semiquantitative PCR employing reverse transcription of RNA into cDNA. Total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol/chloroform method (21). Purified RNA (0.2 μg) was reverse transcribed and PCR was performed with a GeneAmp RNA PCR kit (Perkin–Elmer/Cetus) according to the manufacturer's instructions. Primers synthesized as described above were as follows: TPK I forward and backward, 5′-GGGCCAAGAGAACGAAGTCTT-3′ and 5′-GGCGTTTGCAGGCGGTGAAGC-3′, respectively. After addition of [α-^{32}P]dCTP (0.1 μCi/μl) to the reaction mixture, amplification was carried out in an automated thermal cycler (ALTECH) for 30 cycles of 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1.5 min. PCR products were electrophoresed in a 5% polyacrylamide gel, and the gel was dried and visualized with a laser image analyzer (Fuji BAS 2000).

Alz-50 Immunocytochemistry. Hippocampal cell cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min. Fixed cultures were incubated in Tris-buffered saline containing 0.2% Triton X-100 for 30 min to make the cells permeable. Then, they were immunostained with a 1:5 dilution of Alz-50 primary antibody, a Vectastain ABC avidin–biotin–peroxidase detection kit (Vector Laboratories), and diaminobenzidine tetrahydrochloride as the chromogen. The Alz-50 mouse monoclonal antibody, raised against basal forebrain tissue from four cases of AD, was a gift from Peter Davies (22).

RESULTS

To assess whether TPK I was involved in the events leading to neuron death, experiments were conducted with a TPK I antisense oligonucleotide. TPK I cDNA was previously cloned from a rat brain cDNA library (38), and its sequence coincided with the glycogen synthase kinase 3β isoform found in rat brain (23). Based on this sequence, TPK I sense and antisense oligonucleotides were synthesized.

Treatment of rat hippocampal cultures with Aβ (20 μM) or Aβ plus TPK I sense oligonucleotide (1 μM) was neurotoxic at densities between 5 × 10^4 and 2 × 10^5 cells per cm^2 (Fig. 1 *B* and *C*). Neurotoxicity was significantly reduced in the presence of Aβ plus TPK I antisense oligonucleotide (1 μM) (Fig. 1*D*). Cell survival was not affected by addition of 0.25% bovine serum albumin to the medium as a non-Aβ control, and after 24 hr under these conditions, neurons were indis-

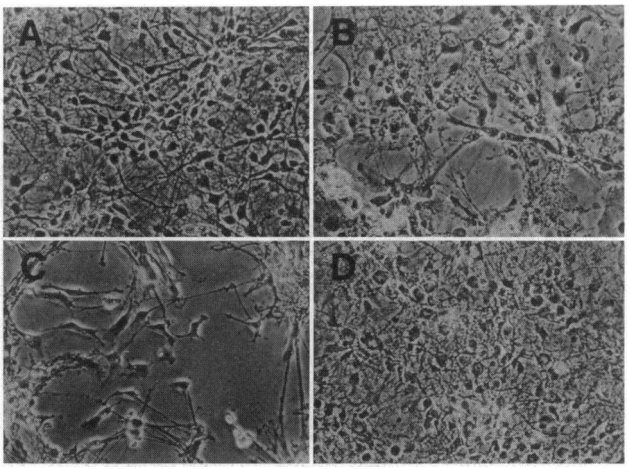

FIG. 1. TPK I antisense oligonucleotide prevents Aβ-induced neurotoxicity in hippocampal cell cultures. Neurotoxicity was recorded 24 hr after various treatments. (*A*) Control. (*B*) Aβ (aa 1–43; 20 μM). (*C*) TPK I sense oligonucleotide (1 μM) added prior to Aβ (20 μM). (*D*) TPK I antisense oligonucleotide (1 μM) added prior to Aβ (20 μM). (Phase-contrast microscopy, ×210.)

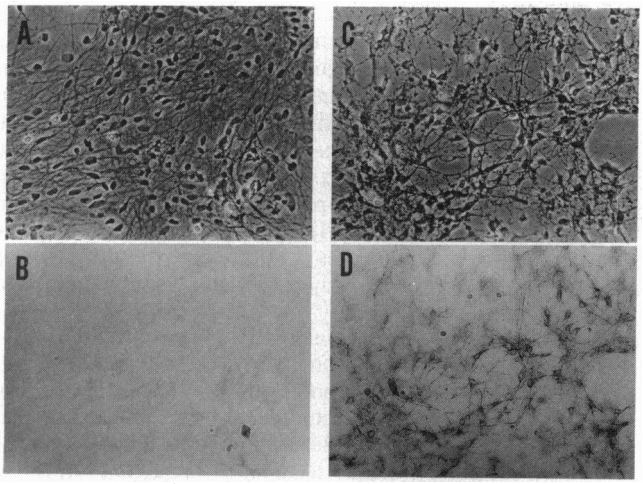

FIG. 2. Immunocytochemical staining of the Alz-50 epitope 24 hr after addition of Aβ. (*A* and *B*) Control culture. (*C* and *D*) Culture treated with Aβ (aa 1–43; 20 μM). Phase-contrast (*A* and *C*) and brightfield (*B* and *D*) micrographs show paraformaldehyde-fixed cells immunostained with Alz-50 as described in *Materials and Methods*. (×100.)

tinguishable from untreated neurons. By contrast, in Aβ-treated cultures the neuron survival was 35% of untreated controls. The preventive effect of TPK I antisense oligonucleotide was reproducible in five independent experiments. In addition, the increase in lactate dehydrogenase activity released into the medium of cultures treated with Aβ plus TPK I antisense oligonucleotide was about 20% of that in cultures exposed to Aβ alone. By comparison, cultures receiving Aβ plus sense oligonucleotide had lactate dehydrogenase levels that were 90% of those in cultures treated with Aβ alone. When cultures were exposed to Aβ alone, neurons showing toxic effects also were immunoreactive with Alz-50 (Fig. 2). In this respect, Aβ-treated rat hippocampal cultures resembled one of the histopathological features of AD (1).

In hippocampal cultures treated with Aβ, the activity catalyzing the phosphorylation of tau proteins was ≈1.6 times that of control cultures (Fig. 3). By contrast, in cultures

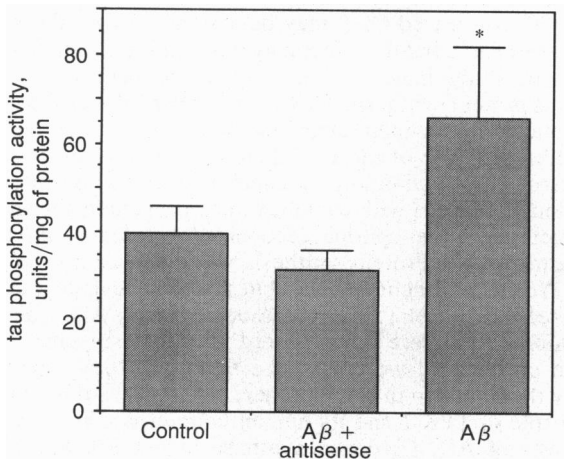

FIG. 3. tau protein phosphorylation activities in hippocampal neuron cultures after 24 hr. Cells were either untreated (control), treated with 20 μM Aβ (aa–43), or treated with Aβ plus 1 μM TPK I antisense oligonucleotide. Activity was normalized to protein in the corresponding supernatant. One unit is equivalent to the intensity of radioactivity measured by fluorometric image analysis (Fuji BAS 2000). Data are expressed as mean ± SD, $n = 3$. *, $P < 0.05$ (two-tailed t test) compared with control. The experiment was performed four times on independent cultures, and similar results were obtained in each case.

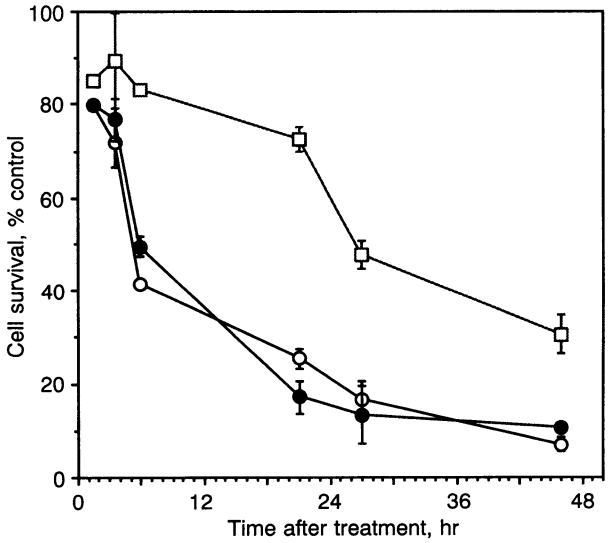

FIG. 4. Time course of Aβ-induced neuron death in cultures treated with Aβ (aa 25–35; 20 μM) (○), Aβ plus 1 μM TPK I sense oligonucleotide (●), or Aβ plus 1 μM TPK I antisense oligonucleotide (□). Numbers of surviving neurons in experimental and control cultures were counted at each time point. Data were combined from triplicate cultures and were plotted as mean ± SD, $n = 3$.

pretreated with TPK I antisense oligonucleotide (1 μM), Aβ did not induce an increase in this kinase activity. These observations suggested that the increase in tau protein phosphorylation induced by Aβ resulted from increased levels of TPK I mRNA and its corresponding protein.

In addition to blocking the increase in kinase activity, introduction of TPK I antisense oligonucleotide (1 μM) into cultures shortly before addition of Aβ also protected the neurons from its neurotoxic effects (Figs. 1, 4, and 5). This was not observed when the antisense oligonucleotide was present at <0.1 μM. Surviving neurons were counted at each time point after the addition of Aβ (20 μM) to cultures containing either antisense or sense oligonucleotide (1 μM) (Fig. 4). Both Aβ alone and Aβ plus TPK I sense oligonucleotide significantly decreased the number of surviving cells between 6 and 48 hr of culture. After 21 hr, the number of

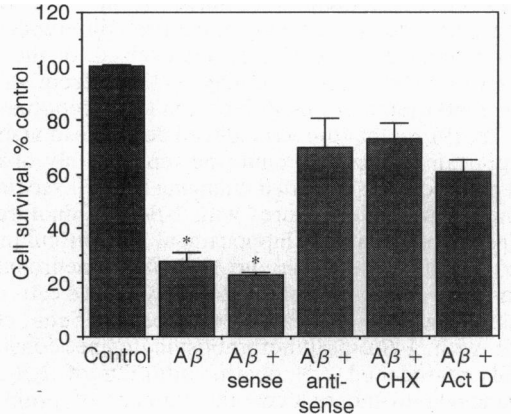

FIG. 5. Neurotoxic effects of various treatments on hippocampal cultures. Cultures were untreated (control) or were treated with Aβ (aa 1–43; 20 μM) alone or in the presence of 1 μM TPK I sense oligonucleotide, 1 μM TPK I antisense oligonucleotide, cycloheximide (CHX, 0.1 μg/ml), or actinomycin D (Act D, 0.01 μg/ml). After 24 hr, surviving neurons in each culture were counted. In each experiment, data were combined from triplicate cultures and were plotted as mean ± SD, $n = 3$. *, $P < 0.05$ (two-tailed t test) compared with control. Experiments were performed twice on independent cultures and showed similar results.

surviving cells was 20–30% that of control for both Aβ treatment alone and Aβ plus TPK I sense oligonucleotide. By contrast, cultures with Aβ plus TPK I antisense oligonucleotide had a level of surviving cells between 70% and 90% of control until 21 hr of culture. During the subsequent 25 hr, the number of surviving cells declined to 30% of control, probably as a result of metabolic degradation of the antisense oligonucleotide. To confirm the protective effect of TPK I antisense oligonucleotide, transcription of TPK I mRNA was quantified by the PCR method. In Aβ (20 μM)-treated cultures, the PCR-amplified cDNA product showed a 20–30% increase over control. Cultures containing Aβ (20 μM) plus TPK I antisense oligonucleotide (1 μM) had levels that were only 49% of those in cultures treated with Aβ (20 μM) alone. TPK I sense oligonucleotide was ineffective in reducing the level of these PCR products. Thus, Aβ-induced expression of TPK I mRNA appeared to correlate with cell death in this system.

Neuron death was also prevented by inhibitors of protein and RNA synthesis (24) (Fig. 5). In preliminary experiments, cycloheximide at >0.1 μg/ml and actinomycin D at >0.01 μg/ml were toxic. At or below these concentrations control cultures were unaffected, and there was no significant difference in cell survival or morphology between treated and untreated cultures. As noted previously, addition of Aβ decreased the number of surviving neurons to ≈30% that of the control culture after 24 hr. In the presence of the protein-synthesis inhibitor cycloheximide (0.1 μg/ml) or the RNA-synthesis inhibitor actinomycin D (0.01 μg/ml), the values were 72.9 ± 5.9% and 60.7 ± 4.6% of the control, respectively. This indicated that protein and RNA synthesis were required for neuron death and further suggested that the process was an active programmed cell death as opposed to passive necrosis.

DISCUSSION

Several studies have reported evidence that Aβ is neurotoxic in cultures of rodent brain cells (18, 19, 25–28). In differentiated hippocampal cell cultures, Aβ peptides (aa 1–42 and 25–35) were toxic. However, it has also been reported that Aβ was not toxic in cortical neuron cultures from mice (29) and humans (30). In the latter instance, although Aβ was not itself neurotoxic, it did make the neurons more vulnerable to excitotoxicity. Apparent contradictions in these findings from various investigators may result from differences in the species examined, the brain regions excised, or the culture conditions employed. Accordingly, in the present study the culture conditions were modified from those reported previously (18, 19), so that the Aβ-induced decrease in viability of the hippocampal neurons could be reproducibly observed. These modifications included changing the cell seeding density and treatment of cultures with 1-β-D-arabinofuranosylcytosine. Embryonic rat hippocampal neuron cultures are primary cultures containing up to 20% nonneuronal cells, such as glial cells, that are mitotically active. At seeding densities between 5×10^4 and 2×10^5 cells per cm^2, cultures treated with 1-β-D-arabinofuranosylcytosine consistently showed Aβ-induced cell death. Addition of 1-β-D-arabinofuranosylcytosine reduced the number of proliferating nonneuronal cells and presumably eliminated their protective effects on neurons.

The molecular mechanisms by which Aβ induces neuronal death require clarification. It is known that PHF form insoluble deposits within the perikaryons of degenerating neurons in the AD brain, and it is thought that progressive accumulation of these deposits may eventually cause cell death. This is the so-called amyloid hypothesis. If so, the problem is how to explain the production of PHF, which are composed of highly phosphorylated tau proteins as well as ubiquitin. A specific PHF kinase designated TPK I has been identified and found to be responsible for phosphorylation of tau proteins (13–16). Consistent with the amyloid hypothesis, the present results show that Aβ treatment is associated with an increased level of kinase activity in rat hippocampal cell cultures. Aβ-treated neurons exhibiting toxic effects were immunoreactive with the Alz-50 monoclonal antibody (25), an antibody that recognizes phosphorylated tau proteins in PHF from brains with AD (31). Thus, the evidence suggests that rat hippocampal cultures may offer a useful model for studying by analogy the early molecular events leading to neurodegeneration in AD.

In the course of these investigations, two additional observations appeared significant. First, TPK I antisense oligonucleotide protected neurons from cell death, presumably by blocking the Aβ-induced increase in TPK I mRNA and kinase activity. Second, inhibitors of protein and RNA synthesis showed effects similar to those of the TPK I antisense oligonucleotide. Accordingly, it is possible that Aβ induces an increase in TPK I, and the increase in kinase activity results in the formation of an epitope of PHF recognized by Alz-50, leading to neuronal death. Although this seems to be the simplest interpretation of the present findings, it cannot be excluded that another substrate of TPK I is responsible for neuron death in the hippocampal cultures. Whether these ideas also apply to the pathogenesis of AD remains to be determined.

The precise way in which Aβ induces the synthesis of TPK I needs to be examined. Native Aβ, which aggregates to form the core of senile plaques, has been shown to be neurotoxic (32), and synthetic Aβ also requires aggregation and deposition for neurotoxicity (33, 34). These polypeptides may directly interact with components of the plasma membrane and stimulate the formation of intracytoplasmic second messengers. Aβ has a high amino acid sequence homology with members of the tachykinin neuropeptide family, and its neurotoxicity is prevented by substance P (18, 19), yet ligand–receptor studies show that Aβ does not bind to the substance P receptor.

Recently it has been reported that Aβ forms ion channels in bilayer membranes (35) and that it may cause an increase in intraneuronal Ca^{2+} levels (1). This may account for some of its neurotoxicity or make neurons sensitive to other toxins (29, 30). Increased Ca^{2+} may be associated with the pathogenesis of AD, but the effect may not be direct. Data from the present study indicate that Aβ-induced neurotoxicity requires protein synthesis. However, Ca^{2+} mediates the action of many intracellular signaling pathways in the nervous system, and one or more of them may be related to TPK I expression or activation. In another investigation, preincubation of neurons with cycloheximide prevented subsequent induction of the epitope recognized by Alz-50 after Aβ treatment (36). Protein synthesis was required not only for the Aβ-induced neurotoxicity but also for the appearance of altered cytoskeletal proteins associated with AD. The findings presented here are in accord with these results.

In conclusion, we emphasize that this study represents only the first step in a comprehensive analysis of the biological role of TPK I and its human counterpart in the pathogenesis of AD. Glycogen synthase kinase 3β, the TPK I homolog in rat brain, has been demonstrated immunohistochemically in cortical pyramidal neurons of both normal and AD tissues (37), but its substrates and reaction products need further biochemical characterization. The complex interplay of factors that must be sorted and defined is daunting. Nevertheless, if the amyloid hypothesis accounts for AD, it appears likely that TPK I is a key enzyme in this process.

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