Inhibition of tau phosphorylating protein kinase cdk5 prevents β-amyloid-induced neuronal death

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Abstract The key target of this study was the tau protein kinase II system (TPK II) involving the catalytic subunit cdk5 and the regulatory component p35. TPK II is one of the tau phosphorylating systems in neuronal cells, thus regulating its functions in the cytoskeletal dynamics and the extension of neuronal processes. This research led to demonstration that the treatment of rat hippocampal cells in culture with fibrillary β-amyloid (Aβ) results in a significant increase of the cdk5 enzymatic activity. Interestingly, the data also showed that the neurotoxic effect of 1–20 μM Aβ on primary cultures markedly diminished with co-incubation of hippocampal cells with the amyloid fibers plus the cdk5 inhibitor butyrolactone I. This inhibitor protected brain cells against Aβ-induced cell death in a concentration dependent fashion. Moreover, death was also prevented by a cdk5 antisense probe, but not by an oligonucleotide with a random sequence. The cdk5 antisense also reduced neuronal expression of cdk5 compared with the random oligonucleotide. The studies indicate that cdk5 plays a major role in the molecular path leading to the neurodegenerative process triggered by the amyloid fibers in primary cultures of rat hippocampal neurons. These findings are of interest in the context of the pathogenesis of Alzheimer's disease.

Key words: Protein kinase cdk5; Tau phosphorylation; Butyrolactone; Cdk5 antisense; Hippocampal cell; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) corresponds to one of the most common types of senile dementia in adults older than 65 years. This disease is characterized by cognitive disorders associated with a loss of memory and orientation and deterioration of the intellectual capacity of patients. Two major histological lesions are observed in AD brains, associated with a neuronal loss: (i) at the intracellular level, formation of neurofibrillary tangles (NFTs) composed of paired helical filaments (PHFs), composed of hyperphosphorylated tau [1,2], and (ii) at the extracellular level by senile plaques formed by the fibrillary β-amyloid (Aβ) [3].

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Abbreviations: Aβ, amyloid peptide; AD, Alzheimer's disease; calcein-AM, calcein acetoxymethyl ester; DMSO, dimethylsulfoxide; cdk5, cyclin dependent protein kinase 5; MT, microtubule; PHF, paired helical filament; PI, propidium iodide; NFT, neurofibrillary tangle

NFTs are mainly deposited in cellular bodies of neurons, apical dendrites, even though lesions are also found in filaments of the neuropil and distrophic neurites around the core of amyloid plaques [1]. PHFs consist of two coiled filaments resulting from self-polymerization of tau subunits arranged in a helical supramolecular structure of 10–20 nm in diameter [4]. Tau, one of the most relevant MAPs, corresponds to a set of six major isoforms containing around 352–441 amino acids, products of the alternative splicing of a single gene [5]. These isoforms differ from each other for the presence of either three or four microtubule (MT) binding motifs [6] and a variable number of 29 amino acid inserts towards the N-terminal moiety [7,8]. Tau co-localizes with MTs and its major cell role is modulating MT assembly and their stabilization in neurons [9], although a role for tau in directing neuritogenesis has also been reported [10]. Tau of the Alzheimer's type contains 3–4 repeats, shows three major bands in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system and is highly insoluble [11,12].

Tau participates in two apparently different functions: to induce MT assembly under normal conditions and to self-associate under pathological conditions like those of AD and other dementia. In this context, tau hyperphosphorylations determined by the effects of different protein kinases and phosphatases causes conformational changes, thus affecting its assembling capacity and interactions with tubulin [13,14]. Several phosphorylation sites have been identified on the tau molecule, including a site on the first tau repeat [15]. Anomalous phosphorylations on tau appear to be involved in its abnormal self-aggregation into PHFs, leading to a loss of the normal neuronal functions [16].

Among kinases involved in tau modifications leading to PHFs, two are the most relevant: tau protein kinase I (TPK I) also named glycogen synthase kinase 3β and tau kinase II (TPK II) [17,18]. TPK II is a complex of two subunits: a catalytic component of 33 kDa (cdk5) and the regulatory subunit of 23 kDa (p35) derived from proteolytic cleavage of a 34 kDa precursor [19]. The neuron-specific activator p35 is involved in axonal growth [20]. TPK II is a proline-directed Ser/Thr kinase that contributes to the phosphorylation of human tau on Ser^{202}, Thr^{205}, Ser^{235} and Ser^{404} [18]. The abnormally phosphorylated tau contains PHF epitopes. TPK I phosphorylates tau at four distinct sites: Ser^{199}, Thr^{231}, Ser^{396} and Ser^{413} [21]. These sites correspond to those phosphorylated in the PHF-tau purified from the AD brains [22]. The initial phosphorylation of tau with TPK II appears to stimulate tau modifications by the TPK I/GSK3β kinase system [23,24]. The results showed that Aβ induces an increase in protein kinase activity of cdk5 on hippocampal cells, a phenomenon associated with neuronal death. It was noteworthy

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to observe that butyrolactone I, a cdk5 inhibitor [25], prevented neuronal death caused by the fibrillary Aβ. Neurons were protected from the toxic effects of Aβ by treatment of cultures with cdk5 antisense oligonucleotides. The result suggest that in rat hippocampal neurons, cdk5 participates in the process of cell death triggered by Aβ, which could contribute to the formation of NFT.

2. Materials and methods

2.1. Hippocampal cultures

Primary rat hippocampal cultures were prepared as described [26,27]. Briefly, hippocampi from Sprague-Dawley rats at embryonic day 18 were dissected in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS, Gibco 14180-020) with 10 mM HEPES, pH 7.4, and 0.5% glucose and rinsed twice with HBSS by allowing for the tissue to settle to the bottom of the tube. After the second wash, the tissue was resuspended in HBSS containing 0.25% trypsin (Gibco 25095-019) and incubated for 5 min at 37°C. After three rinses with HBSS, the tissue was mechanically dissociated in minimal essential medium (MEM, Gibco 61100-053; Earle's salt, sodium bicarbonate-buffered, 600 mg/l glucose), supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine (MEM-10), by gentle passage through pasteur pipettes. Undisrupted tissue fragments were allowed to settle, cells in suspension were transferred to a new tube and viable cells were counted using 0.2% trypan blue. Cells were initially plated in MEM-10 media and maintained at 37°C in a humid atmosphere with 5% CO_2/95% air. Three h after plating, media MEM-10 was removed and serum-free medium N_2/MEM added (MEM supplemented with 2 mM L-glutamine, 750 mg/l glucose, 100 μM putrescine, 20 nM progesterone, 30 nM selenium dioxide, 100 μg/ml transferrin, 5 μg/ml insulin, 1 mM sodium pyruvate and 0.1% ovalbumin).

2.2. Cytotoxicity assays

For cytotoxicity assays, the hippocampal cells were seeded in polylysine-coated 96 well plates at 2.0×10^4 cells/100 μl per well and maintained in N_2/MEM without phenol red media. After day 4 of in vitro culture, neurons were treated with the amyloid fibrils with or without butyrolactone I or N_2/MEM media (control) were added to the wells at different concentrations in a final volume of 10 μl. Cells were incubated for 24 h at 37°C, after which the cell viability was measured by the modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [28]. This involves determining mitochondrial dehydrogenase activity in intact cells by incubation for 4 h at 37°C with MTT (10 μl of 5 mg/ml MTT solution per well). The reaction was stopped by addition of cell lysis buffer (50% dimethylformamide and 20% SDS, pH 7.4). ΔA values at 540–650 nm were determined the following day, using an automatic microtiter plate reader (Metertech Σ960) and the results were expressed as a percentage of control.

2.3. Microscopy of fluorescently stained cells

Neurons were plated at 1×10^6 cells/cm^2 on polylysine-coated coverslips. After 4 days in N_2/MEM media, cells were exposed to Aβ fibrils in the presence or the absence of butyrolactone I and incubated for 24 h. Then, the cells were incubated for 30 min with 20 μM propidium iodide (PI) and 150 nM calcein acetoxymethyl ester (calcein-AM) (Molecular Probes, Eugene, OR, USA), rinsed and visualized by fluorescence microscopy (Zeiss Axiovert). Calcein-AM labels viable cells, whereas PI has access only to cells with plasma membrane damage and accumulates in the nucleus.

2.4. Immunoprecipitation and assay of cdk5 activity

Neurons were plated at 1×10^6 cells/cm^2 on polylysine-coated 35 mm dishes. After 4 days in N_2/MEM media, the cells were exposed to 20 μM Aβ fibrils in N_2/MEM for 24 h. Hippocampal cells were lysed in buffer RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin and 1 μg/ml pepstatin). Two-hundred μg of total cellular protein was used for immunoprecipitation with anti-cdk5 antibody (Santa Cruz, CA, USA) at a final dilution of 1:50. For an in vitro kinase assay, the immunoprecipitates were rinsed three times with RIPA buffer and one time with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) plus 1 μM cold ATP. The washed beads were incubated with kinase buffer containing 2.5 μg of histone H1 or 20 μg microtubular protein or 4 μg purified tau [6] plus 5 μCi [^{32}P]ATP in a final volume of 50 μl for 30 min at 24°C. Enzyme assays using the synthetic tau peptide RTPPKS^{235}PSSAKSR as a kinase substrate were also carried out. After incubation, 50 μl of 2×Laemmli sample buffer [29] was added to each sample and they were analyzed by SDS-PAGE and autoradiography [10].

2.5. Preparation of Aβ fibrils

Synthetic peptide $A\beta_{1-40}$, including residues 1–40 of the human Aβ sequence were purchased from Chiron Corporation (Emeryville, CA, USA). The $A\beta_{1-40}$ peptide was incubated in a stirring aggregation assay as described [30]. Stock solutions were prepared by dissolving lyophilized aliquots of Aβ peptide in dimethylsulfoxide (DMSO) at 15 mg/ml (3.5 mM). Aliquots of peptide stock solution (70 nmol in 20 μl DMSO) were added to PBS, pH 7.2, to give a final volume of 0.725 ml. The solution was stirred continuously and aggregation was measured by the turbidity at 400 nm using buffer as a blank. After 6 h incubation under stirring, the solution was incubated for 4 days after which the formed aggregates were analyzed by electron microscopy. The amyloid nature of fibrils was evaluated by thioflavine T using fluorescence microscopy [31]. The Aβ fibrils were concentrated by centrifugation (14 000 rpm for 30 min) and resuspended at 1 mg/ml in N_2/MEM media, the Aβ concentration was evaluated by the BCA protein assay (Pierce) as described by Smith et al. [32]. Butyrolactone I (Calbiochem, La Jolla, CA, USA) prepared in 10 mM DMSO was added with Aβ fibrils at the concentrations indicated and final DMSO remained less than 0.04%.

2.6. Antisense oligonucleotides

Antisense phosphorothioate oligonucleotide (S-modified) RK2 described by Pigino et al. [10] was used in the present study. This oligonucleotide consists of the sequence 5′-GCATCGCAGCGGCCA-3′ and it is the inverse complement of nucleotides +83/+97 of the cDNA for cdk5 and was purchased from Quality Controlled Biochemicals (Hopkinton, MA, USA). The antisense oligonucleotide was pre-incubated with 2 μl of Lipofectin Reagent (1 mg/ml, Gibco BRL, Gaithersburg, MD, USA) diluted in 100 μl of serum-free medium. The resulting oligonucleotide suspension was then added to primary hippocampal cultures that were grown for 4 days. Four pulses of oligonucleotide (1 μM each) were added every 6 h within a period of 24 h. Control cultures were treated with the same concentration of a scrambled oligonucleotide sequence. Simultaneously with the third pulse of the oligonucleotide (after 12 h treatment), Aβ fibrils (1–10 μM) were added to hippocampal cells, after the cell viability was evaluated. The effects of the cdk5 antisense on expression of the enzyme were assessed by Western blot analysis [20] of the immunoreactive cdk5 in both Aβ-treated and non-treated paired samples.

2.7. Statistical analyses

The program Graph Pad Prism was used for all statistical analyses and evaluation of tests of significance during this study.

3. Results

The expression and activity of cdk5 in hippocampal neurons. Rat primary hippocampal cultures have proved to be an appropriate model for studying amyloid toxicity [27,33]. The involvement of cytoskeletal proteins in the morphological changes associated with neuronal death induced by the amyloid fibrils have been investigated in these cells [34,35]. On the other hand, protein kinase TPK II appears to be a regulatory element of the microtubular cytoskeleton, playing a critical role in neurites [10]. We have taken advantage of this model system to obtain information about the relationships between TPK II and the cellular changes in neuronal cells exposed to amyloid. The cdk5 levels in hippocampal cultures were analyzed by using a rabbit polyclonal antibody against cdk5 as shown in Fig. 1A. The antibody recognized a single band of

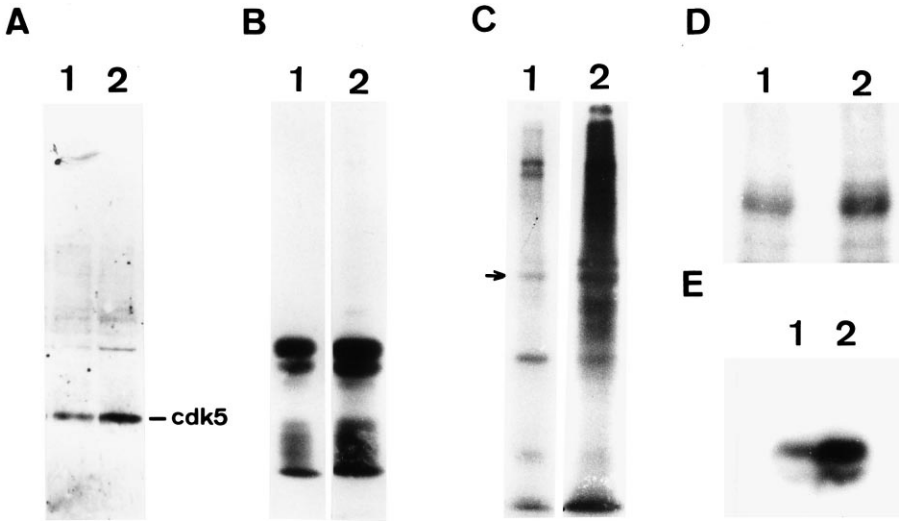

Fig. 1. Effects of Aβ fibrils in the activity and expression of cdk5 in cultured hippocampal neurons. The expression of cdk5 in the cells was revealed by Western blot analysis of cell homogenates reacted with the antibody anti-cdk5 (1:1000). Twenty μg of total protein was loaded in each well. Samples were obtained at 24 h after the treatment (A, lane 1, control cells; lane 2, cells exposed to 15 μM Aβ fibrils). The cdk5 kinase activity in hippocampal neurons exposed to 15 μM Aβ fibrils was analyzed after immunoprecipitation of cell homogenates (200 μg/sample), followed by incubation with [^{32}P]ATP either in the presence of H1 histone as a substrate (B, lane 1 untreated control; lane 2, treated with 15 mM Aβ fibrils) or in the presence of microtubular protein as substrate (C, lane 1, control; lane 2, Aβ-treated) or using purified tau as a substrate (D, lane 1, control; lane 2, Aβ-treated sample). Furthermore, cdk5 activity from hippocampal cells was assessed by using the tau peptide RTPPKS^{235}PSSAKSR as a substrate (E, lane 1, untreated control; lane 2, treated with Aβ).

approximately 35 kDa, when whole cell homogenates were resolved in SDS-PAGE, blotted and immunostained with anti-cdk5 antibody (Fig. 1A, lane 1). A higher level of cdk5 staining was observed in cells incubated with Aβ (Fig. 1A, lane 2). Western blot analysis revealed that the hippocampal neurons express cdk5 after 4 days of plating the cells and that a relatively small increase in its levels occurred when cells were exposed to Aβ fibrils (15 μM) by 24 h. By contrast, immunoprecipitates of cdk5 from hippocampal cells incubated with Aβ fibrils showed that the amyloid induced a significant increase in cdk5 kinase activity (Fig. 1B, lanes 1 and 2), using histone H1 as a substrate. Furthermore, a significant increase in cdk5 activity was observed in cdk5 immunoprecipitates of the cells exposed to amyloid fibrils, when microtubular protein (Fig. 1C, lanes 1 and 2) or pure tau (Fig. 1D, lanes 1 and 2) were used as a substrate. Additional confirmation was obtained by an experiment showing an increased phosphorylation of the synthetic tau peptide RTPPKS^{235}PSSAKSR as related to Aβ-induced cdk5 activation (Fig. 1E, lanes 1 and 2). Concomitant with this effect in activating cdk5, Aβ trig-

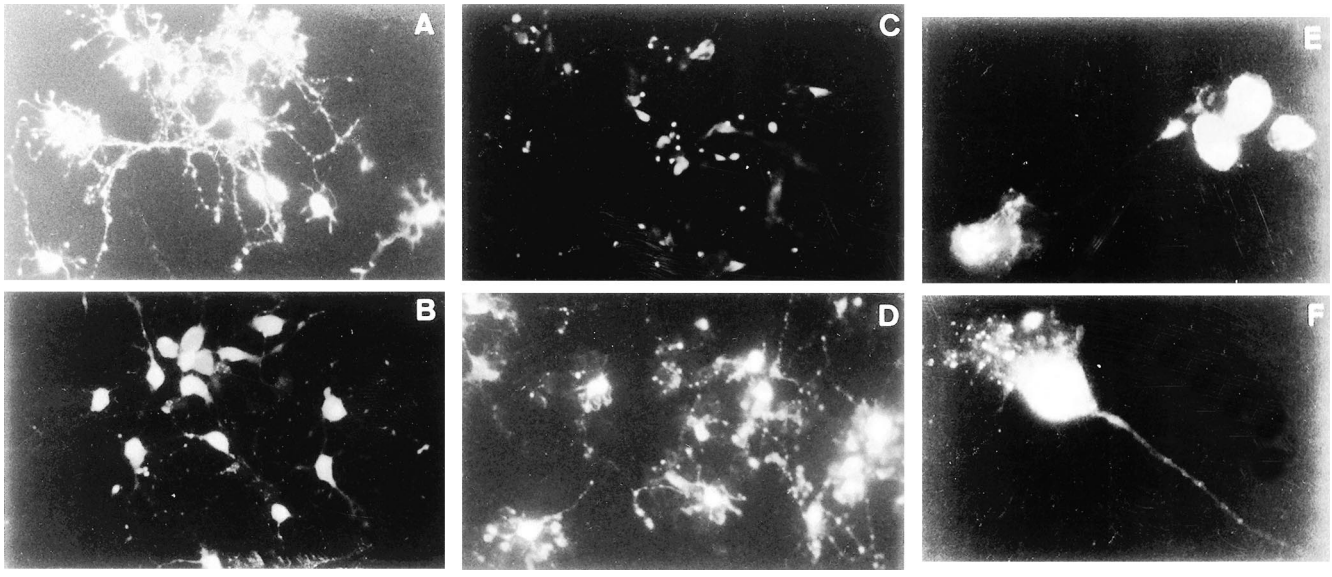

Fig. 2. Effects of Aβ and butyrolactone on the morphological integrity of primary neurons. Aβ peptide was added in the presence or the absence of butyrolactone I and cells were analyzed 24 h later by fluorescence microscopy after labelling with calcein-AM and PI. (A) Control neurons, (B) cells only treated with 5 μM butyrolactone, (C) cells treated with 10 μM Aβ, (D) cells treated with 5 μM butyrolactone I plus 10 μM Aβ. Images are representatives of many cultures in which multiple fields were analyzed under each experimental condition. Higher magnifications of cells treated with Aβ (E) and cells treated with Aβ plus butyrolactone I (F) are also shown.

gered morphological changes of neurons and neuronal death, in a peptide concentration-dependent fashion. In addition, studies were carried out to evaluate expression of cdc2, cdk4 and cdk6 and none of these kinases were detected in the rat hippocampal cells.

In this context, it was of utmost interest to examine the effects of butyrolactone, a cdk5 inhibitor, on the morphology of Aβ-exposed cells. Neurons from a single preparation were plated at the same density, grown for 4 days and exposed to Aβ with or without butyrolactone I (5 μM) for 24 h and visualized using calcein/PI as shown in Fig. 2. Control cells retained the calcein dye (green) and showed extensive neuritic processes (Fig. 2A). No labelling with PI (red) was observed in these cultures, indicating that almost no dead cells were present. After Aβ exposure for 24 h, neuritic processes became shortened or disappeared and a significant proportion of PI-labelled death cells was observed (Fig. 2C,E). However, when butyrolactone was added to cultures with Aβ, degenerative changes were much less evident (Fig. 2D), many neurons still had normal, long processes as shown at higher magnification (Fig. 2F). When butyrolactone was added alone, cells were indistinguishable from the controls (Fig. 2B). These patterns are representative of several experiments ($n = 5$). The percentages of surviving neurons after Aβ treatment were estimated with the calcein/PI assay: neurons treated with 10 μM Aβ had survival rates of 30%, however, viability of neurons treated with 10 μM Aβ in the presence of butyrolactone was over 60%. Butyrolactone I alone for 24 h did not decrease viability.

In order to correlate changes in morphology and viability with the general metabolic state of the cell, MTT reduction was assayed. Fig. 3 shows the concentration-dependent neurotoxic response of hippocampal cells incubated with increasing concentrations of Aβ fibrils alone or in the presence of the cdk5 inhibitor. The increase in the Aβ fibrils concentration incremented cell death, as reflected by the gradual decrease in MTT reduction. However, MTT associated with neuronal survival for cells exposed to Aβ in the presence of 5 μM butyrolactone was much higher, as revealed by the two-tail Student t-test of significance, with $n = 5$. Butyrolactone protects hippocampal cells from neuronal death induced by Aβ. Fig. 4 shows the concentration-dependent protective effects of butyrolactone I on hippocampal cells against Aβ-induced toxicity. Increasing concentrations of butyrolactone I increased the viability of Aβ fibrils-treated neurons, the toxic effect of the Aβ fibrils was almost completely blocked by 20 μM butyrolactone. As control, the inhibitor added in the absence of Aβ did not modify cell survival.

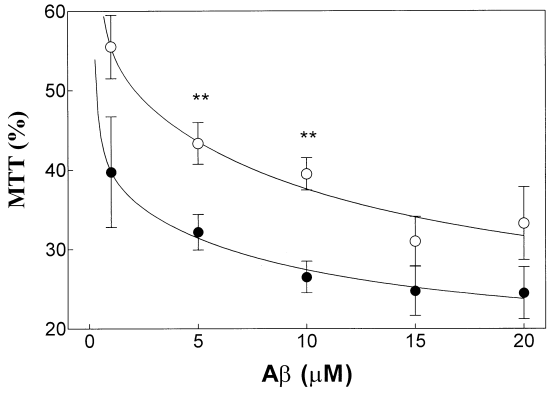

Fig. 3. Effects of butyrolactone I on Aβ-induced neurotoxicity in hippocampal cell cultures. Cultures were treated with increasing concentrations of amyloid fibrils. Aβ fibrils alone (●), Aβ fibrils plus 5 μM butyrolactone I (○). Cell viability was evaluated using the MTT reduction assay. Cell viability after treatment was measured by the MTT reduction assay ($n = 5$). The two-tail t-test revealed significant differences between data of cells treated with Aβ and those treated with Aβ plus butyrolactone. $^{**}P < 0.001$ by non-paired Student's t-test.

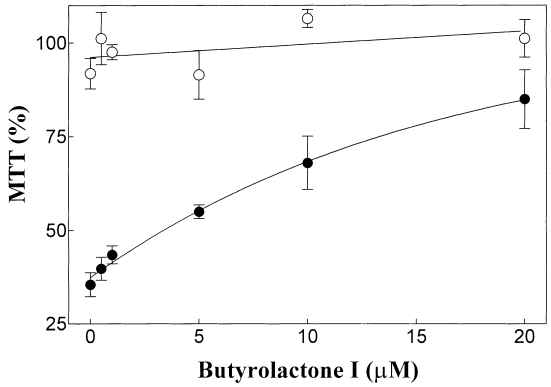

Fig. 4. Concentration-dependent protective effects of butyrolactone I on hippocampal cells against Aβ-induced toxicity. Increasing concentrations of butyrolactone I (●), increased MTT reduction as an expression of viability of neurons treated with Aβ. The toxic effect of the Aβ fibrils diminished as the concentration of butyrolactone (1–20 μM) increased. As a control, the inhibitor added in the absence of Aβ (○) did not modify cell survival. Data are expressed with the respective S.D.s.

To further analyze whether cdk5 was involved in the events leading to neuronal death, experiments were conducted with a cdk5 antisense oligonucleotide, that inhibits cdk5 expression [10]. In the negative control, the treatment of rat hippocampal cells cultured with either 1 or 10 μM Aβ fibrils, with an oligonucleotide with a scrambled sequence (4 μM) did not prevent neurotoxicity caused by the amyloid. However, in the presence of Aβ fibrils plus cdk5 antisense oligonucleotide (4 μM), neurotoxicity was significantly reduced (Fig. 5). The protection effect of the antisense against Aβ-promoted neuronal death was correlated with a decrease in cdk5 expression by more than 50% (Fig. 5, insert, lane 3) as compared with controls incubated with Aβ but in the absence of antisense (lane 1) or controls treated with a scrambled oligonucleotide (lane 2).

4. Discussion

The amyloidogenic theory states that Aβ is a factor triggering a series of molecular events leading to neuronal death [3]. On the other hand, it has been described that Aβ has a neurotoxic effect on neuronal cell lines and that this effect is directly related with the level of aggregation of the amyloid [33]. It was also reported that neuronal cells treated with Aβ result in a selective phosphorylation of specific epitopes on PHFs [35]. The focus of the present study was to explore the links between the action of fibrillary Aβ and tau phosphorylations, in the context of molecular changes leading to neuronal death. Previous studies have shown that amyloid fibrils, but not amorphous aggregates of Aβ, are toxic to neuronal cells in culture [33] and the existence of a strong correlation between Aβ neurotoxicity and the aggregation state of the Aβ peptide

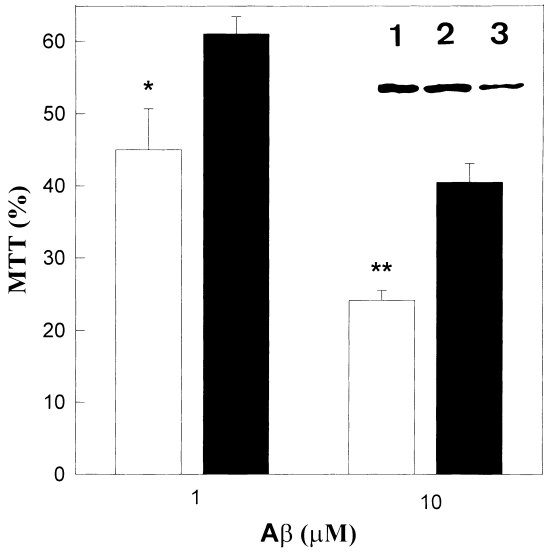

Fig. 5. Effects of cdk5 antisense probes on cdk5 expression and the viability of hippocampal neurons treated with Aβ. Viability was evaluated on the basis of the MT reduction assay on cells cultured with either 1 or 10 μM Aβ. The open bars denote viability of cells treated with the amyloid fibrils plus the scrambled oligonucleotide. The closed bars (dark) correspond to the hippocampal cells treated with Aβ in the presence of the cdk5 antisense probe. The S.D.s are shown for each histogram. Every bar corresponds to the mean of three determinations. *Significant differences ($P < 0.05$), **Very significant differences ($P < 0.001$). The insert shows the Western blots of cdk5 from the Aβ-treated hippocampal cells in the absence of the cdk5 antisense (lane 1) or in the presence of the random oligonucleotide (lane 2) or in the presence of cdk5 antisense oligonucleotide (lane 3).

[34,36]. In the present study, it was noteworthy that the addition of pre-formed amyloid fibrils to neuronal cultures caused an activation of protein kinase cdk5, as measured using histone and tau as substrates. This protein kinase has been found distributed in both the nucleus and cytoplasm of neurons from normal and Alzheimer's brains [37]. According with previous findings, Ser^{235} is a main residue phosphorylated by cdk5 on human tau [38]. The present findings are of interest, since they provide a suggestion that TPK II appears to mediate the effects of Aβ on neuronal tau behavior. In this context, previous studies have shown that glycogen synthase kinase (TPK I) seems to be involved in mediating Aβ-induced neurotoxicity [38]. The treatment with amyloid increased TPK I, thus affecting the neuronal tau capacity to interact with the microtubular cytoskeleton [38]. On the other hand, TPK I phosphorylates tau threonine-231 on a pT^{231}-P bond, containing a proline residue susceptible to prolyl isomerization by Pin-1 [39]. In the line of these observations, it has also been reported that tau from COS cells transfected with GSK3β and cdk5 activator (p35) exhibits phosphorylations at almost all Alzheimer's epitopes [22]. In this context, it is interesting to point out that, in contrast with Aβ, the excitatory amino acid glutamate and the drug colchicine appear to prevent anomalous phosphorylations on tau in cortical neurons [40].

Besides the action of Aβ on tau phosphorylation, the present studies show that direct blockage of cdk5 by the enzyme inhibitor, butyrolactone [25], or alterations on cdk5 expression by using an antisense probe for cdk5 [10] reduced significantly the signs of neurotoxicity and prevented neuronal death. Since no expression of other cdks was detected under the present experimental conditions, data suggest that butyrolactone I-induced neuroprotection appears to be mainly due to inhibition of cdk5. These data were supported by the effects of cdk5 antisense in reducing neuronal death promoted by Aβ, as correlated with a decrease in the expression of this kinase by around 50%. These findings suggest that cdk5 activation is a target in the process triggered by Aβ and that it leads to neuronal degeneration and death.

On the other hand, data suggest that anomalous tau phosphorylations that appear to be derived from the Aβ-induced activation of cdk5 could also be involved in neuronal alterations that lead to cell death. These studies are also in agreement with the idea that tau protein modified by the cdk5 system becomes sensitive to subsequent phosphorylations by GSK3, a sequence of events in which neurodegeneration may involve coordinated activation of both cdk5 and GSK3 systems [41].

In the context of this study, it is interesting to point out that previous findings revealed that an activation of cdk5 induces apoptosis in neuronal cells [42,43]. Thus, the marked decrease in neuronal death caused by cdk5 inhibition by butyrolactone I or the cdk5 antisense could be related with its reduction of apoptotic effects. However, at this stage, it is difficult to ascertain how the changes in cdk5 promoted by Aβ would contribute to neuronal death via alteration on the cell cytoskeleton or by a direct effect in stimulating the apoptotic pathway or a combination of both phenomena. On the other hand, the contribution of cytoskeletal alteration on the sequence of events leading to neuronal death is strongly supported by the effects of MT stabilizing drugs like paclitaxel in protecting neurons against the neurofibrillary pathology observed in AD [44]. The involvement of cdk5 in the cytoskeletal changes resulting after the abnormal phosphorylations on tau is strongly supported by the presence of this protein kinase in NFTs from AD brains [45]. Taken together all this information, this study provides new clues to understand how fibrillary Aβ promotes hyperphosphorylations at tau epitopes related with AD and the involvement of cdk5, a component of the TPK II system, in mediating the sequence of molecular changes occurring in neurons affected by the amyloid.

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