Neurotoxicity of acetylcholinesterase amyloid β-peptide aggregates is dependent on the type of Aβ peptide and the AChE concentration present in the complexes

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Abstract Alzheimer's disease (AD) is a neurodegenerative disorder whose hallmark is the presence of senile plaques and neurofibrillary tangles. Senile plaques are mainly composed of amyloid β-peptide (Aβ) fibrils and several proteins including acetylcholinesterase (AChE). AChE has been previously shown to stimulate the aggregation of $A\beta_{1-40}$ into amyloid fibrils. In the present work, the neurotoxicity of different amyloid aggregates formed in the absence or presence of AChE was evaluated in rat pheochromocytoma PC12 cells. Stable AChE-Aβ complexes were found to be more toxic than those formed without the enzyme, for $A\beta_{1-40}$ and $A\beta_{1-42}$, but not for amyloid fibrils formed with $A\beta_{Val18\rightarrow Ala}$, a synthetic variant of the $A\beta_{1-40}$ peptide. Of all the AChE-Aβ complexes tested the one containing the $A\beta_{1-40}$ peptide was the most toxic. When increasing concentrations of AChE were used to aggregate the $A\beta_{1-40}$ peptide, the neurotoxicity of the complexes increased as a function of the amount of enzyme bound to each complex. Our results show that AChE-$A\beta_{1-40}$ aggregates are more toxic than those of AChE-$A\beta_{1-42}$ and that the neurotoxicity depends on the amount of AChE bound to the complexes, suggesting that AChE may play a key role in the neurodegeneration observed in Alzheimer brain.

Key words: Alzheimer's disease; Amyloid β_{1-40}-peptide; Amyloid β_{1-42}-peptide; Acetylcholinesterase; Neurotoxicity; PC12 cell

1. Introduction

Alzheimer's disease (AD) is characterized by a selective neuronal degeneration, intracellular accumulation of neurofibrillary tangles and extracellular local deposits of amyloid in the form of senile plaques [1,2]. Senile plaques are mainly composed of an amyloid β-peptide (Aβ) of 40–43 amino acids [3] which is neurotoxic when it forms amyloid fibrils in vitro [4] and in vivo [5], although this by itself cannot explain the cascade of events which underlies this disease [6]. In fact, the presence of senile plaques in the brains of individuals without AD symptoms [7] suggests that specific agents could transform innocuous plaques into neuropathogenic aggregates. The factors that contribute to the putative transformation of the Aβ amyloid from a relatively inert to a pathogenic state remain unknown and may involve interactions with additional plaque constituents. Several macromolecules have been reported to be associated with senile plaques including apolipoprotein E, α_1-antichymotrypsin, α_2-macroglobulin, perlecan, laminin, complement factors and acetylcholinesterase (AChE) [8–11]. Nonetheless, the role of these molecules in the development of AD is unknown.

AChE, the enzyme which catalyzes the hydrolysis of the neurotransmitter acetylcholine [12], has been implicated in different non-cholinergic activities [13] including a possible role in the development of AD [14,15]. AChE has been found to co-localize with Aβ in mature senile plaques and cerebral blood vessels [16]. Moreover, most of the brain areas which are susceptible to plaque formation are strongly AChE positive [17]. In our laboratory, the ability of various AChE molecular forms to promote the aggregation of $A\beta_{1-40}$ was demonstrated in vitro [9]. Furthermore, the presence of AChE in the $A\beta_{1-40}$ pre-formed fibrils renders the amyloid fibrils more toxic [18,19].

Considering that senile plaques are mainly composed of $A\beta_{1-40}$ and $A\beta_{1-42}$ [2,11,20], the aim of the present work was to evaluate the relative contribution of AChE to the neurotoxicity of amyloid fibrils formed with different Aβ peptides, and to establish whether the potential difference in toxicity is induced by changes in the AChE concentration in the enzyme-Aβ complexes.

2. Materials and methods

2.1. Materials

Synthetic Aβ peptides corresponding to residues of the human wild-type sequence $A\beta_{1-40}$ and $A\beta_{1-42}$, as well as a variant $A\beta_{1-40}$ peptide containing a valine to alanine substitution $A\beta_{Val18\rightarrow Ala}$ with a very low fibrillogenetic capacity [21], were used in the present work. All the peptides were purified by HPLC and obtained from Chiron Corp. Inc. (Emeryville, CA, USA).

2.2. AChE purification

The tetrameric G_4 AChE form (sedimentation coefficient 10.7 S) was purified from bovine caudate nucleus, using acridine-affinity chromatography as described previously [22]. Both specific activities (6000 U/mg protein) and staining intensities following SDS-polyacrylamide gel electrophoresis (PAGE) (a single band of 66 kDa) were used to verify purity. AChE activity was determined by the method of Ellman et al. [23].

2.3. Aggregation assays

2.3.1. Turbidity. The aggregation assay was carried out as previously described [10,18]. Specifically, stock solutions were prepared by

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Abbreviations: AChE, acetylcholinesterase; Aβ, amyloid β-peptide; AD, Alzheimer's disease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CR, Congo red; Th-t, thioflavine-t; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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dissolving freeze-dried aliquots of $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{Val18\rightarrow Ala}$ in dimethyl sulfoxide (DMSO) at 15 μg/ml (3.5 mM). Aliquots of peptide stock (70 nmol in 20 μl of DMSO) were added to aqueous buffer (725 μl total volume; 0.1 M Tris-HCl, pH 7.4). In order to avoid initial aggregation an 'alkaline shock' was induced by 2.5 N NaOH up to 8.5 pH and neutralized by 2.5 N HCl down to 7.2 pH. For the aggregation assays with AChE, peptide stock (70 nmol in DMSO) was added to buffer containing AChE (100 nM). The solutions were stirred continuously (210 rpm) at room temperature for 48 h. Aggregation was measured by turbidity at 405 nm vs. buffer blank. In a second series of experiments, $A\beta_{1-40}$ was induced to aggregate in the same conditions with increasing concentrations of AChE (25, 100 and 250 nM). The fibrils obtained were characterized by Congo red (CR) binding and thioflavine-t (Th-t) based fluorometric assays.

2.3.2. Th-t based fluorometric assay. Th-t binds specifically to β-pleated structures such as amyloid and this binding produces a shift in its emission spectrum and fluorescent signal related to the amount of amyloid formed [24]. Fibril aliquots (3 μl) from the aggregation assays were added to 50 mM phosphate (pH 6.0) and 3 μM Th-t to a final volume of 500 μl [25]. A fluorescence time scan was performed and fluorescence was monitored at excitation 450 nm and emission 485 nm using a Shimadzu spectrofluorometer. For co-incubation experiments the fluorescence of AChE alone was determined as a blank, giving values of 2 or less.

2.3.3. CR assay. The binding of CR to amyloid fibrils was used to quantify the amount of fibrils in an aggregation assay [26]. Fibril aliquots (40 μl) were added to 960 μl of a solution containing 25 μM CR, 100 mM phosphate buffer (pH 7.4) and 150 mM NaCl, and incubated for 30 min [18]. Absorbance was measured at 480 nm and 540 nm, and CR binding was determined by CR $(M) = (A_{540}/25\,295) - (A_{480}/46\,306)$.

2.4. Amyloid fibril isolation

Pre-formed fibrils were washed four times with phosphate buffered saline (PBS) at 14 000 rpm for 30 min to remove the soluble Aβ and AChE [18,25]. Pellets were homogenized in PBS. Aliquots were put on a denaturing buffer and subjected to Tris-Tricine SDS-PAGE [27] to quantify the concentrations of Aβ peptide contained in the fibrils by densitometric scanning using Aβ and AChE with known concentrations as controls. Data were processed by the GS365W program from Hoefer Scientific Instruments (San Francisco, CA, USA).

2.5. Identification of AChE in the complexes

The presence of AChE in the complexes was identified using a modified histochemical method [28], as previously described [18,25]. Briefly, AChE-Aβ complexes were incubated in a solution (pH 6.0) containing acetylthiocholine iodide as an enzymatic substrate for 30 min at room temperature in the presence of 0.1 mM iso-OMPA (butyrylcholinesterase specific inhibitor). AChE activity produced a characteristic brown stain which was visualized under a light microscope.

2.6. Cytotoxicity assays

Rat pheochromocytoma (PC12) cells [29] were grown on collagen coated dishes in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 5% horse serum and 100 U/ml penicillin and 100 mg/ml streptomycin. Cytotoxicity assays were performed as previously described [19,30,31]. Cells were dissociated using a Pasteur pipette and washed with serum-free medium for 10 min at 1500 rpm. Cells were seeded in 96 well plates in serum-free medium with 2 μM insulin at a density of 4×10^3 cells/100 μl/well [32]. 10 μl PBS (control), fibrils and AChE-Aβ complexes were added to reach a final concentration of 1, 10 or 25 μM Aβ peptide. Cells were incubated for 48 hours at 37°C, after which cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-

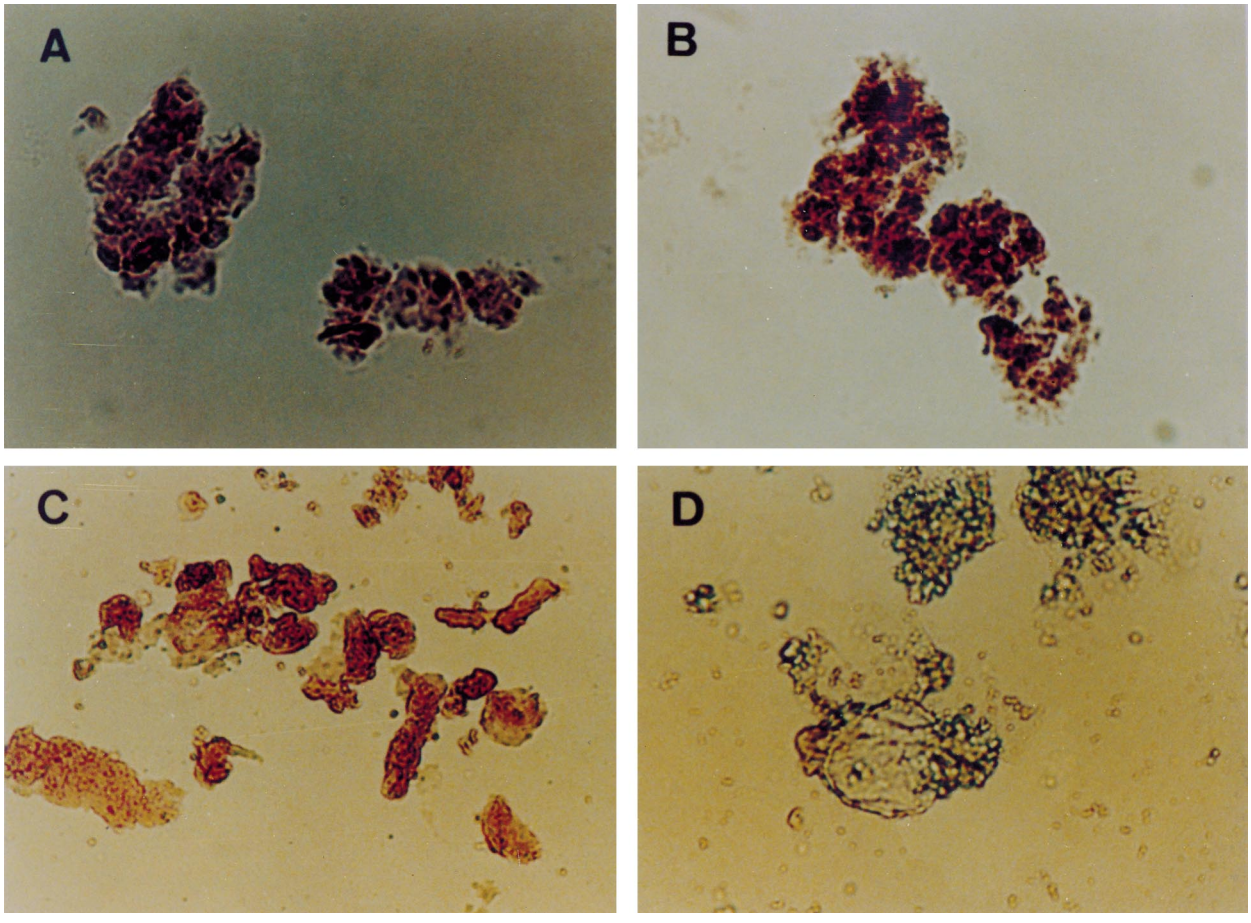

Fig. 1. Histochemical staining of AChE bound to amyloid fibrils. Amyloid fibrils containing AChE from aggregation assays with $A\beta_{1-40}$ (A), $A\beta_{1-42}$ (B) and $A\beta_{Val18\rightarrow Ala}$ (C) were washed three times with PBS and stained by the Karnovsky and Roots method [28]. The fibrils containing $A\beta_{1-40}$ (or the other peptides) alone did not present AChE activity (D). Photographs were taken with a Nikon Optiphot light microscope at 600× magnification.

2,5-diphenyltetrazolium bromide (MTT) method [33] by adding 11 μl MTT stock (5 mg/ml). The reaction was stopped 4 h later by adding 110 μl stop solution (50% dimethylformamide and 20% SDS at pH 4.7). MTT reduction was determined in a Labsystems Uniskam I spectrophotometer (Finland) at 540 and 650 nm. Identical experiments were performed with 10 μM fibrils and complexes and were followed by lactic dehydrogenase (LDH) release test using the Cytotox 96 kit from Promega (Madison, WI, USA), as previously described [30,31].

3. Results

3.1. AChE is present on amyloid fibril complexes formed with different Aβ peptides

The presence of AChE in amyloid fibrils formed with the peptides $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{Val18\rightarrow Ala}$ was evidenced histochemically by the method of Karnovsky and Roots [28]. Fig. 1 shows positive stains for AChE activity in all three cases. The fibrils obtained for $A\beta_{1-40}$ and $A\beta_{1-42}$ form large compact aggregates (Fig. 1A,B), while $A\beta_{Val18\rightarrow Ala}$ produced smaller and more disperse fibrils (Fig. 1C). No staining was observed for $A\beta_{1-40}$ fibrils pre-formed without AChE (Fig. 1D), and for $A\beta_{1-42}$ and $A\beta_{Val18\rightarrow Ala}$ alone (data not shown).

3.2. Cytotoxicity of AChE-Aβ complexes depends on the type of Aβ peptides

To evaluate whether AChE-Aβ complexes formed from different Aβ peptides ($A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{Val18\rightarrow Ala}$) play a role in cell damage, the effect of exogenously applied complexes on rat pheochromocytoma PC12 cells was examined. Neurotoxicity was evaluated using the MTT reduction assay, an indicator of cell redox activity [30,33,31]. The experiments were carried out using 100 nM AChE to form the complexes, which were processed as described in Section 2. Final concentrations per well of 1, 10 and 25 μM Aβ peptide, both in the fibrils and in the complexes, were used [18,19]. For 10 and 25 μM $A\beta_{1-40}$ and $A\beta_{1-42}$, the fibrils containing AChE were more toxic than those without the enzyme (Fig. 2A,B). For instance, at a concentration of 25 μM $A\beta_{1-40}$ fibrils with AChE were two-fold more toxic than $A\beta_{1-40}$ fibrils alone. The highest cytotoxicity in either the presence or absence of AChE was obtained with the $A\beta_{1-40}$ peptide. On the other hand, $A\beta_{Val18\rightarrow Ala}$ fibrils, formed by a peptide with low fibrillogenetic capacity [21], showed almost no toxicity, in either the presence or absence of AChE (Fig. 2C).

3.3. Cytotoxicity depends on the concentrations of AChE bound to the fibrils

Considering that $A\beta_{1-40}$ fibrils were found to be more toxic than the other Aβ peptides tested, this peptide was used to assess the AChE concentration dependence of the cytotoxicity observed with the AChE-Aβ complex. $A\beta_{1-40}$ peptide was incubated with different AChE concentrations (25, 100 and 250 nM) and fibril formation was followed by turbidity. Fig. 3A shows that the aggregation of the $A\beta_{1-40}$ peptide increased as a function of AChE concentration. In order to verify that the fibrils formed corresponded to amyloid, Th-t fluorescence (Fig. 3B) and CR binding (Fig. 3C) assays were carried out. In both cases, the quantity of amyloid increased as a function of the AChE concentration. SDS-PAGE was carried out to demonstrate the presence of AChE in the amyloid fibrils. As expected, increasing amounts of AChE bound to the Aβ fibrils were observed by SDS-PAGE, and the AChE

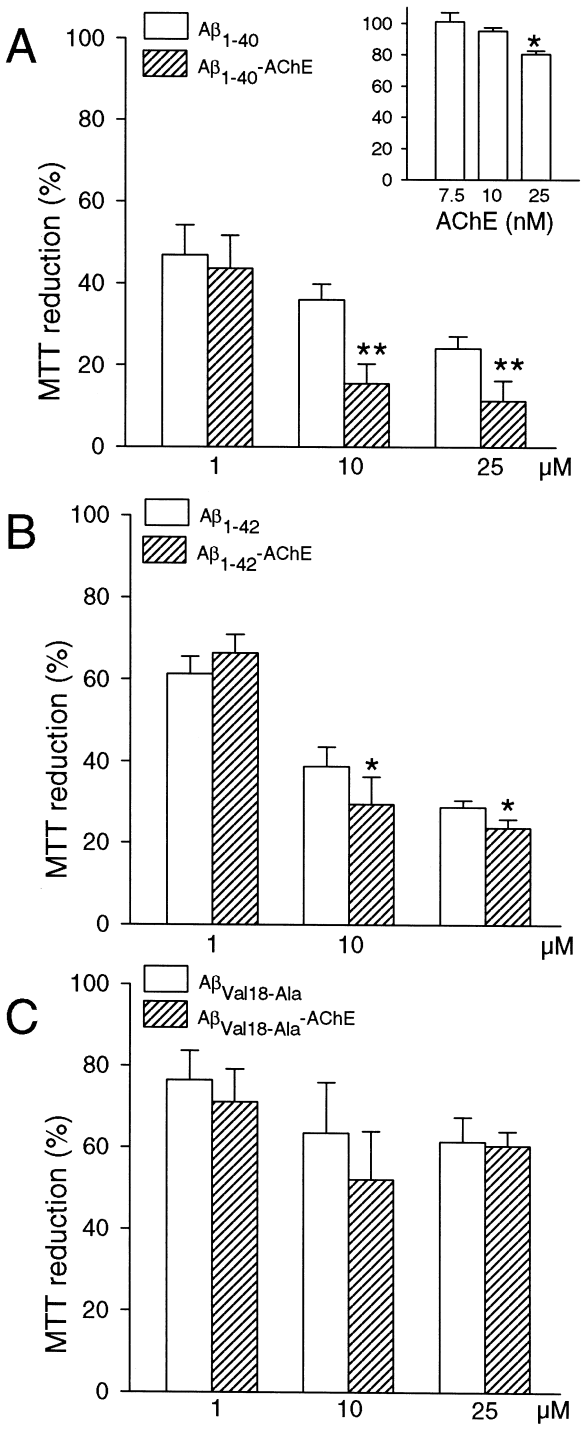

Fig. 2. Inhibition of MTT reduction in PC12 cells after 48 h of treatment with three concentrations of pre-formed fibrils (1, 10 and 25 μM) with and without AChE. The different peptides were $A\beta_{1-40}$ (A), $A\beta_{1-42}$ (B) and $A\beta_{Val18\rightarrow Ala}$ (C). MTT reduction assay values were expressed as percentage of control values. Data are means ± S.E.M. (bars) of 6–9 separate experiments performed in triplicate. $^{*}P<0.05$; $^{**}P<0.005$ by non-paired Student's *t*-test. The AChE bound to fibrils was determined by SDS-PAGE gels and corresponds to 7.5, 10 and 25 nM AChE bound to 1, 10 and 25 μM $A\beta_{1-40}$ and $A\beta_{1-42}$. The inset shows the MTT reduction percentages obtained with AChE alone, data are means ± S.E.M. (bars) of six separate experiments performed in triplicate. $^{*}P<0.05$ by non-paired Student's *t*-test.

amount was proportional to the initial concentration of AChE used in each case (Fig. 3D). Finally, when Aβ fibrils (25 μM) formed in the presence of different concentrations of AChE were used for cytotoxicity assays, fibril toxicity was found to increase in relation to the amount of AChE bound to the fibrils (Fig. 4). Fibrils incubated with the highest concentration of AChE were the most cytotoxic, whereas no difference was observed between fibrils without AChE and those pre-formed with a low amount of AChE. In order to verify the observed difference in the neurotoxicity induced by the AChE-Aβ complexes respect to Aβ fibrils, LDH release test was carried out, as indicated in the inset of Fig. 4, and similar results to those obtained with the MTT reduction assay were observed with this test.

4. Discussion

Previous studies in our laboratory have shown that AChE accelerated the assembly of the $A\beta_{1-40}$ peptide into amyloid fibrils, through the formation of a stable AChE-Aβ complex which turns amyloid fibrils more toxic [9,18,19]. In the present work we have found that $A\beta_{1-42}$ peptide increases its neurotoxicity when it forms aggregates with AChE, and the cytotoxicity produced by AChE-$A\beta_{1-40}$ complexes depends on the concentration of enzyme bound to the complex, those complexes which contain higher amounts of AChE being more toxic.

Considering that the presence of senile plaques in the brain of aging individuals does not necessarily lead to symptoms of AD [7], the presence of AChE in some critically located senile plaques could play a key role in triggering the cytotoxic events which occur around mature plaques in AD. $A\beta_{1-42}$ has been suggested to be important for inducing Aβ deposits [2,11,34,35]. Fibril formation has been suggested to be important for Aβ cytotoxicity [36], and previous studies have found that once formed, aggregates of $A\beta_{1-42}$ and $A\beta_{1-40}$ appear to be equally neurotoxic [37]. We have obtained the highest cytotoxicity in either the presence or absence of AChE with the $A\beta_{1-40}$ peptide, although $A\beta_{1-42}$ showed a high cytotoxicity on PC12 cells with Aβ fibrils alone or with AChE complexes. AChE seems to act by an enhancement of the fibril toxicity, but not by converting low- or non-toxic peptides into toxic ones, as was the case for the synthetic variant $A\beta_{Val18\rightarrow Ala}$.

AChE has been found to be associated with Aβ deposits from the beginning of plaque formation. In fact both pre-amyloid diffuse deposits as well as compact senile plaques contain the enzyme [38]. Moreover, AChE forms very stable enzyme-Aβ complexes in vitro and remains tightly bound to Aβ aggregates after washing them with high salt solutions and detergents [25]. These results indicate that AChE may participate by providing heterogeneous nuclei during the seeding and growth phase of amyloid fibrils [18,39].

In conclusion, extensive evidence has accumulated indicating that Aβ aggregation into amyloid deposits is a central event in the pathogenesis of AD [2,4]. Our present findings indicate that AChE was able to modulate the Aβ aggregation into Alzheimer fibrils, and to increase the neurotoxicity of the AChE-Aβ complexes towards neuronal cells in culture, despite the fact that AChE, by itself, was not able to render inert peptides toxic.

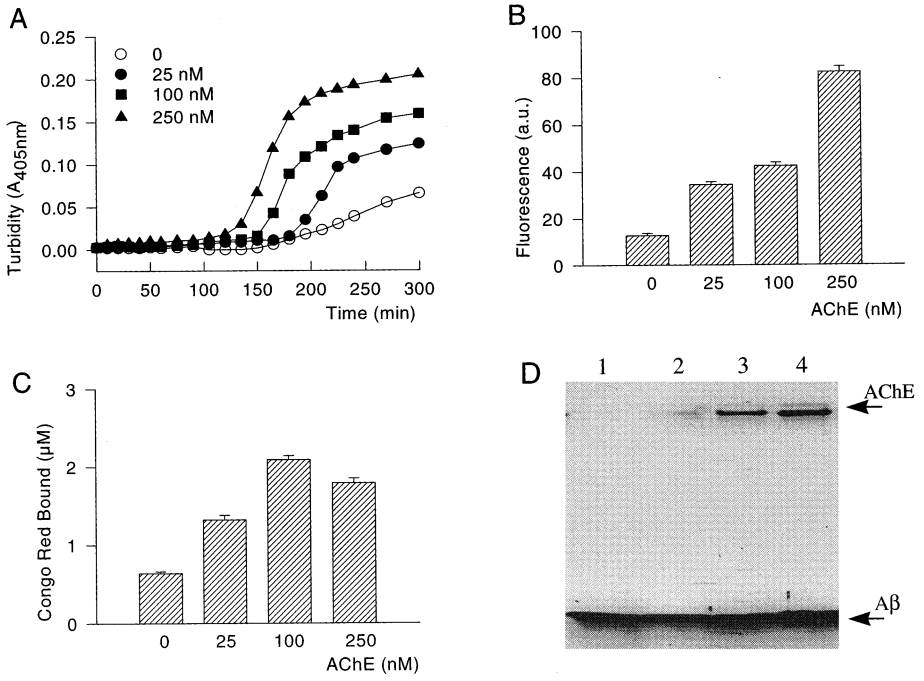

Fig. 3. Effect of increasing concentrations of AChE (25, 100 and 250 nM) on the formation of $A\beta_{1-40}$ fibrils by using 70 nmol $A\beta_{1-40}$. AChE-Aβ complexes were obtained after turbidity experiments (A) up to 300 min when the different aggregation kinetics reached a plateau. At the end of the assays (24 h), aliquots were taken to quantify amyloid by Th-t fluorescence (B) and CR binding (C). Data are from a representative experiment of three separate ones performed. D shows a SDS-PAGE with samples from the aggregation assays which were washed three times to remove all the soluble components of $A\beta_{1-40}$ aggregates incubated with and without increasing concentrations of AChE. An increasing amount of AChE was bound to the fibrils depending on the initial concentration of AChE. The different lanes were occupied as follows: lane 1, 10 μl of purified $A\beta_{1-40}$ fibrils; lanes 2, 3 and 4, with 10 μl of purified AChE-$A\beta_{1-40}$ fibrils aggregated with 25, 100 and 250 nM AChE respectively.

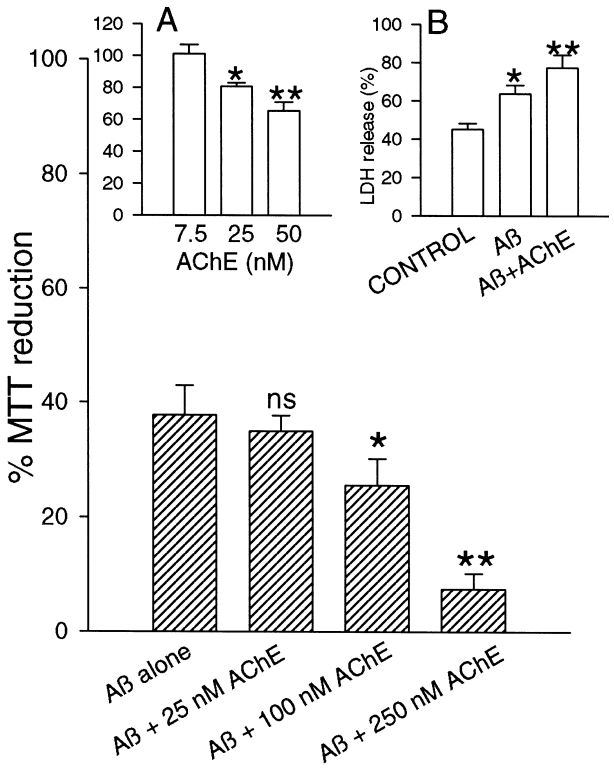

Fig. 4. Effects of fibrils with increasing concentrations of AChE on MTT reduction in PC12 cells. Cells were incubated with 25 μM fibrils without or with AChE for 48 h. Control values with PBS were taken as 100%. Data are means ± S.E.M. (bars) of five different experiments performed in triplicate. ns: not statistically different; $*P < 0.05$; $**P < 0.005$ by non-paired Student's *t*-test. AChE bound to 25 μM $A\beta_{1-40}$ was 7.5, 25 and 50 nM when 25, 100 and 250 nM AChE, respectively, were used in the incubations. Inset A shows the effect of AChE alone on MTT reduction and data are means ± S.E.M. (bars) of six different experiments performed in triplicate. $*P < 0.05$; $**P < 0.005$. Inset B shows the effect of 10 μM $A\beta_{1-40}$ fibrils and AChE-Aβ complexes (having incubated the peptide with 100 nM AChE) on LDH release, total released enzyme was taken as 100%. Data are means ± S.E.M. (bars) of three different experiments performed in triplicate. $*P < 0.05$; $**P < 0.005$.

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