Alzheimer disease is characterized by the accumulation of aggregated amyloid β-peptide (Aβ) in the brain. The physiological mechanisms and factors that predispose to Aβ aggregation and deposition are not well understood. In this report, we show that calcium can predispose to Aβ aggregation and fibril formation. Calcium increased the aggregation of early forming protofibrillar structures and markedly increased conversion of protofibrils to mature amyloid fibrils. This occurred at levels 20-fold below the calcium concentration in the extracellular space of the brain, the site at which amyloid plaque deposition occurs. In the absence of calcium, protofibrils can remain stable in vitro for several days. Using this approach, we directly compared the neurotoxicity of protofibrils and mature amyloid fibrils and demonstrate that both species are inherently toxic to neurons in culture. Thus, calcium may be an important predisposing factor for Aβ aggregation and toxicity. The high extracellular concentration of calcium in the brain, together with impaired intraneuronal calcium regulation in the aging brain and Alzheimer disease, may play an important role in the onset of amyloid-related pathology.

Amyloid β-peptide (Aβ) is the primary constituent of amyloid plaques in Alzheimer disease (AD). Cleavage of the amyloid β precursor protein leads to the production of Aβ peptides of varying lengths, of which the 40-amino acid peptide is the major species (Aβ_{1–40}) (1). Mutations that cause familial Alzheimer disease generally lead to an increase in the level of the more fibrilligenic 42-amino acid peptide Aβ_{1–42} (1, 2). The aggregation of Aβ into mature amyloid fibrils occurs through a number of intermediate structural forms, variously referred to as oligomers or protofibrils. Soluble oligomeric species and protofibrillar structures may affect neuronal function and viability (3–5), and mature amyloid fibrils are toxic to neurons in vitro and in vivo (6, 7). Identifying factors that promote Aβ aggregation may thus be important for understanding the pathophysiology of AD and developing therapeutic strategies.

The mechanism by which Aβ aggregates in the brain is not fully understood, although there is increasing evidence that metal ions may play a role. Physiological levels of copper and zinc have been shown to accelerate Aβ aggregation (8–10), and trace levels of copper and zinc may initiate seeding and oligomerization of Aβ (11). It has also been suggested that the binding of Aβ to the membrane sialoglycolipid GM1 ganglioside can lead to a conformational change in Aβ and seeding of aggregation (12). Recent evidence suggests that Aβ_{1–42} is the Aβ species required for the seeding of amyloid fibril formation and plaque deposition in vivo, as mice overexpressing only Aβ_{1–42} developed amyloid plaques, whereas mice overexpressing only Aβ_{1–40} did not (13). In this report we show that calcium can accelerate aggregation of Aβ_{1–42} with marked effects on the formation of early protofibrillar structures, as well as the protofibril to fibril conversion event. Both protofibrillar and fibrillar forms of Aβ are neurotoxic. These effects of calcium on Aβ aggregation occurred at concentrations 20-fold lower than the 2 mM calcium concentration present in the extracellular space of the brain, implicating a potential role for calcium in Aβ aggregation and toxicity in the AD brain.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Reagents*—All cell culture reagents were purchased from Invitrogen, and chemicals were from Sigma-Aldrich, unless stated otherwise.

*Preparation of Aβ—Aβ_{1–42} was from either rPeptide, BIO-SOURCE, or Bachem, and all gave similar results (Aβ_{1–42} was used for all the experiments described and will be referred to as Aβ from here on). Aβ was solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mM. HFIP was allowed to evaporate off in a fume hood, followed by drying under vacuum in a Savant SpeedVac. The Aβ HFIP film was stored at −20°C until use. For experiments in which Aβ was aggregated in the presence of 150 mM NaCl or in Dulbecco’s modified Eagle’s medium (DMEM), Aβ was resuspended at 200 μM in reverse osmosis-purified water (MP Biomedicals) and taken to pH 11.5 with 1 N NaOH to solubilize the peptide. Aβ was then filtered through a 30-kDa Ultrafree-MC with UltraCel-PL membrane centrifugal filter (Millipore). The concentra-
tion of Aβ postfiltration was determined using the Bio-Rad Dc protein assay kit with unfiltered Aβ as a standard.

**Aβ Aggregation**—Aβ solubilized in HFIP was resuspended at 2 mM in dimethyl sulfoxide (Me2SO). 2 mM Aβ in Me2SO was diluted to 100 μM in 75 mM MOPS pH 7.4 buffer, and metal ions were added as described in the text. pH was confirmed to be 7.4 by using an Orion Micro pH electrode (Thermo Electron Corp.) or by the addition of phenol red. Aggregation was at 37 °C without stirring. For investigation of Aβ aggregation at early time points, Aβ was brought to 100 μM in MOPS buffer as described above and immediately centrifuged at 320,000 × g for 1.5 h in an Optima TLX desktop ultracentrifuge (Beckman Coulter) to remove pre-existing aggregates. Aβ concentration was determined using the Bio-Rad Dc protein assay kit, with non-centrifuged Aβ as a standard, and the Aβ was aged at 50 μM at 37 °C without stirring. For aging in the presence of 150 mM NaCl or in DMEM, 30-kDa filtered Aβ was brought to pH 7.4 with 75 mM MOPS buffer and aged at 50 μM at room temperature or 10 μM at 37 °C, without stirring. All aggregation experiments were performed at least 3 times per condition.

**Congo Red Binding Assay**—The Congo Red assay was performed according to Klunk et al. (14). Aβ and Congo Red dye content 97%) were diluted to 8–10 μM and 25 μM, respectively, in phosphate-buffered saline (PBS) to a final volume of 150 μl. Aβ aged at 10 μM was used without dilution. Absorbance was read at 405 nm and 540 nm in a Titertek Multiskan Plus 96-well plate spectrophotometer. Congo Red binding by Aβ fibrils was calculated using Equation 13 of Klunk et al. (14).

**Thioflavin T Binding Assay**—The thioflavin T binding assay was performed according to Naiki and Nakakuki (15). 0.5 μM Aβ and 5 μM thioflavin T diluted in 50 mM glycine buffer, pH 8.5, in a total volume of 2 ml were analyzed in a F-4500 fluorescence spectrophotometer (Hitachi). Excitation was set at 446 nm, and emission data were collected at 485 nm.

**Electron Microscopy**—For protofibril and fibril visualization, Aβ was centrifuged at 14,000 rpm in a desktop centrifuge (Eppendorf) at 4 °C for 30 min. The supernatant was removed, and the pellet was resuspended at 10 μM in reverse osmosis-purified water. For preformed fibrils and protofibrils that had been added to culture medium for 24 h at 20 μM, the medium was centrifuged as above, and the pellet was resuspended at 100 μM for use in the Congo Red assay and 10 μM for electron microscopy. Aβ aged at 10 μM was diluted 1:1 with reverse osmosis-purified water. For electron microscopy of early forming Aβ species no spin was performed and samples were diluted 1:1 with reverse osmosis-purified water. All samples were then prepared for negative staining as follows: 2–5 μl of the sample was adsorbed to a carbon-coated grid that was glow-discharged in an Edwards Auto 306 vacuum evaporator for 30 s. Excess liquid was removed with a filter paper (Whatman No. 1), and the samples were stained with 1% uranyl acetate or 1% uranyl formate for 1 min. The grids were examined using a JEOL 1200EX transmission electron microscope.

**Inductively Coupled Plasma Mass Spectrometry**—Samples were acidified to 5% HNO3 using trace metal grade concentrated acid (J. T. Baker Inc.). The amount of metal in each sample was quantified by inductively coupled plasma mass spectrometry (PerkinElmer Life Sciences Elan 6100). Standards were prepared from a concentrated multi-element stand-
ard (SPEX CertiPrep Inc, Metuchen, NJ) that was serially diluted in 5% HNO₃ to obtain working standards in the range of 0.5–25 µg/liter. Indium (2 µg/liter) was used as an internal standard.

SDS-PAGE and Western Blotting—0.5 µl of a 100 µM Aβ sample was diluted with reverse osmosis-purified water and 2× sample buffer to a final volume of 20 µl, and 5 µl was run on a 10–20% Tricine gel (Invitrogen), transferred to polyvinylidene difluoride (Whatman), and probed with monoclonal antibody 6E10 (Signet) for the detection of Aβ.

Neuronal Cultures—E18 rat hippocampal neurons were plated at 1.25 × 10⁵ per well on coverslips coated with lysine and laminin in 24-well plates or at 2 × 10⁴ in 96-well plates coated with lysine. Neurons were plated in DMEM supplemented with 10% bovine calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively). After 2 days in culture the medium was replaced with neurobasal medium supplemented with B27-AO, and a half-medium change performed every 3–4 days. Neurons were treated after 6–7 days in vitro.

Detection of Cell Death—Apoptosis was detected by the characteristic condensed and fragmented morphology of neuronal nuclei observed by Hoechst staining. Cell death due to loss of membrane integrity was measured by the release of lactate dehydrogenase (LDH) into the cell culture medium. Aβ was aged at 100 µM in the presence or absence of 1 or 2 mM calcium for 24–48 h at 37 °C, and fibril and protofibril content was measured by Congo Red and thioflavin T as described. Aβ was then added to 6–7 days in vitro neurons on coverslips in 24-well plates for Hoechst staining or to 96-well plates for LDH measurement for 24 h. For Hoechst staining, the culture medium was removed and the neurons were fixed in 4% paraformaldehyde for 15 min at room temperature, washed in PBS for 5 min, stained with Hoechst dye for 5 min in PBS, and washed in PBS and then water. At least 500 cells were counted per well by an investigator blind to the treatment, and triplicate wells were used per condition. The results shown are the average of at least three independent experiments. For LDH measurement, the CytoTox non-radioactive cytotoxicity assay kit (Promega) was used according to the manufacturer’s instructions. Three independent experiments were performed using 6 wells per condition.

RESULTS

Calcium Accelerates Aβ Aggregation—We observed that Aβ aggregates more rapidly in tap water than reverse osmosis-purified water and that calcium was the main agent in tap water that correlated with this effect (supplemental Fig. 1). We therefore decided to investigate the effects of calcium on Aβ aggregation. Aβ was incubated at 100 µM for up to 120 h at 37 °C in 75 mM MOPS buffer in the presence or absence of 2 mM calcium. The formation of fibrils, as determined by

![Figure 3. Time course of fibril formation in the presence and absence of calcium.](image-url)

![Figure 4. Zinc and copper do not accelerate protofibril to fibril conversion.](image-url)

![Graph](image-url)

* p < 0.001 using ANOVA and Student-Newman-Keuls post hoc test. Each data point comprises 3 independent experiments, error bars are S.E.
Congo Red binding, was significantly accelerated in the presence of calcium. Fibrils start to form within 6 h in the presence of calcium but require 48 h for a similar amount of fibril formation in the absence of calcium (Fig. 1A). In contrast, thioflavin T binding did not show a difference in Aβ aggregation kinetics in the presence or absence of calcium (Fig. 1B). This suggested that calcium was accelerating the formation of an Aβ species that was recognized by Congo Red but not thioflavin T. To further investigate this difference in Aβ structure, Aβ was Western-blotted after incubation for 24 h in either the presence or absence of 2 mM calcium. A species of ~80–120 kDa was identified only in the absence of calcium, and Aβ species of 12 kDa and 16 kDa were also increased in the absence of calcium (Fig. 2A). The sample aged in the presence of calcium had little Aβ signal due to the formation of high molecular weight aggregated protein that was unable to enter the gel. We performed electron microscopy on the Aβ species produced after 48 h in the presence or absence of calcium. When Aβ was incubated with calcium, mature amyloid fibrils formed (Fig. 2B). When Aβ was incubated without calcium for 48 h, small curvilinear structures formed that resembled previously described protofibrils (16, 17), as well as a few longer fibrils (Fig. 2B). The protofibrillar structures had lengths less than 200 nm, also consistent with the dimensions of previously described protofibrils (16, 17). Therefore, it appeared that protofibrils were forming in either the presence or absence of calcium, giving rise to a positive thioflavin T signal and the presence of the 80–120 kDa Aβ species identified by immunoblotting. However, in the presence of calcium, protofibrils were rapidly converted to mature amyloid fibrils. To confirm this sequence of events, an electron microscopy time course was performed. After 6 h of incubation in the presence of calcium, both protofibrils and fibrils were present but by 24 h only mature amyloid fibrils were detected (Fig. 3). In contrast, Aβ incubated in the absence of calcium became protofibrillar by 6 h and remained protofibrillar for up to 72 h of incubation before converting to fibrils (Fig. 3). These results suggest that calcium promotes the conversion of protofibrils to mature amyloid fibrils.

**Zinc and Copper Do Not Accelerate Protofibril to Fibril Conversion**—It has previously been shown that physiological levels of copper and zinc can accelerate Aβ aggregation (8, 9). We therefore tested whether these concentrations of copper and zinc had an effect on protofibril to fibril conversion. Aβ was aged for 24 h in the absence of metal ions or in the presence of 2 mM calcium, 20 μM copper, or 20 μM zinc. Congo Red and
Calcium Accelerates Aβ Aggregation

The effects of calcium were then assessed in the presence of a physiological concentration of sodium chloride at lower Aβ concentrations. Aβ was first passed through a 30-kDa filter to remove preformed larger aggregates. Aβ was aged at 50 μM at room temperature or at 10 μM at 37 °C. Congo Red binding, thioflavin T binding, and electron microscopy showed that under these conditions calcium accelerated protofibril to fibril conversion (Fig. 6A–D). These experiments show that calcium accelerates Aβ aggregation in the presence of physiological concentrations of salt.

Calcium Accelerates the Formation of Early Aβ Aggregates—To determine if calcium can also accelerate the formation of early Aβ aggregates, we analyzed the aggregation state of Aβ in the presence or absence of calcium during the first 2 h of incubation. Freshly solubilized Aβ was centrifuged for 1.5 h at 320,000 × g to remove preformed aggregates. Electron microscopy performed following centrifugation showed that just a few small spherical Aβ aggregates remained (Fig. 7B), which did not lead to a thioflavin T-positive signal (Fig. 7A). After 30 min of incubation, there was a small increase in thioflavin T-positive species in the presence of calcium (Fig. 7A). By electron microscopy, the effect of calcium was more striking. Short individual protofibrils were observed as well as aggregates of protofibrils (Fig. 7B).

After 1 h of incubation in the presence of calcium, thioflavin T binding was significantly greater than that observed in the absence of calcium (Fig. 7A), and protofibrillar and fibrillar structures were apparent by electron microscopy (Fig. 7B). These results suggest that calcium accelerates the formation of protofibrillar as well as fibrillar Aβ aggregates.

Protofibrils and Fibrils Are Both Neurotoxic—To investigate the neurotoxicity of fibrils and protofibrils, these two forms were selectively generated by incubating Aβ in the presence or absence of calcium, respectively. Preformed fibrils and protofibrils were then added to primary rat hippocampal neuronal cultures. The extent of protofibril to fibril conversion in the culture medium of neurons after a 24-h incubation was determined by Congo Red binding and electron microscopy (Fig. 8A and B). There was no significant conversion to fibrils during this time period suggesting that protofibrils are stable in the cell culture medium. Thus, neurobasal/B27-AO medium, which contains 1.8 mM calcium (18), did not promote the conversion of protofibrils to fibrils.

thioflavin T binding showed that only calcium was able to accelerate protofibril to fibril conversion (Fig. 4). We also measured the levels of copper and zinc in our 2 mM calcium solution by inductively coupled plasma mass spectrometry to ensure that contaminating trace copper and zinc were not having an effect. Copper was below our detection limits of 0.5 μg/liter, and zinc was at the level of our detection limits, 0.5 μg/liter ± S.D. of 0.039. This is equivalent to an upper limit of 140 nm zinc. These results suggest that copper and zinc do not have an effect on protofibril to fibril conversion in this Aβ aging paradigm.

Calcium Accelerates Aβ Aggregation at a Range of Calcium and Aβ Concentrations—We then determined the concentration range for calcium-induced acceleration of protofibril to fibril conversion. A standard curve shows that the EC_{50} for calcium-mediated fibril formation was ~4 mg/liter (100 μM) (Fig. 5). This is 20-fold lower than the 2 mM calcium concentration in the extracellular space of the brain, the site at which amyloid plaque deposition occurs. Thus, calcium in the extracellular space of the brain may provide a favorable milieu for amyloid fibril formation and plaque deposition.

FIGURE 7. Calcium accelerates the formation of early Aβ aggregates. Aβ was incubated at 50 μM in 75 mM MOPS at 37 °C with or without 2 mM calcium. A, thioflavin T (ThT) binding was measured at 0, 0.5, 1, and 2 h. B, electron microscopy was performed at 0, 0.5, and 1 h. Small spherical structures (closed arrows) are observed at 0 and 0.5 h with and without calcium. Short protofibrils (open arrows) and larger protofibrillar aggregates (boxed inset) are observed at 0.5 h only in the presence of calcium. The scale bar is 100 nm. *, p < 0.05; **, p < 0.001 using ANOVA and Student–Neuman-Keuls post hoc test. Each data point comprises 4–6 independent experiments, error bars are S.E.
Cell death was measured after 24 h to ensure that the protofibrils had not converted to fibrils. Hoechst dye was used to identify the characteristic nuclear morphology of apoptotic cells. The LDH assay was used to measure cell death via loss of membrane integrity through release of LDH into the cell culture medium. The LDH assay gave a very small but statistically significant increase in cell death with both protofibrils and fibrils at 20 μM (Fig. 8F). The insensitivity of the LDH assay for measuring protofibril and fibril toxicity over the relatively short time period of 24 h has been described previously, with 2–3 days required to see significant cell death (4). We therefore focused on the more sensitive method of scoring apoptotic cells using Hoechst dye. Both protofibrils and fibrils induced neuronal apoptosis, with a trend toward increased toxicity of fibrils versus protofibrils that was statistically significant at a concentration of 10 μM (Fig. 8G). To ensure that small amounts of soluble Aβ species that might remain in our fibrillar preparations after in vitro aggregation were not responsible for the observed toxicity, we centrifuged the fibrillar Aβ preparation and compared the toxicity of the pellet and supernatant. Only the pelletable insoluble Aβ was neurotoxic whereas the supernatant did not elicit any neurotoxicity, indicating that small amounts of contaminating soluble Aβ are not responsible for the observed toxicity (Fig. 8H). These results suggest that both protofibrils and fibrils are inherently neurotoxic.

DISCUSSION

The aggregation of Aβ is a characteristic pathological feature of AD that may contribute to neurodegeneration. These experiments indicate that physiological concentrations of calcium markedly accelerate Aβ aggregation. Calcium had significant effects on the generation of both early protofibrillar forms of Aβ, as well as the conversion of protofibrils to mature amyloid fibrils. The concentration of calcium in the extracellular space of the brain is ~2 mM, which is 20-fold higher than the calcium concentration required to promote fibril formation in vitro. Thus, calcium may play an important role in Aβ aggregation and amyloid dep-
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osition in AD. Moreover, the increased formation of early protofibrillar aggregates suggests that calcium may play a role in the initiation of Aβ aggregation.

A role for metal ions in accelerating Aβ aggregation has previously been reported for zinc and copper (8, 9). We did not observe an effect of zinc and copper on protofibril to fibril conversion under the conditions used. The effects of copper and zinc may occur at earlier stages of Aβ aggregation. This would be consistent with evidence that trace levels of copper and zinc can initiate Aβ aggregation and increase oligomer formation (11). The studies on zinc and copper were performed with Aβ40 (8, 9), whereas this study used Aβ42, which could also explain the differences observed. Further, the copper effect required a slightly acidic pH (8) that was not required for the effects of calcium, which occur at physiological pH. In addition, the effect of zinc on Aβ aggregation is salt-dependent and reversible by zinc chelation (10). In contrast, the effects of calcium are independent of salt concentration and were not reversed by calcium chelation (data not shown). This difference in the stability of zinc- and calcium-induced aggregates suggests that they may have different conformations. The conformation of different Aβ species clearly has an effect on their ability to bind the dyes Congo Red and thioflavin T. The inability of protofibrils to bind Congo Red that we observed is consistent with another report (4). However, a different study reported variable binding of Congo Red to protofibrils (19).

The stability of protofibrils in the absence of calcium enabled an assessment of their effects on cultured neurons, providing evidence that both protofibrils and fibrils are neurotoxic, consistent with previous reports (4, 6, 7). The toxicity of protofibrils appears to be inherent to the protofibrillar structure, as we did not detect significant conversion to fibrils in the conditioned medium of cells treated with protofibrils. This lack of conversion in tissue culture medium appears to be a property of neurobasal/B27-AO medium, as protofibril conversion did occur in DMEM. The reason for this is presently unclear but may relate to the presence of albumin in B27 medium (18), which has been shown to bind Aβ (33). Fibrils also appear to be inherently neurotoxic, as we did not detect protofibrillar contamination in fibrillar preparations generated in the presence of calcium. As a further control, we centrifuged fibrillar Aβ and compared the toxicity of the peptide in the pellet and supernatant. Only the insoluble pelleted fraction that contained the fibrils was toxic, indicating that fibrils alone can mediate toxicity. The supernatant is likely to contain very small amounts of soluble Aβ, as most of the Aβ converts to fibrils during aging in the presence of calcium. Therefore, the lack of neurotoxicity of the supernatant does not suggest that soluble Aβ per se is not toxic but that it cannot explain the toxicity of the fibrils in this case. Previous reports suggest that soluble oligomeric Aβ can be neurotoxic (3, 4). Another study, however, reported that neither fibrils nor soluble Aβ were toxic unless they were combined, suggesting that ongoing polymerization of Aβ is required for Aβ toxicity (34). Although this is likely to be an important mechanism, our results suggest it is not required, as Aβ fibrils alone are neurotoxic.

Several lines of evidence suggest that calcium homeostasis may become altered in the aging brain. Using microarray analysis, we have shown that mRNA levels of the calcium binding proteins calbindin and calretinin decrease significantly in the aging human brain, as does PMCA2, a plasma membrane calcium ATPase responsible for pumping calcium out of the cell (21). Decreases in calbindin and calretinin have also been detected by immunohistochemistry in the brains of aged humans, monkeys, and rodents (22–25). These changes would be expected to lead to an increase in intracellular free calcium, which could potentially initiate Aβ aggregation. Calcium homeostasis may also be altered by AD-causing mutations in presenilin 1 (PS1) (26, 27), and several lines of evidence suggest that Aβ itself can increase intracellular calcium levels (28–31). Moreover, memantine, an N-methyl-D-aspartate receptor antagonist that reduces calcium influx, can improve cognition in moderate to severe AD patients (32). Thus, calcium dysregulation may contribute to the pathogenesis of AD by increasing the formation of neurotoxic aggregated forms of Aβ.

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