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Involvement of Endoplasmic Reticulum Ca^{2+} Release Through Ryanodine and Inositol 1,4,5-Triphosphate Receptors in the Neurotoxic Effects Induced by the Amyloid-β Peptide

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Studies with in-vitro-cultured neurons treated with amyloid-β (Aβ) peptides demonstrated neuronal loss by apoptosis that is due, at least in part, to the perturbation of intracellular Ca^{2+} homeostasis. In addition, it was shown that an endoplasmic reticulum (ER)-specific apoptotic pathway mediated by caspase-12, which is activated upon the perturbation of ER Ca^{2+} homeostasis, may contribute to Aβ toxicity. To elucidate the involvement of deregulation of ER Ca^{2+} homeostasis in neuronal death induced by Aβ peptides, we have performed a comparative study using the synthetic peptides $A\beta_{25-35}$ or $A\beta_{1-40}$ and thapsigargin, a selective inhibitor of Ca^{2+} uptake into the ER. Incubation of cortical neurons with thapsigargin (2.5 μM) increased the intracellular Ca^{2+} levels and activated caspase-3, leading to a significant increase in the number of apoptotic cells. Similarly, upon incubation of cortical cultures with the Aβ peptides ($A\beta_{25-35}$, 25 μM; $A\beta_{1-40}$, 0.5 μM), we observed a significant increase in $[Ca^{2+}]_i$, in caspase-3-like activity, and in number of neurons exhibiting apoptotic morphology. The role of ER Ca^{2+} release through ryanodine receptors (RyR) or inositol 1,4,5-trisphosphate receptors (IP_3R) in Aβ neurotoxicity has been also investigated. Dantrolene and xestospongin C, inhibitors of ER Ca^{2+} release through RyR or IP_3R, were able to prevent the increase in $[Ca^{2+}]_i$ and the activation of caspase-3 and to protect partially against apoptosis induced by treatment with $A\beta_{25-35}$ or $A\beta_{1-40}$. In conclusion, our results demonstrate that the release of Ca^{2+} from the ER, mediated by both RyR and IP_3R, is involved in Aβ toxicity and can contribute, together with the activation of other intracellular neurotoxic mechanisms, to Aβ-induced neuronal death. This study suggests that Aβ accumulation may have a key role in the pathogenesis of AD as a result of deregulation of ER Ca^{2+} homeostasis.

Key words: Alzheimer's disease; amyloid-β peptide; apoptosis; endoplasmic reticulum; Ca^{2+} homeostasis; thapsigargin

A defining feature of Alzheimer's disease (AD), the most common form of senile dementia, is the accumulation of amyloid-β (Aβ; Mattson, 1997), a 39–43-amino-acid peptide derived from the amyloid precursor protein (APP; Wisniewski et al., 1997). The importance of Aβ in the pathogenesis of AD is suggested by several findings. Notably, mutations in APP or presenilin, two proteins that are implicated in familiar forms of AD, lead to an increase in the amyloidogenic forms of Aβ (Selkoe, 1999). In addition, $A\beta_{1-40}$ and $A\beta_{1-42}$ (the principal products resulting from the sequential proteolytic processing of APP by β- and γ-secretases) and the toxic fragment $A\beta_{25-35}$ have been shown to be toxic to a wide variety of cells, including neurons (Yankner, 1996). The mechanism(s) by which these peptides lead to neuronal loss is not well understood, so greater understanding is required and is necessary to future therapeutic intervention. Studies with in-vitro-cultured neurons treated with amyloidogenic Aβ peptides demonstrated neuronal loss by an apoptotic pathway (Loo et al., 1993; Estus et al., 1997). Furthermore, there is good agreement that Aβ-induced apoptosis is due, at least in part, to the perturbation of intracellular Ca^{2+} homeostasis (Scorzielo et al., 1996; Huang et al., 2000).

Several studies have suggested that changes in intracellular Ca^{2+} homeostasis play an important role in the modulation of apoptosis. Many cell death stimuli are known to alter the concentration of Ca^{2+} in the cytosol and the storage of Ca^{2+} in the intracellular organelles (Baffy et al., 1993; Bian et al., 1997). In addition, com-

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pounds that directly affect the intracellular Ca^{2+} homeostasis have been shown to induce apoptosis (Jiang et al., 1994; Reynolds et al., 1996; Wei et al., 1998). Ca^{2+} release channels in the endoplasmic reticulum (ER), i.e., ryanodine receptors (RyR) or inositol 1,4,5-trisphosphate receptors (IP_3R), seem to participate in the signal transduction pathway of apoptosis (Guo et al., 1997; Jayaraman and Marks, 1997; Pan et al., 2000).

It was recently demonstrated a novel ER-specific apoptosis pathway in which caspase-12 acts as the initiator caspase in response to a toxic insult to the ER (Nakagawa et al., 2000). Caspase-12 is specifically involved in apoptosis that results from stress in the ER and seems to be activated upon perturbation of ER Ca^{2+} homeostasis (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). ER stress has received growing attention because it is considered a cause of pathologically relevant apoptosis, and it is implicated particularly in neurodegenerative disorders (Paschen and Frandsen, 2001). Recent evidence points to the involvement of the ER in AD pathogenesis (Ferri and Kroemer, 2000; Mattson and Chan, 2001; Mattson et al., 2001) owing to the fact that it is an important site for generating $A\beta_{1-40}$ and $A\beta_{1-42}$ in neurons (Hartmann et al., 1998) and that both presenilin proteins are localized predominantly in this cellular compartment (Walter et al., 1996).

The goal of the present study was to determine the role of ER Ca^{2+} release in the neurotoxic effects induced by the AD-associated Aβ peptides. For that purpose, primary cultures of rat brain cortical neurons were exposed to the synthetic peptides $A\beta_{25-35}$ or $A\beta_{1-40}$, which have been shown to be toxic to a wide variety of cells, including neurons (Mattson, 1997; Pereira et al., 1999; Cardoso et al., 2002; Casley et al., 2002). In addition, cortical neurons were treated with thapsigargin, a selective ER Ca^{2+}-ATPase inhibitor that leads to the loss of Ca^{2+} homeostasis and to apoptotic cell death (Jiang et al., 1994). Agents that suppress ER Ca^{2+} release through the RyR or the IP_3R were used to investigate the involvement of ER Ca^{2+} release channels in the deregulation of Ca^{2+} homeostasis and in the apoptotic death process that occurs upon Aβ exposure. The results support a mechanistic link among Aβ accumulation, ER dysfunction, and apoptotic neuronal death in AD.

MATERIALS AND METHODS

Materials

Neurobasal medium and B27 supplement were purchased from Gibco BRL, Life Technologies (Paisley, United Kingdom). Trypsin, DNase I, trypsin inhibitor type II-S-soybean, dantrolene, and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO). SYTO-13, propidium iodide (PI), and Indo-1 acetoxymethyl ester (Indo-1/AM) were purchased from Molecular Probes (Leiden, The Netherlands). The synthetic $A\beta_{25-35}$ and $A\beta_{1-40}$ peptides and the respective reverse sequences $A\beta_{35-25}$ and $A\beta_{40-1}$ were from Bachem (Bubendorf, Switzerland). Ionomycin and xestospongin C were purchased from Calbiochem (Darmstadt, Germany). The colorimetric substrate for caspase-3 [N-*acetyl*-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA)] was also from Calbiochem. All the others chemicals were obtained from Sigma or from Merck kgaA (Darmstadt, Germany).

Primary Cortical Neuronal Cultures and Experimental Treatments

Primary cultures of cortical cells were prepared from 15–16-day embryos of Wistar rats according to the method described by Hertz et al. (1989), with some modifications (Agostinho and Oliveira, 2003). Briefly, the neocortices of embryos were dissected and placed in Ca^{2+}- and Mg^{2+}-free Krebs buffer (in mM): NaCl 120, KCl 4.8, KH_2PO_4 1.2, glucose 13, Hepes 10 (pH 7.4), supplemented with 0.3% (wt/vol) bovine serum albumin (BSA). Minced cortical tissues were washed and incubated in Krebs buffer containing 0.05% (wt/vol) trypsin and 0.004% (wt/vol) DNase I, for 10 min at 37°C. The digestion was stopped with Krebs buffer containing 0.05% (wt/vol) trypsin inhibitor (type II-S) and 0.004% (wt/vol) DNase I, and the tissue was centrifuged at 140*g* for 5 min. After the pellet was washed once with Krebs buffer, the cells were dissolved in fresh Neurobasal medium supplemented with 2 mM L-glutamine, 2% (vol/vol) B27 supplement, penicillin (100 U/ml), and streptomycin (100 μg/ml) and were dissociated mechanically. Under these culture conditions, 90% of the cells are neurons as evaluated using microtubule-associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP) immunoreactivity (data not shown). Cortical cells were plated on poly-L-lysine (0.1 mg/ml)-coated plates or coverslips at a density of 0.10×10^6 cells/cm^2 for fluorescence microscopy experiments or at 0.50×10^6 cells/cm^2 for measurement of $[Ca^{2+}]_i$ and caspase-3-like activity. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and were used for experiments after 5–7 days in vitro. Cortical neurons were treated with $A\beta_{25-35}$ or $A\beta_{35-25}$ (25 μM), $A\beta_{1-40}$ or $A\beta_{40-1}$ (0.5 μM), or thapsigargin (2.5 μM, a concentration that induces 50% decrease in cell viability at 24 hr) in serum-free Neurobasal medium supplemented with B27. The concentrations of Aβ peptides used were chosen based on previous results demonstrating that 25 μM $A\beta_{25-35}$ and 0.5 μM $A\beta_{1-40}$ induced a maximal toxic effect (50% decrease in cell viability) on cortical neurons. Before the addition of Aβ peptides, cells were preincubated for 1 hr with dantrolene (10 μM) or xestospongin C (1 μM), concentrations that were shown to exert maximal protective effects, without being toxic by themselves (data not shown).

Assessment of Neuronal Injury

After treatment of cortical cells with thapsigargin or with Aβ peptides, neuronal injury was assessed by analysis of the nuclear morphology by fluorescence microscopy of live cells using the stains PI, which fluoresces red and enters the cells only when membrane integrity is lost, and SYTO-13, which fluoresces green and enters the cells with intact cytoplasmic membrane (Rego et al., 2001). Cortical cells cultured on glass coverslips were treated with thapsigargin or with Aβ peptides in the absence or in the presence of dantrolene or xestospongin C. After washing, cells were incubated in sodium medium (in

mM): NaCl 132, KCl 4, NaH_2PO_4 1.2, $MgCl_2$ 1.4, glucose 6, HEPES-Na 10, and $CaCl_2$ 1, pH 7.4, containing 3.8 μM SYTO-13 and 2.5 μg/ml of PI for 3 min at 37°C. Viable, necrotic, or apoptotic neurons were visualized by nuclear morphology criteria. Viable neurons display a normal nuclear size and green fluorescence. Necrotic neurons manifest red fluorescence without chromatin condensation. Scored apoptotic neurons include neurons that display pyknotic nuclei with condensed or fragmented chromatin and fluoresce green and secondary necrotic neurons that display condensed or fragmented chromatin and fluoresce red. Cells were examined and scored with a Nikon Diaphot TMP microscope. All experiments were performed in duplicate, and at minimum 300 cells were scored for each coverslip. The number of viable, necrotic, and apoptotic cells was expressed as the percentage of the total number of cells in the microscope field.

Measurement of Intracellular Ca^{2+} Concentration

Control or treated cortical cells cultured on glass coverslips were incubated with 3 μM Indo-1/AM in a sodium medium containing (in mM): NaCl 132, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1.4, glucose 6, HEPES-Na 10, pH 7.4, for 45 min at 37°C. The cells were further incubated in sodium medium for 15 min at 37°C to ensure a complete hydrolysis of the acetoxymethyl ester of Indo-1. After being washed, cells were mounted in a special holder, and the Indo-1 fluorescence was measured with excitation of 335 nm and 410 nm emission. The free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated as previously described (Bandeira-Duarte et al., 1990).

Caspase-3-Like Activity Assay

Untreated or treated cortical cells were lysed with a buffer containing (in mM): HEPES-Na 25, $MgCl_2$ 2, EDTA 1, EGTA 1, supplemented with 100 μM phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), and a protease inhibitor cocktail (containing 1 μg/ml leupeptin, pepstatin A, chymostatin, and aprotinin). The cellular suspension was rapidly frozen/defrosted three times and then centrifuged for 10 min at 20,200*g*. The supernatant was collected and assayed for protein content by using the Bio-Rad protein dye assay reagent. To measure caspase-3-like activity, aliquots of cell extracts containing 25 μg of protein were incubated for 2 hr at 37°C, in a reaction buffer containing 25 mM HEPES-Na, 10 mM DTT, 10% (wt/vol) sucrose, and 0.1% (wt/vol) CHAPS (pH 7.4), with 100 μM Ac-DEVD-pNA, a chromogenic substrate for caspase-3 (Cregan et al., 1999). Caspase-3-like activity was determined by measuring substrate cleavage at 405 nm with a microplater reader. Results were expressed as the increase above control absorbance at 405 nm.

Statistical Analysis

Results are expressed as means ± SEM of the number of experiments indicated in the figure legends. Statistical significance was analyzed by analysis of variance (ANOVA), followed by Dunnett's post hoc tests for multiple comparisons or by the unpaired two-tailed Student's *t*-test. $P < .05$ was considered statistically significant.

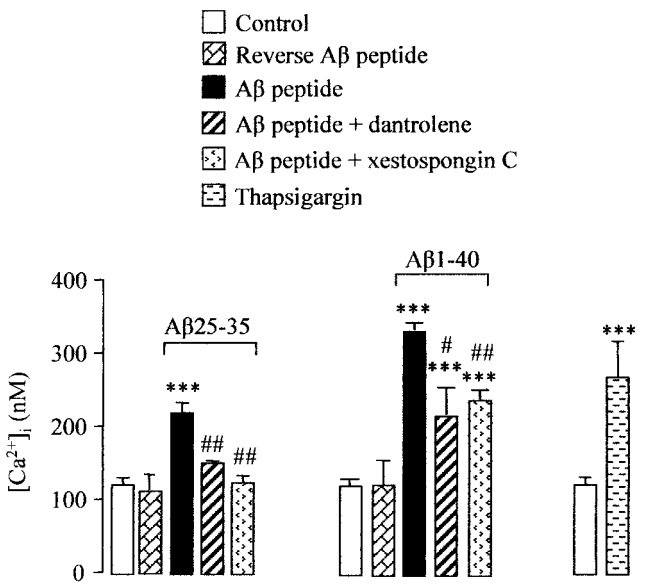

Fig. 1. Effect of $A\beta_{25-35}$, $A\beta_{1-40}$, and thapsigargin on basal intracellular Ca^{2+} levels in cortical neurons. Cells cultured on coverslips were treated, for 24 hr, with $A\beta_{25-35}$ (25 μM), $A\beta_{1-40}$ (0.5 μM), in the absence or in the presence of dantrolene or xestospongin C, or with thapsigargin (2.5 μM). The fluorescence of Indo-1-loaded cells was measured, and the $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. The results are the means ± SEM of values corresponding to at least three experiments, each value being the mean of duplicate assays. ***$P < .001$ with respect to control values. $^{\#}P < .05$, $^{\#\#}P < .01$ with respect to Aβ addition.

RESULTS

Intracellular Calcium Levels Are Increased in Cortical Neurons Treated With $A\beta_{25-35}$, $A\beta_{1-40}$, and Thapsigargin; Dantrolene and Xestospongin C Prevent Aβ-Induced $[Ca^{2+}]_i$ Rise

To analyze the involvement of ER Ca^{2+} release in the toxic effects of $A\beta_{25-35}$ or $A\beta_{1-40}$ on cortical neuronal cultures, the intracellular Ca^{2+} levels were determined in the absence or in the presence of dantrolene or xestospongin C, which inhibit the release of Ca^{2+} through the ER RyR or IP_3R, respectively. In addition, cytosolic Ca^{2+} concentration was determined in neurons exposed to thapsigargin, a known inhibitor of the ER Ca^{2+}-ATPase that deregulates ER Ca^{2+} homeostasis.

Treatment of cells with 25 μM $A\beta_{25-35}$ increased the intracellular Ca^{2+} levels, as shown in Figure 1. After 24 hr, the $[Ca^{2+}]_i$ increased from 122.60 ± 9.78 nM in control to 201.90 ± 20.65 nM in $A\beta_{25-35}$-treated cells. When cells were preincubated for 1 hr with dantrolene (10 μM) or xestospongin C (1 μM) before $A\beta_{25-35}$ addition, the intracellular Ca^{2+} levels decreased significantly and were similar to those determined for controls (Fig. 1). The $[Ca^{2+}]_i$ measured after incubation with $A\beta_{25-35}$ during 24 hr, in the presence of dantrolene, was 152.30 ± 3.11 nM and the $[Ca^{2+}]_i$ measured after $A\beta_{25-35}$ exposure during 24 hr, in the presence of xestospongin C, was

125.60 ± 9.64 nM. Cells treated with $A\beta_{1-40}$ (0.5 μM) also showed an increament in intracellular Ca^{2+} levels in comparison with controls (Fig. 1). After treatment for 24 hr with $A\beta_{1-40}$, $[Ca^{2+}]_i$ increased 2.7-fold above control values (Ca^{2+} levels increased from 122.60 ± 9.78 nM in controls to 336.10 ± 9.37 nM in cells treated with $A\beta_{1-40}$). When cells were pretreated with dantrolene (10 μM) or xestospongin C (1 μM) before $A\beta_{1-40}$ addition, intracellular Ca^{2+} levels decreased significantly (Fig. 1). Intracellular Ca^{2+} levels decreased from 336.10 ± 9.37 nM to 219.50 ± 36.90 nM vs. 240.80 ± 12.60 nM in cells treated with $A\beta_{1-40}$ in the absence or in the presence of dantrolene vs. xestospongin C, respectively. Incubation of cortical neurons with dantrolene or xestospongin C alone during 24 hr did not affect the $[Ca^{2+}]_i$ compared with control. Unlike $A\beta_{25-35}$ and $A\beta_{1-40}$, the reverse peptides $A\beta_{35-25}$ (25 μM) and $A\beta_{40-1}$ (0.5 μM) were not able to increase intracellular free Ca^{2+} levels.

When cells were treated with 2.5 μM thapsigargin, an increase in intracellular Ca^{2+} levels was also observed (Fig. 1). After incubation of cortical neurons with thapsigargin during 24 hr, the $[Ca^{2+}]_i$ increased 2.2-fold above control values (Ca^{2+} levels increased from 122.60 ± 9.78 nM in controls to 269.30 ± 8.69 nM in thapsigargin-treated cells at 24 hr).

Caspase-3 Activation Occurs After $A\beta_{25-35}$ or $A\beta_{1-40}$ and Thapsigargin Treatment; Dantrolene and Xestospongin C Protect From Aβ-Induced Caspase-3 Activation

Cells treated with $A\beta_{25-35}$ (25 μM) for 24 or 48 hr showed a significant increase in caspase-3-like activity (Fig. 2), this increase being higher after 24 hr than after 48 hr of treatment. A 3.58-fold increase above control in the activity of caspase-3 was determined in cells exposed to $A\beta_{25-35}$ during 24 hr, whereas an increment of 2.23-fold was measured in cells treated with this peptide during 48 hr. When cells were treated with $A\beta_{1-40}$ (0.5 μM), a significant increase in caspase-3 activation was observed at 48 hr of treatment, but no caspase-3 activation was demonstrated after 24 hr of incubation (Fig. 2). $A\beta_{1-40}$ (0.5 μM) induced a 1.67-fold increase above control values. Caspase-3-like activity was not significantly affected by treatment with the reverse peptides ($A\beta_{35-25}$, 25 μM, and $A\beta_{40-1}$, 0.5 μM).

Cells treated with thapsigargin also demonstrated caspase-3 activation (Fig. 2). An activation of 7.20-fold was determined for caspase-3 upon 24 hr incubation with 2.5 μM thapsigargin. No significant differences were detected in caspase-3 activity between 24 and 48 hr incubation in the presence of thapsigargin.

Because caspase-3 activation is higher after incubation for 24 hr than for 48 hr in the presence of Aβ25–25 (25 μM) and because caspase-3 is only activated upon exposure of $A\beta_{1-40}$ (0.5 μM) for 48 hr, the protective effect of dantrolene and xestospongin C against Aβ-induced caspase-3 activation was analyzed under these experimental conditions. As shown in Figure 3A, preincubation of cortical cells with dantrolene or xestospongin C before $A\beta_{25-35}$ (25 μM) addition significantly reduced caspase-3 activation induced by this peptide (Ac-DEVD-pNA cleavage decreased from 3.58 ± 0.72 to 1.53 ± 0.20 vs. 1.48 ± 0.04 in cells treated with $A\beta_{25-35}$ in the absence or in the presence of dantrolene vs. xestospongin C, respectively). Cells pretreated with dantrolene or xestospongin C before $A\beta_{1-40}$ addition showed a significant decrease in caspase-3 like activity (Ac-DEVD-pNA cleavage decreased from 1.67 ± 0.33 to 1.01 ± 0.05 vs. 0.86 ± 0.14 in cells treated with $A\beta_{1-40}$ in the absence or in the presence of dantrolene vs. xestospongin C, respectively; Fig. 3B).

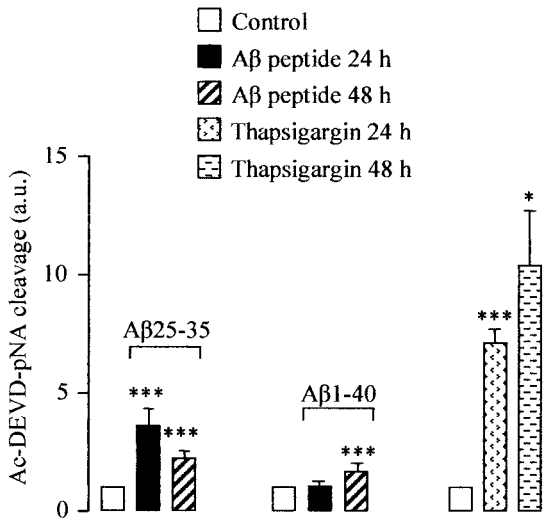

Fig. 2. Caspase-3 activation in cortical neurons induced by $A\beta_{25-35}$, $A\beta_{1-40}$, or thapsigargin. After incubation for 24 or 48 hr with 25 μM $A\beta_{25-35}$, 0.5 μM $A\beta_{1-40}$, or 2.5 μM thapsigargin, the cultured cortical cells were lysed, and the protein was extracted. Caspase-3-like activity was determined by measuring the cleavage of the chromogenic substrate Ac-DEVD-pNA at 405 nm, as described in Materials and Methods. The results, expressed as the increase above control values, are the means ± SEM of values corresponding to at least to three experiments, each value being the mean of duplicate assays. $\star P < .05$, $\star\star\star P < .001$ with respect to control values.

$A\beta_{25-35}$, $A\beta_{1-40}$, and Thapsigargin Reduce Cell Viability and Induce Apoptosis; Dantrolene and Xestospongin C Protect From Aβ Toxicity

In cortical neurons, the number of viable, necrotic, and apoptotic cells upon exposure to Aβ peptides or thapsigargin was analyzed by fluorescence microscopy after SYTO-13/PI labeling. Exposure of cortical neurons to $A\beta_{25-35}$ (25 μM) revealed a reduction in the number of viable cells after 48 hr of incubation (Fig. 4A). Dantrolene (10 μM) and xestospongin C (1 μM) protected cortical neurons against $A\beta_{25-35}$-induced toxicity (the number of viable cells was 46.89% ± 1.94%, 71.3% ± 2.22%, and 81.78% ± 3.01% in cells treated with $A\beta_{25-35}$ alone, with $A\beta_{25-35}$ plus dantrolene, or with $A\beta_{25-35}$ plus xestospongin C, respectively). Incubation of cortical neurons with $A\beta_{25-35}$ peptide during 48 hr increased significantly the

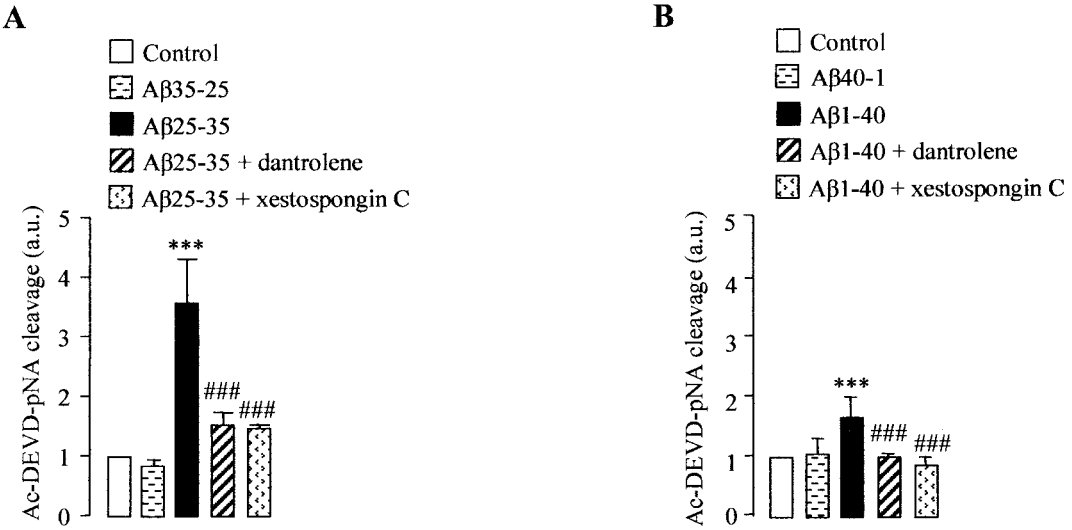

Fig. 3. Effect of dantrolene and xestospongin C on the increase in caspase-3-like activity induced by $A\beta_{25-35}$ (**A**) or $A\beta_{1-40}$ (**B**). After incubation for 24 hr with the peptide $A\beta_{25-35}$ (25 μM) and incubation for 48 hr with the peptide $A\beta_{1-40}$ (0.5 μM), the cultured cortical cells were lysed, and the protein was extracted. Caspase-3-like activity was determined by measuring Ac-DEVD-pNA cleavage at 405 nm, as described in Materials and Methods. The results, expressed as the increase above control values, are the means ± SEM of values corresponding to at least to three experiments, each value being the mean of duplicate assays. $^{***}P < .001$ with respect to control values. $^{\#\#\#}P < .001$ with respect to Aβ addition.

number of necrotic cells, the increase being prevented by dantrolene or xestospongin C (35.24% ± 2.39%, 15.68% ± 3.01%, and 9.26% ± 2.76% in cells treated with $A\beta_{25-35}$ alone, with $A\beta_{25-35}$ plus dantrolene, or with $A\beta_{25-35}$ plus xestospongin C, respectively). Furthermore, in the presence of $A\beta_{25-35}$ a higher number of neurons exhibiting apoptotic morphology was observed (from 2.76% ± 0.47% in controls to 25.68% ± 1.48% in $A\beta_{25-35}$-treated cells; Fig. 4A). Dantrolene was shown to prevent significantly, although partially, the $A\beta_{25-35}$-induced increase in the number of apoptotic cells (8.21% ± 1.15% or 25.68% ± 1.48% apoptotic cells after treatment with $A\beta_{25-35}$ in the presence or in the absence of dantrolene). Similarly, xestospongin C prevented the increase in the number of apoptotic cells induced by $A\beta_{25-35}$ (8.51% ± 0.91% or 25.68% ± 1.48% apoptotic cells after treatment with $A\beta_{25-35}$ in the presence or in the absence of xestospongin C). Treatment of cortical cells with $A\beta_{35-25}$ (25 μM) for 48 hr did not affect the number of viable, necrotic, or apoptotic cells in comparison with the control situation (Fig. 4A).

Similar analysis was performed for the peptide $A\beta_{1-40}$ (0.5 μM). As observed for the $A\beta_{25-35}$ peptide, the SYTO-13/PI labeling also revealed a reduction in cell viability in cortical neurons treated with $A\beta_{1-40}$ (0.5 μM) for 48 hr and the protective effect of dantrolene and xestospongin C (the number of viable cells was 50.95% ± 2.43%, 77.74% ± 1.98%, or 73.33% ± 3.00% in cells treated with $A\beta_{1-40}$ alone, with $A\beta_{1-40}$ plus dantrolene, or with $A\beta_{1-40}$ plus xestospongin C, respectively; Fig. 4B). Furthermore, the number of necrotic cells after $A\beta_{1-40}$ exposure (28.09% ± 1.46%) is higher compared with control (13.95% ± 1.24%). The incubation with dantrolene and $A\beta_{1-40}$ or with xestospongin C and $A\beta_{1-40}$ reduced the number of necrotic cells, exerting its protective effect over cortical neurons (10.05% ± 1.45% or 11.25% ± 0.65%, respectively). Likewise, upon treatment of cortical neurons with $A\beta_{1-40}$, the number of apoptotic cells increased above control (2.76% ± 0.48% and 20.96% ± 1.80% in control cells and $A\beta_{1-40}$-treated cells, respectively). Once again, both dantrolene and xestospongin C show a protective effect evident in the reduced number of apoptotic cells (5.68% ± 0.76% or 5.71% ± 0.95% with $A\beta_{1-40}$ in the presence of dantrolene or xestospongin C, respectively). Treatment of cortical cells with the reverse peptide, $A\beta_{40-1}$ (0.5 μM), for 48 hr did not affect the number of viable, necrotic, or apoptotic cells (Fig. 4B). Incubation of cortical neurons with dantrolene or xestospongin C alone did not affect the SYTO-13/PI fluorescence in comparison with controls.

Finally, data obtained also demonstrated a decrease in the number of viable cells and an increase in the number of apoptotic cells after incubation with thapsigargin (Fig. 4C). The number of viable cells decreased from 79.36% ± 2.26% in controls to 55.32% ± 4.47% or 35.05% ± 2.54% in thapsigargin-treated cells and the number of apoptotic cells increased from 2.76% ± 0.47% in controls to 24.25% ± 2.67% or 54.40% ± 2.54% upon treatment with thapsigargin during 24 or 48 hr, respectively. The number of necrotic cells was not significantly affected upon thapsigargin treatment.

DISCUSSION

Our results with primary cultures of rat brain cortical neurons showed that incubation with the $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides significantly increases the $[Ca^{2+}]_i$, activates caspase-3, and leads to apoptotic cell death. On the

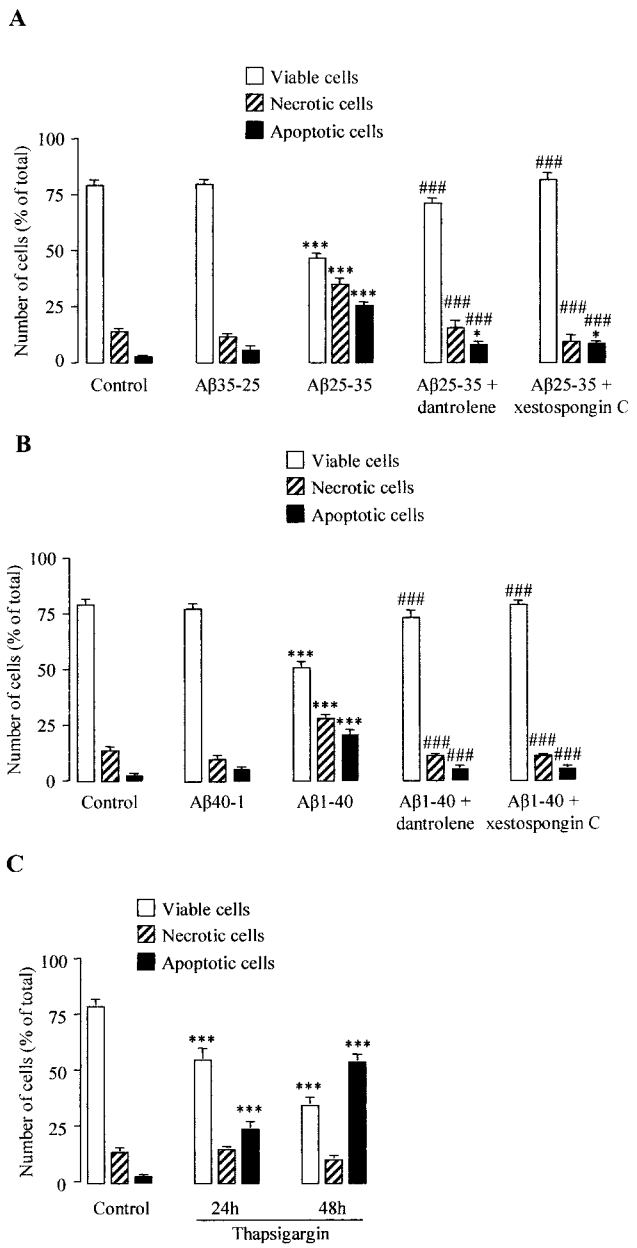

Fig. 4. DNA fragmentation induced by $A\beta_{25-35}$, $A\beta_{1-40}$, or thapsigargin. Cultured cortical neurons were treated, for 48 hr, with $A\beta_{25-35}$ (25 μM; **A**) or $A\beta_{1-40}$ (0.5 μM; **B**), in the absence or in the presence of dantrolene or xestospongin C, or with thapsigargin (2.5 μM) for 24 and 48 hr (**C**). The cells were then incubated with SYTO-13 and PI to visualize nuclei morphology and were scored with a fluorescence microscope. The results, expressed as the percentage of the total number of cells, are the means ± SEM of values corresponding to at least to three experiments, each value being the mean of duplicate assays. $^{\star}P < .05$, $^{\star\star\star}P < .001$ with respect to control values. $^{\#\#\#}P < .001$ with respect to Aβ addition.

other hand, the reversed peptides, $A\beta_{35-25}$ or $A\beta_{40-1}$, were not able to induce neuronal injury. The inhibitors of the ER Ca^{2+} release through the RyR and IP_3R, dantrolene and xestospongin C, respectively, prevented the $[Ca^{2+}]_i$ rise, the activation of caspase-3, or the increase in number of cells exhibiting apoptotic morphology triggered by the Aβ peptides. Therefore, the present study demonstrates that Ca^{2+} release through the ER RyR or IP_3R is induced by the Aβ peptides and is involved in Aβ-induced apoptotic death. The comparative study performed with thapsigargin, a selective ER Ca^{2+}-ATPase inhibitor, which was shown to lead to the loss of Ca^{2+} homeostasis, to caspase-3 activation, and to apoptotic cell death, further supports this hypothesis.

Several studies have suggested that changes in intracellular Ca^{2+} homeostasis play an important role in the modulation of apoptosis. Compounds that directly affect the intracellular Ca^{2+} homeostasis have been shown to induce apoptosis (Jiang et al., 1994; Reynolds et al., 1996). The antiapoptotic protein Bcl-2 protects against thapsigargin-induced increases of Ca^{2+} and apoptosis and was shown to suppress Ca^{2+} release from ER (Guo et al., 1997; Wei et al., 1998). Recently, degradation of caspase-3, cleavage of poly(ADP-ribose) polymerase, DNA fragmentation, and cell death were observed in human neuroblastoma SH-SY5Y cells treated with thapsigargin (Kitamura et al., 2003). Previous studies with in-vitro-cultured neurons treated with Aβ peptides demonstrated neuronal loss by apoptosis (Loo et al., 1993; Estus et al., 1997) that was due at least in part to the perturbation of intracellular Ca^{2+} homeostasis (Scorzielo et al., 1996; Huang et al., 2000). However, the routes of Ca^{2+} homeostasis deregulation in Aβ-treated cells remain controversial. Ca^{2+} influx through voltage-sensitive Ca^{2+} channels has been reported to be involved in the neurotoxic effects of $A\beta_{25-35}$ (Guo et al., 1996; Ekinci et al., 2000). Others have hypothesized that the ability of Aβ peptides to form amyloid channels, and the subsequent disruption of Ca^{2+} homeostasis, could be the molecular basis of the neurotoxicity of these peptides (Kawahara and Kuroda, 2000). Guo et al. (1996, 1997) have demonstrated that Ca^{2+} release from RyR is involved in $A\beta_{25-35}$ toxicity in PC12 cells. In the present study, with cortical neurons in culture, we have shown that, in addition to Ca^{2+} release through RyR, IP_3R also contributes to $A\beta_{25-35}$- and also $A\beta_{1-40}$-induced cell death, insofar as the increase in $[Ca^{2+}]_i$ and the induction of apoptotic cell death were significantly prevented by dantrolene and also by xestospongin C, a specific inhibitor of IP_3R-mediated Ca^{2+} release. IP_3-mediated Ca^{2+} release from the ER was previously demonstrated to trigger apoptosis in response to diverse signals (Distelhorst and Roderick, 2003). Many cell death stimuli are known to alter the concentration of Ca^{2+} in the cytosol and the storage of Ca^{2+} in the intracellular organelles (Baffy et al., 1993; Bian et al., 1997). Lam et al. (1993) showed that glucocorticoids induce release of calcium from ER, which is correlated with subsequent DNA fragmentation and apoptosis. Moreover, Ca^{2+} release channels in the ER, i.e., IP_3R and RyR, seem to participate in the signal transduction pathway of apoptosis (Guo et al., 1997; Jayaraman and Marks, 1997; Pan et al., 2000). Dubrez et al. (2001) have

shown that caffeine, a well-known inducer of Ca^{2+} release through ryanodine-sensitive Ca^{2+} channels expressed in ER, induces translocation of the proapoptotic protein Bax to the mitochondria, leading to caspase-3 activation and apoptotic cell death. Recent studies indicate that IP_3R-mediated Ca^{2+} spikes trigger opening of the mitochondrial permeability transition pore and, in turn, the release of cytochrome c (Szalai et al., 1999; Brustovetsky et al., 2002). The effect of added extracellular Aβ peptides on intracellular Ca^{2+} stores located at the ER could involve a direct action of Aβ on surface membrane constituents, which could modulate the activity of intracellular constituents. It was previously demonstrated that Aβ toxicity involves the early induction of membrane lipid peroxidation, which liberates 4-hydroxynonenal that binds to ion channels at the ER and thereby alters their activities, leading to the perturbation of ER Ca^{2+} homeostasis (Mattson, 1998). Furthermore, Aβ peptides could be internalized and act directly on the ER Ca^{2+} channel, increasing its Ca^{2+} sensitivity, as occurs with caffeine (Zhao et al., 2001), or could form a pore permeable to Ca^{2+} in the ER membrane.

ER Ca^{2+} release has been shown to have immediate effects on mitochondrial function, leading to rapid Ca^{2+} accumulation in mitochondria, which promotes cytochrome c release and activation of downstream caspase pathways in cells exposed to proapoptotic agents (Nutt et al., 2002). The $A\beta_{25-35}$- or $A\beta_{1-40}$-induced release of Ca^{2+} from ER might stimulate mitochondrial Ca^{2+} uptake, leading to cytochrome c release and activation of a caspase-dependent apoptotic cascade. In our laboratory, it was recently demonstrated that incubation of cortical neurons with $A\beta_{25-35}$ (25 μM) increases mitochondrial cytochrome c release and activates caspase-3 (Agostinho and Oliveira, 2003). Moreover, mitochondrial dysfunction was previously demonstrated upon $A\beta_{25-35}$ or $A\beta_{1-40}$ treatment (Pereira et al., 1998). Taken together, these observations suggest that ER stress induced by the disruption of Ca^{2+} homeostasis in neuronal cells exposed to Aβ peptides may be associated with mitochondrial dysfunction. The disruption of Ca^{2+} homeostasis mediated through Ca^{2+} release by ER IP_3R and RyR may be intermediate between ER stress and mitochondrial dysfunction. In human neuroblastoma cells, both ER- and mitochondrial-dependent pathways were shown to be involved in thapsigargin-induced apoptosis (Kitamura et al., 2003).

One of the mechanisms involved in the activation of apoptotic cell death upon $A\beta_{25-35}$- and $A\beta_{1-40}$-induced Ca^{2+} release from ER could be related to calcineurin activation. It was recently demonstrated in our laboratory that this Ca^{2+}/calmodulin-dependent phosphatase is involved in $A\beta_{25-35}$ toxicity in cortical neurons (Agostinho and Oliveira, 2003). Calcineurin has previously been proposed as a link between Ca^{2+} deregulation and neuronal cell death (Wang et al., 1999; Foster et al., 2001). Jayaraman and Marks (2000) have shown that calcineurin is downstream of the IP_3R in the apoptotic pathway, supporting the involvement of ER Ca^{2+} homeostasis deregulation in calcineurin-mediated apoptosis. When activated, calcineurin dephosphorylates the proapoptotic BAD protein, allowing its translocation to the mitochondria, where it binds to antiapoptotic Bcl-2 family members and induces cytochrome c release (Springer et al., 2000). In the cytosol, cytochrome c binds Apaf-1 and dATP to form a complex that activates caspase-9, which can activate the executioner caspase-3 (Desagher and Martinou, 2000).

Several studies have shown that caspase-3 activation is an initial event in neurodegeneration induced by $A\beta_{25-35}$ (Mattson et al., 1998; Harada and Sugimoto, 1999; Xiao et al., 2002). Under our experimental conditions, caspase-3 activity increased upon treatment with $A\beta_{25-35}$ or $A\beta_{1-40}$, this increase being prevented by the ER Ca^{2+} release inhibitors dantrolene and xestospongin C, which suggests that perturbation of ER Ca^{2+} homeostasis mediated by Ca^{2+} release from RyR and IP_3R is involved in the activation of caspase-3. Furthermore, thapsigargin significantly increased caspase-3 activity, supporting the activation of this caspase executioner of apoptosis under conditions of perturbed ER Ca^{2+} homeostasis. Increased Ca^{2+} release from ER has been previously associated with caspase-3 activation (Kim et al., 2002). In addition, activation of caspase-3 has been shown to be completely suppressed in cells overexpressing Bcl-2 targeted specifically to the ER (Häcki et al., 2000).

In conclusion, our results show that $A\beta_{25-35}$ and $A\beta_{1-40}$ induce Ca^{2+} homeostasis deregulation, caspases activation, and apoptotic neuronal death. Dantrolene and xestospongin C, inhibitors of the ER Ca^{2+}-release channels RyR and IP_3R, respectively, prevented the $A\beta_{25-35}$- or $A\beta_{1-40}$-induced rise in $[Ca^{2+}]_i$, caspases activation, and apoptotic cell death, indicating that Ca^{2+} release through the ER RyR and IP_3R is involved in the neurotoxicity induced by these peptides. This hypothesis is further supported by the data obtained with thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase. Therefore, ER stress resulting from deregulation of ER Ca^{2+} homeostasis seems to play a crucial role in neurodegeneration triggered by the AD-associated Aβ peptide. However, because of the partial protective effect of dantrolene and xestospongin C against Aβ-induced neuronal death, it cannot be ruled out the hypothesis that other intracellular neurotoxic mechanisms could be triggered by the Aβ peptides and contribute to apoptotic death, in a manner independent of or synergistic with Ca^{2+}-sensitive ER stress. Given the role of perturbed ER function in AD (Mattson and Chan, 2001; Mattson et al., 2001), the regulation of ER Ca^{2+} homeostasis can be a target for the treatment of this neurodegenerative disorder.

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