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Involvement of calcineurin in the neurotoxic effects induced by amyloid-beta and prion peptides

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Abstract

It is usually accepted that prion and amyloid-beta (Aβ) peptides induce apoptotic cell death. However, the mechanisms that trigger neuronal death, induced by these amyloidogenic peptides, remain to be clarified. In the present study we analysed the neurotoxic effects of the synthetic prion and Aβ peptides, $PrP_{106\text{-}126}$ and $A\beta_{25-35}$, in primary cultures of rat brain cortical cells. $PrP_{106\text{-}126}$ and $A\beta_{25-35}$ incubated at a concentration of 25 μM for 24 h, did not affect cell membrane integrity, but decreased the metabolic capacity of the cells. The intracellular free Ca^{2+} concentration and reactive oxygen species levels increased significantly after 24 h treatment with $PrP_{106\text{-}126}$ and $A\beta_{25-35}$. Furthermore, these peptides (after 24 h exposure) also induced cytochrome *c* release from mitochondria and increased caspase-3-like activity. FK506, an inhibitor of the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, was able to prevent cytochrome *c* release, caspase-3 activation and cell death induced by $A\beta_{25-35}$ or $PrP_{106\text{-}126}$ peptides. Taken together these data suggest that calcineurin is involved in $A\beta_{25-35}$ and $PrP_{106\text{-}126}$ neurotoxicity.

Introduction

The scrapie isoform of prion protein (PrP^{Sc}) and the amyloid-beta protein (Aβ), resulting from the abnormal proteolytic cleavage of amyloid precursor protein, are involved in the pathogenesis of prion-related encephalopathies (PRE) and in Alzheimer's disease (AD), respectively. These are protease-resistant proteins that have a structure enriched in β-sheet conformations, and which aggregate extracellulary in brain as amyloid fibrils (Prusiner, 1996; Wisniewski *et al*., 1997). The amyloid plaques formed in AD and in PRE have the same basic structure, consisting of a core surrounded by degenerated neurites, microglia and astrocytic processes (Selkoe, 1993; Forloni, 1996; Prusiner, 1998). The PRE, that include scrapie and bovine spongiform encephalopathy in animals and Creutzfeldt–Jakob disease in humans, are a group of fatal neurodegenerative disorders neuropathologically characterized by deposition of PrP^{Sc} amyloid plaques, gliosis, vacuolation and neuronal loss. The symptoms of these diseases are, usually, motor and cognitive dysfunction, loss of memory leading to dementia and finally death (Forloni *et al*., 1996; Prusiner, 1996). AD, the most common form of dementia in the elderly, is characterized by the accumulation of intracellular neurofibrillary tangles, formed by hyperphosphorylated tau protein, and extracellular senile plaques, mainly composed by Aβ peptides, which have been shown to be associated to neuronal death (Selkoe, 1993; Wisniewski *et al*., 1997).

An apoptotic pathway of neuronal death appears to be a prominent feature of AD and PRE (Smale *et al*., 1995; Brown *et al*., 1997; Gray *et al*., 1999). Certain caspases (cysteine aspartate-specific proteases), such as caspase-3, may play a role in executing apoptosis (Cohen, 1997; Allen *et al*., 2001). The caspase-3 apoptotic cascade can be regulated by calcineurin. This Ca^{2+}/calmodulin-dependent phosphatase, when activated, dephosphorylates the proapoptotic BAD protein allowing its translocation to the mitochondria, where it binds to antiapoptotic Bcl-2 and Bcl-x_L family proteins and induces cytochrome *c* (cyt *c*) release (Zhang *et al*., 1999; Springer *et al*., 2000). In the cytosol, cyt *c* binds Apaf-1 and dATP to form a complex that activates caspase-9, which can activate the executioner caspase-3 (Desagher & Martinou, 2000).

Synthetic peptides homologous to Aβ and PrP^{Sc} have been extensively used in order to clarify the mechanisms of neurodegeneration occurring in AD and PRE (Forloni *et al*., 1996; Brown *et al*., 1997). The synthetic fragment $A\beta_{25-35}$ (amino acids 25–35 of Aβ) contains the residues essential for aggregation and toxicity including methionine 35 (Pike *et al*., 1995), and it has been used in several studies as a model of Aβ cytotoxicity (Brown *et al*., 1997; Pereira *et al*., 2000; Casley *et al*., 2002). The $PrP_{106\text{-}126}$ peptide, corresponding to residues 106–126 of prion protein, is partially resistant to proteinase-K, has a high ability to aggregate into amyloid fibrils and is neurotoxic (Selvaggini *et al*., 1993; Forloni, 1996; Brown *et al*., 1997). In this study we analysed the toxic effect of the synthetic peptides $PrP_{106\text{-}126}$ and $A\beta_{25-35}$ in rat brain cortical neurons. The inhibitor of calcineurin activity, FK506, was used to investigate whether this phosphatase is involved in cyt c release and caspase-3 activation induced by these peptides.

Materials and methods

Materials

Neurobasal medium and B27 supplement were purchased from GIBCO BRL, Life Tecnologies (Scotland, UK). Trypsin, DNase I, trypsin inhibitor type II-S-soybean and FK506 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Synthetic peptides $A\beta_{25-35}$, $A\beta_{35-25}$ and $PrP_{106\text{-}126}$ were from Bachem (Bubendorf, Switzerland). 2′,7′-Dichlorofluorescin diacetate, Indo-1 acetoxymethyl ester

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(Indo-1/AM), MitoTracker-Green, Prolong Antifade Kit, Alexafluor 594 goat anti-mouse IgG conjugate were obtained from Molecular Probes (Leiden, Netherlands). N-*acetyl*-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) was from Calbiochem (Darmstadt, Germany). Reagents and apparatus used in immunoblotting assays were obtained from Bio-Rad (Hercules, CA, USA), whereas polyvinylidene difluoride (PVDF) membranes, alkaline phosphatase-linked anti-mouse secondary antibody and the enhanced chemifluorescence (ECF) reagent were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Cyt *c* antibodies, against native or denatured forms, were obtained from PharMingen (San Diego, CA, USA). All other reagents were from Sigma Chemical Co. (St Louis, MO, USA) or from Merck kgaA (Darmstadt, Germany).

Preparation of rat brain cortical neurons

Primary cultures of cortical cells were prepared from 15 to 16 days embryos of Wistar rats (the mothers were killed by quick cervical dislocation) according to the method described by Hertz *et al*., (1989), with some modifications. In brief, the neocortices of embryos were dissected and placed in Ca^{2+}- and Mg^{2+}-free Krebs buffer (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; Glucose, 13; HEPES, 10; (pH 7.4) and 0.3% BSA. After removal of meninges, the tissues were washed and incubated in Krebs buffer containing 0.02% trypsin and 0.004% DNase I, for 10 min at 37 °C. The digestion was stopped with Krebs buffer containing 0.05% trypsin inhibitor (type II-S) and 0.004% DNase I, and the tissue was centrifuged at 140 ***g*** for 5 min. After washing the pellet once with Krebs buffer, the cells were dissociated mechanically through a large-bore 5 mL glass pipette. Cortical cells were cultured in Neurobasal medium supplemented with 2 mM L-glutamine, 2% B27 supplement, penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were plated on poly-L-lysine (0.1 mg/mL) coated plates or coverslips at a density of 0.15 or 0.6×10^6 cells/cm^2, and the cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. In serum-free Neurobasal medium supplemented with B27, glial growth was reduced (less than 1%, authors' unpublished data) and an excellent viability of cells was achieved after 12 days in culture (see also Brewer *et al*., 1993).

Incubation with the peptides

Cultured cortical cells were treated with $PrP_{106\text{-}126}$ or $A\beta_{25-35}$ at a final concentration of 25 μM, for 24 h or 5 days. The peptides were added to culture medium at the third culturing day. FK506 (1 μM) was added just before the peptides.

Assessment of neuronal injury

After treatment with the peptides, cell viability was evaluated by determining the leakage of the cytosolic enzyme lactate dehydrogenase (LDH) and the capacity of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) to formazan. LDH released to the extracellular medium was expressed as a percentage of the total LDH activity in the cells. The activity of LDH was measured spectrophotometrically following the rate of conversion of NADH to NAD^+ in the presence of pyruvate at 370 nm, according to the method described by Bergmeyer & Brent (1974). The MTT reduction to formazan, by metabolic active cells, was determined according to the method described by Mosmann (1983) with some modifications. In brief, MTT (0.5 mg/mL) dissolved in sodium medium (in mM): NaCl, 132; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 1.4; H_3PO_4, 1.2; glucose, 6; and HEPES-Tris, 10; (pH 7.4) was incubated with the cells for 2 h at 37 °C. After this period, the blue formazan crystals formed were dissolved in an equal volume of 0.04 M HCl in isopropanol, and quantified by measuring the absorbance at 570 nm. MTT reduction was expressed as a percentage of control cells absorbance.

Measurement of intracellular reactive oxygen species

The levels of intracellular peroxides were detected using a nonfluorescent compound, 2′,7′-dichlorofluorescin diacetate $DCFH_2$-DA, which permeates the cells and is desterified by esterases to the acid 2′,7′-dichlorofluorescin. This ionized acid is trapped into the cells and can be oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by hydroperoxides (Cathcart *et al*., 1983). Cortical cells cultured in glass coverslips, after either being treated or not with the peptides, were incubated with 5 μM $DCFH_2$-DA in sodium medium for 30 min at 37 °C. Then, the cells were washed and further incubated in sodium medium for 15 min, to allow desterification of 2′,7′-dichlorofluorescin diacetate. After this period, the glass coverslips containing the loaded cells were mounted in a special holder (Perkin-Elmer L225008) inside a temperature controlled cuvette (37 °C) containing sodium medium, with an alignment of 60° to the excitation beam in order to minimize the effect of light reflection. DCF fluorescence was measured at 502 nm excitation and 550 nm emission.

Assessement of lipid peroxidation

The extent of lipoperoxidation was evaluated by measuring the levels of thiobarturic acid reactive substances (TBARS) in cells treated and not treated with the peptides, as previously described by Agostinho *et al*. (1997b). Briefly, the cells were lysed and diluted three times with 15% trichloroacetic acid, 0.375% thiobarturic acid, 0.25 M HCl, 0.015% 2,6-di-*ter*t-butyl-4-methylphenol, and incubated for 15 min at 100 °C. The samples were centrifuged at 1 200 ***g*** for 10 min, the supernatants were collected and the absorbance measured at 530 nm. The amount of TBARS formed was expressed as nmol/mg protein. Protein concentration was determined by the method of Sedmak & Grossberg (1977).

Intracellular Ca^{2+} concentration measurements

Cortical cells cultured on glass coverslips, in the presence or in the absence of peptides, were incubated with 3 μM Indo-1/AM in neurobasal medium buffered with 20 mM HEPES, for 45 min at 37 °C. The cells were further incubated in a salt solution without phosphate containing (in mM): NaCl, 132; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 1.4; glucose, 6; and HEPES-Tris, 10; (pH 7.4) for 15 min, to ensure a complete hydrolysis of the acetoxymethyl ester of Indo-1. They were then washed and 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 by Agostinho *et al*. (1997a).

Measurement of caspase-3 activity

Cultured cortical cells, which were either treated or untreated with the peptides, were lysed with a buffer containing (in mM): HEPES, 25; $MgCl_2$, 2; EDTA, 1; EGTA, 1; DTT, 2; (pH 7.4), phenylmethylsulphonyl fluoride (PMSF), 100; and a protease inhibitor cocktail (containing 1 μg/mL leupeptin, 1 μg/mL pepstatin A and 1 μg/mL 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 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 with a reaction buffer (in mM): HEPES, 25; 10% sucrose (pH 7.4), DTT, 10; 0.1% CHAPS and 100 μM N-*acetyl*-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA), a chromogenic substrate for caspase-3-like activity, for 2 h at 37 °C (Cregan *et al*., 1999). Caspase activity was determined

by measuring DEVD-pNA cleavage at 405 nm using a microplater reader.

Immunocytochemistry

Cortical cells grown in glass coverslips in the presence or absence of peptides were harvested with sodium medium and incubated with 500 nM MitoTracker-green for 45 min at 37 °C. After washing with phosphate buffer solution (PBS), the cells were fixed with 4% paraformaldehyde (pH 7.4) for 15 min at room temperature. Then, the cells were incubated in PBS containing 20 mM glycine for 15 min, and permeabilized with 0.1% saponin in PBS buffer, for 30 min at room temperature. The cells were incubated with a mouse anti-cyt *c* monoclonal antibody (6H2-B4, 1 : 100 dilution in 0.1% saponin/PBS), that recognizes the native form of this protein, for 30 min at room temperature. After incubation, they were washed and incubated with Alexa Fluor 594 goat anti-mouse IgG antibody conjugated (1 : 50 dilution in 0.1% saponin/PBS). Finally, the cells were treated with mounting solution, Prolong Antifade Kit, on a microscope slide and examined by confocal microscopy (Bio-Rad MRC 600).

Western blotting

The detection of cyt *c* by immunoblotting was performed in cytosolic and mitochondrial fractions that were obtained after the treatment of cortical cells with the peptides. Mitochondrial and cytosolic fractions were prepared according to the method described by Rodrigues *et al*., 2000) with some modifications. Briefly, the cells were lysed with ice-cold isolation buffer (in mM): sucrose, 250; HEPES, 20; KCl, 10; $MgCl_2$, 1.5; (pH 7.4), DTT, 1; PMSF, 1; and protease inhibitor cocktail. The lysates were homogenized with 20 strokes using a Thomas (O872) homogenizer, at 4 °C. Nuclei and intact cells were removed by centrifugation at 700 ***g***, for 12 min at 4 °C, followed by a centrifugation at 17 000 ***g*** during 20 min, to pellet the mitochondria. The resulting supernatant (S2-cytosolic fraction) was treated with 5% trichloroactetic acid and centrifuged at 20 200 ***g*** for 10 min at 4 °C, to precipitate cytosolic proteins. The resulting pellet was resuspended in the isolation buffer, and neutralized using KOH. The mitochondrial pellet, after washing once, was also resuspended in isolation buffer. Bio-Rad protein dye assay reagent was used to determine protein concentration in the samples.

Equal amounts of protein from cytosolic and mitochondrial fractions were separated by electrophoresis on 15% SDS-polyacrylamide gels (SDS-PAGE), after denaturation at 100 °C for 5 min in a 2x concentrated sample buffer (mM): Tris, 100; dithiothreitol, 200; 4% SDS, 0.2% bromophenol blue and 20% glycerol. To facilitate the identification of proteins a prestained precision protein standard (Bio-Rad) was used. The proteins were transferred electrophoretically to PVDF membranes, which were incubated in Tris-buffer (mM): NaCl, 150; Tris-HCl, 25; (pH 7.6) with 0.1% Tween 20 (TBS-T) and 5% nonfat milk for 1 h at room temperature. Cyt *c* was detected by immunodetection using a mouse monoclonal antibody (7H8.2C12) against the denatured form of cyt *c* (1 : 500 dilution in TBS-T 1% nonfat milk) and an alkaline phosphate-conjugated anti-mouse secondary antibody (1 : 20000 dilution in TBS-T 1% nonfat milk). Bands of immunoreactive protein were visualized, after membrane incubation with ECF reagent for 5 min, on a Storm 860 Gel and Blot Imaging System (Amersham Pharmacia Biotech).

Statistical analysis

Results are expressed as means ± SEM of the number of experiments indicated in the figure captions. Statistical significance was determined using an analysis of variance (ANOVA), followed by Dunnett's post-tests or by two-tailed Student's *t*-test.

Results

In the present study we analysed the neurotoxic effect of the synthetic peptides $PrP_{106-126}$ (peptide fragment that mimics PrP^{Sc} toxicity) and $A\beta_{25-35}$ (toxic sequence of Aβ peptide) in primary cultures of rat brain cortical cells. The viability of both treated and untreated (control) cells was assessed by measuring the levels of LDH in the extracellular milieu (a cytosolic enzyme released when membrane cell integrity is disrupted), and by MTT assay. The amount of LDH released by the cells treated with $A\beta_{25-35}$ (25 μM) or $PrP_{106-126}$ (25 μM), for 24 h, was not significantly different ($P > 0.05$) from that observed in control cells (Table 1), indicating that cell membrane integrity was preserved. However, the cellular capacity to reduce the tetrazolium salt MTT was slightly decreased upon $A\beta_{25-35}$ and PrP_{106126} treatment (Table 1). $A\beta_{25-35}$ and $PrP_{106-126}$, incubated at 25 μM for 24 h, induced a moderate neuronal injury, whereas at longer incubation time periods

TABLE 1. Effect of $A\beta_{25-35}$- and $PrP_{106-126}$-treatment on cortical neurons viability

	LDH leakage (% of total)	MTT reduction (% of control)
Control	15.8 ± 0.21	100.2 ± 0.8
$A\beta_{25-35}$	16.2 ± 3.2	87.5 ± 1.2
$PrP_{106-126}$	14.9 ± 5.9	90.3 ± 2.4

Cultured cortical cells were treated with $A\beta_{25-35}$ (25 μM) or $PrP_{106-126}$ (25 μM) for 24 h. The cell viability of control cells (not treated with peptides) and of cells treated with the peptides was evaluated by measuring LDH leakage and the capacity of the cells to reduce MTT. The amount of LDH leakage into the extracellular medium was expressed as a percentage of total enzyme activity in the cells. The MTT assay was used to evaluate the metabolic capacity of cells, and the values were expressed as percentage of the absorbance determined for control cells. The data are means ± SEM of duplicates from three to six independent experiments.

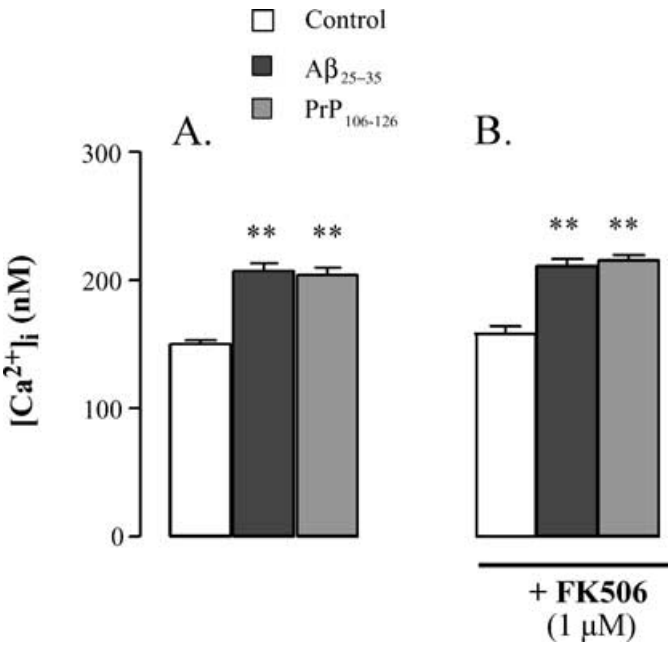

FIG. 1. Effect of $A\beta_{25-35}$ (25 μM) and $PrP_{106-126}$ (25 μM) on basal $[Ca^{2+}]_i$ in the presence (B) or absence (A) of FK506. Cortical cells cultured in coverslips were treated or not treated with the peptides in the presence or in the absence of 1 μM FK506, for 24 h. The fluorescence of Indo-1 loaded cells was measured, and the $[Ca^{2+}]_i$ was calculated as described in material and methods section. The data are means ± SEM of four to six independent experiments. $^{**}P < 0.01$, compared with control cells under the same experimental conditions.

TABLE 2. Oxidative injury induced by $A\beta_{25-35}$ and $PrP_{106-126}$ in cortical neurons

	ROS (arbitrary units)	TBARS (nmol/mg protein)
Control	1.21 ± 0.21	1.10 ± 0.08
$A\beta_{25-35}$	$1.62 \pm 0.32^{*}$	$1.32 \pm 0.02^{*}$
$PrP_{106-126}$	$1.41 \pm 0.59^{*}$	$1.53 \pm 0.02^{*}$

Cultured cortical cells were incubated or not (control cells) with the β_{25-35} (25 μM) or $PrP_{106-126}$ (25 μM) for 24 h. The intracellular reactive oxygen species (ROS) levels were determined by DCF fluorescence, and the extent of lipid peroxidation was evaluated by measuring the production of TBARS, as described in material and methods section. The results are expressed as arbitrary units of DCF fluorescence and as nmol TBARS/mg protein, and represent the means ±SEM of four to six independent experiments. $^{*}P < 0.05$ compared with control cells.

(> 48 h) with $A\beta_{25-35}$ and $PrP_{106-126}$ the cell survival was drastically compromised (see Fig. 2). Therefore, in order to study the molecular events that precede the cell death induced by these peptides, a 24 h incubation time period was used.

The cytotoxic effects of Aβ and PrP peptides have been attributed, in different studies, to Ca^{2+} homeostasis deregulation and oxidative stress (Brown *et al*., 1997; Silei *et al*., 1999; Herms *et al*., 2000; Pereira *et al*., 2000; Xiao *et al*., 2002). Therefore, to investigate whether, under our experimental conditions, these cellular events are involved in the $A\beta_{25-35}$ and $PrP_{106-126}$ induced neurotoxicity, the $[Ca^{2+}]_i$ and oxidative stress parameters were evaluated. The basal $[Ca^{2+}]_i$ was significantly higher in cells treated with $A\beta_{25-35}$ (207.1 ± 15.0 nM) and $PrP_{106-126}$ (203.5 ± 14.2 nM), for 24 h, than in control cells (150.0 ± 7.1 nM) (Fig. 1A). It should be pointed out that the $[Ca^{2+}]_i$ rise, induced by these peptides, occurred before the cell survival was significantly compromised. Table 2 shows that the treatment of cortical cells with $A\beta_{25-35}$ and $PrP_{106-126}$ for 24 h also induced a significant ($P < 0.05$) increase in intracellular ROS levels, measured with DCF, and in the extent of lipoperoxidation, given by the amount of TBARS formed. These results indicate that $A\beta_{25-35}$ and $PrP_{106-126}$ induced oxidative stress, in an early phase of neuronal injury. Therefore, the deregulation of Ca^{2+} homeostasis and oxidative stress may be two sequential events involved in the neurotoxicity induced by these peptides.

In order to evaluate whether calcineurin, a phosphatase activated by Ca^{2+} and calmodulin, is involved in neuronal injury induced by $A\beta_{25-35}$ and $PrP_{106-126}$ the effect of the calcineurin inhibitor, FK506, was evaluated. As shown in Fig. 2, FK506 significantly reduced ($P < 0.05$) LDH leakage induced by $A\beta_{25-35}$ and $PrP_{106-126}$. The amount of LDH released by the cells treated with $A\beta_{25-35}$ or $PrP_{106-126}$ in the presence of FK506 (1 μM), for 5 days, was similar ($P > 0.05$) to that released by control cells. FK506 (1 μM) did not induce any significant change in LDH release *per se*, as compared with control cells.

Calcineurin modulates the activity of voltage-sensitive Ca^{2+} channels (VSCC) and of endoplasmic reticulum (ER) ryanodine (RyR) and inositol 1,4,5-trisphosphate receptors (IP_3R) (Lukyanetz *et al*., 1998; Snyder *et al*., 1998). Therefore, to analyse whether the neuroprotection afforded by FK506 was due to influence on Ca^{2+} homeostasis deregulation induced by $A\beta_{25-35}$ and $PrP_{106-126}$, we tested the effect of 1 μM FK506 on the $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ of cells treated with $A\beta_{25-35}$ (210.8 ± 10.9 nM) or $PrP_{106-126}$ (215.1 ± 9.6 nM) in the presence of FK506 (Fig. 1B) was not significantly ($P > 0.05$) different from that determined in cells treated with the peptides in the absence of this inhibitor (Fig. 1A).

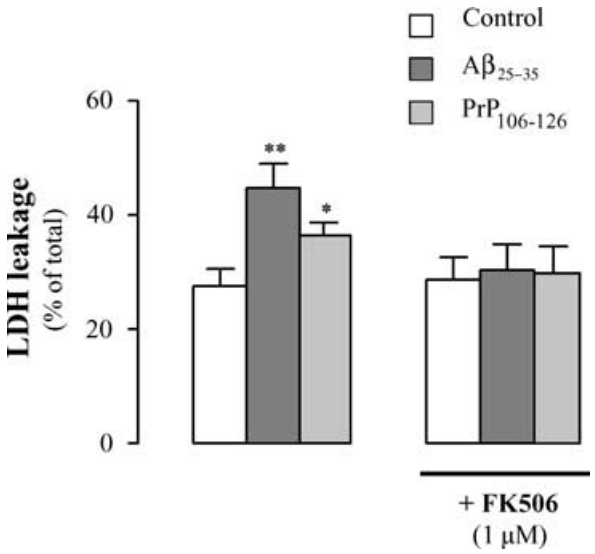

FIG. 2. Effect of FK506 on neuronal injury induced by $A\beta_{25-35}$ (25 μM) and $PrP_{106-126}$ (25 μM). Cortical cells were treated with the peptides in the presence of 1 μM FK506 for 5 days, because a significant reduction on cell viability caused by the peptides was observed at 5th day of incubation. LDH leakage was determined as described in material and methods section. The results are expressed as percentage of total LDH leakage, and represent the means ± SEM of four to six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control cells under the same experimental conditions.

The caspase-3 apoptotic cascade, which is activated by cyt *c* leakage from mitochondria, can be regulated by calcineurin-mediated BAD dephosphorylation (Springer *et al*., 2000). Therefore, we determined if $A\beta_{25-35}$ and $PrP_{106-126}$, incubated at 25 μM for 24 h, induced cyt *c* release and caspase-3 activation, and if calcineurin was involved in these events. The caspase-3-like activity, measured by the cleavage of substrate DEVD-pNA, was significantly higher in cells treated with $A\beta_{25-35}$ or $PrP_{106-126}$, for 24 h, than in control cells. FK506 (1 μM) prevented the increase in caspase-3 activity induced by these peptides. This calcineurin inhibitor, by itself, did not affect caspase-3-like activity (Fig. 3). These results suggest that calcineurin is involved in the caspase-3 activation induced by $A\beta_{25-35}$ and $PrP_{106-126}$. Cyclosporin A (CsA), which is also an inhibitor of calcineurin, induced cell death at a concentration range of 0.5–2 μM, and at nontoxic concentrations (< 0.5 μM) did not prevent caspase-3 activation induced by $A\beta_{25-35}$ and $PrP_{106-126}$ (data not shown).

The results obtained by immunoblotting showed that cyt *c* levels in the mitochondrial fraction of cells treated with $A\beta_{25-35}$ ($51.6 \pm 11.2\%$ of control) and $PrP_{106-126}$ ($56.5 \pm 11.5\%$ of control) were significantly ($P < 0.05$) lower than those determined in control cells. In the presence of FK506, cyt *c* levels of cells treated or not treated ($81.5 \pm 11.0\%$ of control, $P = 0.15$) with $A\beta_{25-35}$ and $PrP_{106-126}$ were not significantly different from those observed in untreated cells (Fig. 4A). In Fig. 4B it can be seen that $A\beta_{25-35}$ and $PrP_{106-126}$ significantly increased ($P < 0.05$) cyt *c* levels in cytosol and that FK506 abolished this effect. These results suggest that the calcineurin inhibitor FK506 prevented the cyt *c* release induced by these peptides. To provide additional evidence that $A\beta_{25-35}$ and $PrP_{106-126}$ induced cyt *c* release from mitochondria, immunocytochemistry studies were performed to determine the localization of cyt *c* in cells treated with these peptides in the presence or absence of FK506. Figure 5C shows a higher overlay of mitotracker green and anti-cyt *c* fluorescence in control cells than in cells treated with $A\beta_{25-35}$ and $PrP_{106-126}$ for 24 h. In cells treated with $A\beta_{25-35}$ and $PrP_{106-126}$ in the presence of FK506 a colocalization of

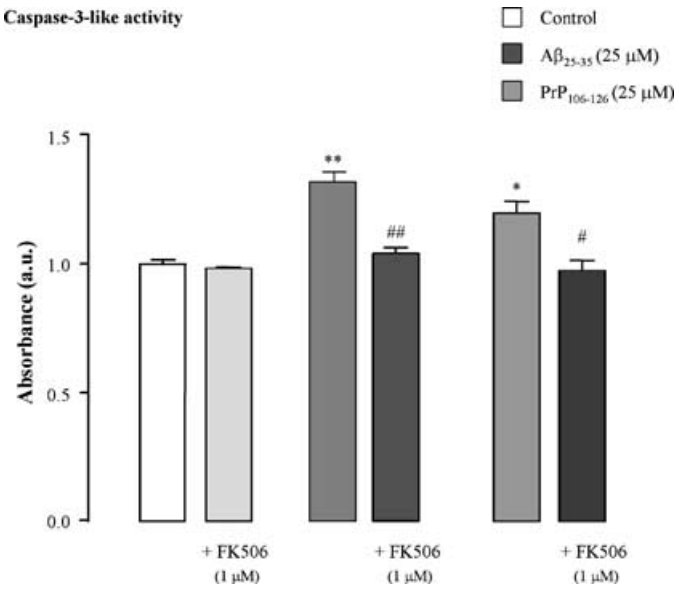

FIG. 3. Effect of FK506 on the increase of caspase-3-like activity induced by $A\beta_{25-35}$ and $PrP_{106-126}$. After 24 h of incubation with the peptides (at a concentration of 25 μM) in the presence or in the absence of 1 μM FK506, the cultured cortical cells were lysed and the protein was extracted. The caspase activity was determined by measuring DEVD-pNa cleavage at 405 nm, as described in material and methods section. Caspase-3-like activity was expressed as arbitrary units of absorbance. Data are means ± SEM of duplicates from three to six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control cells under the same experimental conditions. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with cells treated with the peptides in the absence of FK506, Student's *t*-test.

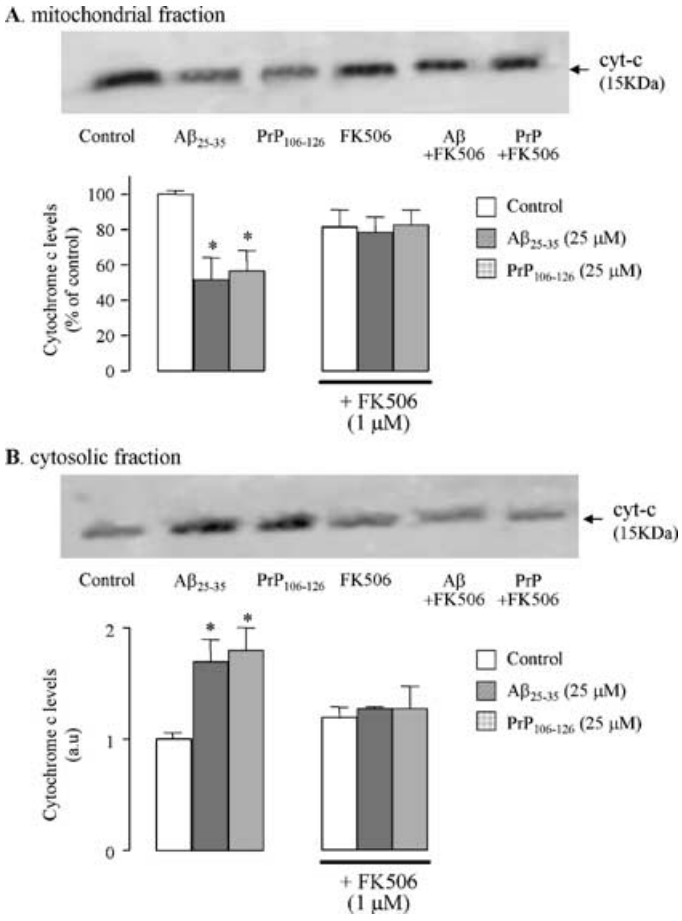

FIG. 4. Cyt *c* levels in mitochondria (A) and in cytosol (B) fractions of cortical cells treated with $A\beta_{25-35}$ and $PrP_{106-126}$ in the presence or in the absence of FK506. Mitochondrial and cytosolic fractions were obtained after cell treatment with the peptides (25 μM) in the presence or in the absence of 1 μM FK506, for 24 h (see material and methods section). The detection of cyt *c* (15 KDa) was performed by immunoblotting using a monoclonal antibody against the denatured form of this protein. Immunoreactive bands were visualized by scanning on a Storm 860. Graphic bars represent the levels of cyt *c* determined using the Image Quant analyser, and are the means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with control cells.

fluorescence similar to that found in control cells (Fig. 5C, 1) was observed, suggesting that both peptides induced cyt *c* release, which was prevented by FK506. These results are in agreement with those obtained by immunoblotting (Fig. 4), showing that calcineurin is involved in the release of cyt *c* due to $A\beta_{25-35}$- and $PrP_{106-126}$-treatment of cortical cells.

Discussion

Our results, using primary cultures of rat brain cortical neurons, showed that 24 h incubation with $A\beta_{25-35}$ and $PrP_{106-126}$ caused cyt *c* release from mitochondria and caspase-3 activation, events that are known to activate the apoptotic cell death cascade, and which were prevented by the calcineurin inhibitor FK506. Therefore, the present study suggests that calcineurin activation is involved in neuronal death induced by $A\beta_{25-35}$ and $PrP_{106-126}$ peptides.

The exposure of cortical cells to $A\beta_{25-35}$ and $PrP_{106-126}$, for 24 h, significantly increased the $[Ca^{2+}]_i$ (Fig. 1), ROS levels and lipoperoxidation (Table 2), before cell viability became significantly reduced, raising the idea that deregulation of Ca^{2+} homeostasis and oxidative stress are two events that precede the neuronal death induced by these peptides. Accordingly, other studies have shown that $A\beta_{25-35}$ and $PrP_{106-126}$ peptides induce Ca^{2+} homeostasis deregulation and oxidative stress, although the relative contribution and sequence of these events remain controversial (Mattson & Goodman, 1995; Brown *et al*., 1997; Florio *et al*., 1998; Ekinci *et al*., 2000; Kawahara *et al*., 2000). Ca^{2+} influx through L-type VSCC has been reported to be involved in the neurotoxic effects of $A\beta_{25-35}$ and $PrP_{106-126}$ (Brown *et al*., 1997; Silei *et al*., 1999; Ekinci *et al*., 2000). The toxicity of $PrP_{106-126}$, unlike $A\beta_{25-35}$ toxicity, has been reported to be associated with Ca^{2+} entry through *N*-metyl-D-aspartate (NMDA) receptors (Brown *et al*., 1997). However, other routes of Ca^{2+} homeostasis deregulation could not be ruled out, such as the entry of Ca^{2+} across amyloid channels (Lin *et al*., 1997; Kawahara *et al*., 2000) or Ca^{2+} leakage from internal stores, including mitochondria and ER, as Ca^{2+} release from these organelles may occur under conditions of oxidative stress (Zhou *et al*., 1996; Mattson *et al*., 2001). Recently, it was shown that $PrP_{106-126}$ induces Ca^{2+} release from mitochondria (O'Donovan *et al*., 2001), and studies performed in our laboratory have shown that $A\beta_{25-35}$ and $PrP_{106-126}$ induce Ca^{2+} leakage from ER through dantrolene-sensitive RyR in cortical neurons (Pereira *et al*. unpublished data).

The $[Ca^{2+}]_i$ rise has been suggested to initiate neurodegeneration through a number of mechanisms, some of which require the activation of calcineurin, a Ca^{2+}/calmodulin dependent phosphatase (Wang *et al*., 1999; Foster *et al*., 2001). An increase in calcineurin activity, due to Ca^{2+} homeostasis deregulation, has been reported to be involved in neuronal injury and memory impairment (Zhuo *et al*., 1999; Springer *et al*., 2000; Foster *et al*., 2001). In this study we show that $A\beta_{25-35}$ and $PrP_{106-126}$ significantly increased the $[Ca^{2+}]_i$ in cortical cells after 24 h

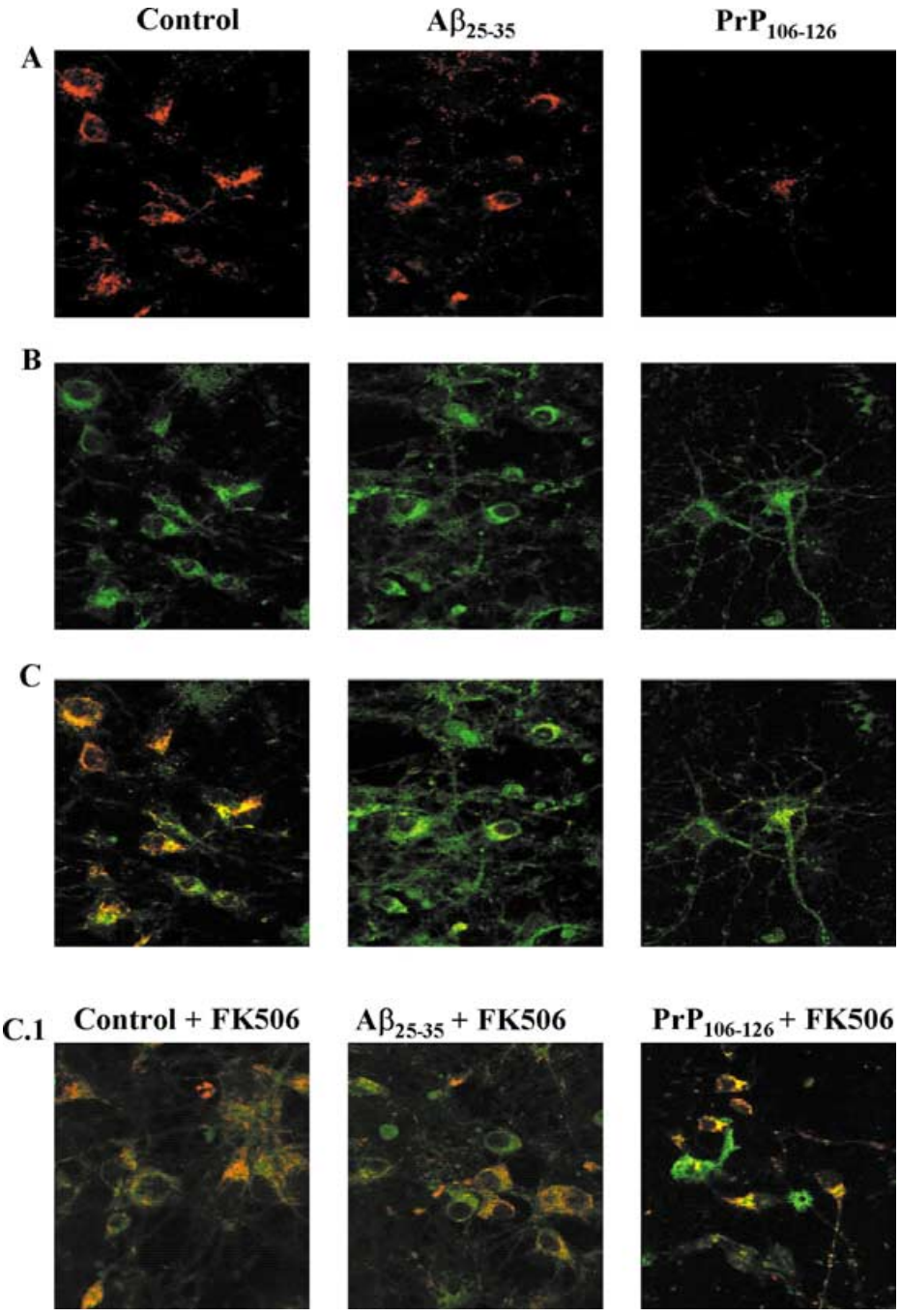

FIG. 5. Representative confocal images of cytochrome *c* in cortical cells treated with $A\beta_{25-35}$ and $PrP_{106-126}$ in the presence or absence of FK506. After 24 h of incubation with the peptides (at a concentration of 25 μM) in the presence or in the absence of 1 μM FK506, for 24 h. Cells were co-labelled with anti-cyt *c* (A, red) and Mitotracker-green (B, green). (C) Merged images from A and B provide evidence about the co-localization of cyt *c* immunoreactivity with mitochondria. Overlay of fluorescence (yellow-orange) indicates retention of cyt *c* in mitochondria. (C1) Localization of cyt c in control cells and cells treated with $A\beta_{25-35}$ and $PrP_{106-126}$ in the presence of FK506. The same pattern of labelling was obtained in three to four independent experiments.

incubation (Fig. 1A), and that cell death induced by $A\beta_{25-35}$ and $PrP_{106-126}$ upon 5 days of incubation can be prevented by the calcineurin inhibitor, FK506 (Fig. 2). Therefore, it is likely that the $[Ca^{2+}]_i$ rise, induced these peptides, is involved in calcineurin activation and cortical cell death.

In our experimental conditions, the caspase-3-like activity increased upon 24 h treatment with $A\beta_{25-35}$ or $PrP_{106-126}$, this effect being prevented by the inhibitor of calcineurin FK506 (Fig. 3). The early time point of caspase-3 activation, before the compromise in cell survival, is in agreement with other studies performed in cortical neurons showing that caspase-3 activation is an initial event of neurodegeneration induced by $A\beta_{25-35}$ and $PrP_{106-126}$ (Sáez-Valero *et al.*, 2000; White *et al.*, 2001). The activation of this caspase executioner of apoptosis has been shown to be regulated, at least in part, by calcineurin-mediated BAD dephosphorylation (Springer *et al.*, 2000). BAD is a pro-apoptotic protein that exists in the cytosol in a

phosphorylated state bound to 14-3-3 protein. When this protein is dephosphorylated by calcineurin, it translocates to mitochondria and binds to Bcl-x_L family protein members, promoting cyt *c* release. As Ca^{2+} can activate calcineurin, the interaction of calcineurin with BAD provides a Ca^{2+}-inducible mechanism for controlling the phosphorylation state and activity of this pro-apoptotic protein, thereby linking calcineurin to apoptosis (Wang *et al*., 1999).

The data obtained, by immunoblotting (Fig. 4) and immunocytochemistry (Fig. 5), showed that treatment of cortical neurons for 24 h with $A\beta_{25-35}$ and $PrP_{106-126}$ lead to cyt *c* release from mitochondria and that the specific calcineurin inhibitor FK506, prevented this release. These results suggest that, in an initial phase of neuronal injury, $A\beta_{25-35}$ and $PrP_{106-126}$ increase calcineurin activity and that this phosphatase, via BAD, promotes the release of cyt *c* from mitochondria and activation of caspase-3. These results are in agreement with those reported in a cell line, with high levels of endogenous phospho-BAD, where it was shown that BAD translocation to mitochondria occurs within 1 h and is maximal 6 h after an apoptotic stimulus (Wang *et al*., 1999). An excessive calcineurin activation due to an alteration in $[Ca^{2+}]_i$ homeostasis (Wang *et al*., 1999), or even to oxidation (Ferri *et al*., 2001), may be one of the pathways that are involved in the neurotoxicity induced by $A\beta_{25-35}$ and $PrP_{106-126}$. Accordingly, a recent study, performed in a human neuronal cell line, also shows that $PrP_{106-126}$ induces cyt *c* release parallel to a $[Ca^{2+}]_i$ rise (O'Donovan *et al*., 2001). Although calcineurin can modulate Ca^{2+} channels activity (Lukyanetz *et al*., 1998; Snyder *et al*., 1998), FK506 had no effect on $[Ca^{2+}]_i$ rise induced by $A\beta_{25-35}$ and $PrP_{106-126}$ in our experimental conditions (Fig. 1B), indicating that neuroprotection afforded by this calcineurin inhibitor was mediated mainly via blockade of cyt *c* release and caspase-3 activation rather than by an effect on Ca^{2+} fluxes.

Similar to FK506, CsA is also an inhibitor of calcineurin and both have to form a complex with immunophilins to block this phosphatase. CsA binds to cyclophilin proteins, whereas FK506 forms a complex with FKBP (FK506 binding proteins) family proteins (Snyder *et al*., 1998). In contrast to FK506, CsA did not show a neuroprotective effect against $A\beta_{25-35}$ and $PrP_{106-126}$ toxicity in cortical neurons (see Results section). According to these data, it has been reported that FK506 is more efficient than CsA at suppressing L-glutamate inducing BAD translocation and apoptosis in hippocampal neurons (Wang *et al*., 1999), and that CSA, unlike FK506, blocks the mitochondrial permeability transition pore (Friberg *et al*., 1998) and is not able to prevent the cortical cell death caused by serum deprivation (Yardin *et al*., 1998). Other studies have demonstrated that CsA and FK506 are able to prevent apoptosis of cerebellar granule cells induced by glutamate (Ankarcrona *et al*., 1996) and both these agents improve the survival of grafted dopaminergic neurons (Castilho *et al*., 2000). All these data suggest that FK506 and CsA may have different effects depending on the cell type and on the activated apoptotic pathway.

In conclusion, our results show that $A\beta_{25-35}$ and $PrP_{106-126}$ induce Ca^{2+} homeostasis deregulation, oxidative stress, cyt *c* release from mitochondria and caspase-3 activation. These events, that precede the neuronal death induced by $A\beta_{25-35}$ and $PrP_{106-126}$, may contribute to an alteration of neuronal homeostasis, in a sequential and additive manner. The calcineurin inhibitor, FK506, prevented cyt *c* release and caspase-3 activation, indicating that this phosphatase is involved in the neurotoxic mechanisms induced by $A\beta_{25-35}$ and $PrP_{106-126}$. Alterations in protein phosphorylation may represent an important marker for Aβ- and PrP^{Sc}-neurodegeneration. Furthermore, given the role of calcineurin for cell survival and regulation of neuronal function, this phosphatase can be an useful target for the treatment of neurodegenerative disorders.

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Abbreviations

Aβ, amyloid-beta protein; AD, Alzheimer's disease; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration, CsA, cyclosporin A; Cyt c, cytochrome c; DCF, 2′,7′-dichlorofluorescein; DEVD-pNA, N-*acetyl*-Asp-Glu-Val-Asp-*p*-nitroanilide ECF, enhanced chemifluorescence; ER, endoplasmic reticulum; Indo-1/AM, Indo-1 acetoxymethyl Ester; IP_3R, inositol 1,4,5-trisphosphate receptor; LDH, lactate dehydrogenase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide); PBS, phosphate buffer solution; PMSF, phenylmethylsulphonyl fluoride; PRE, prion-related encephalopathies; PrP^{Sc}, scrapie isoform of prion protein; ROS, reactive oxygen species, RyR, ryanodine receptor; TBARS, thiobarturic acid reactive substances; VSCC, voltage-sensitive Ca^{2+} channels.

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