Overactivation of calcineurin induced by amyloid-beta and prion proteins

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Abstract

Amyloid-beta protein (Aβ) and the scrapie isoform of prion protein (PrP^{Ss}) have a central role in the pathogenesis of Alzheimer's disease (AD) and prion-related encephalopathies (PRE), respectively. In both disorders, the deposition of these misfolded proteins is accompanied by apoptotic neuronal loss. However, the pathogenesis and molecular basis of Aβ- and PrP^{Sc}-neurotoxic effects are not completely understood. The Ca^{2+}/calmodulin-dependent phosphatase calcineurin (CaN), through the dephosphorylation of the proapoptotic protein BAD, may be the link between Ca^{2+} homeostasis deregulation and apoptotic neuronal death. In this study we used primary cultures of rat brain cortical neurons in order to investigate whether Aβ and PrP affect CaN activity. We observed that synthetic peptides of Aβ (Aβ$_{25-35}$ and Aβ$_{1-40}$) and PrP (PrP$_{106-126}$) increased CaN activity, but did not affect the levels of this protein phosphatase. Moreover, we found that these peptides reduced the levels of BAD phosphorylated at serine residue 112, and this effect was prevented by the CaN inhibitor FK506. Since dephosphorylated BAD translocates to mitochondria, where it triggers cytochrome c release, we determined the levels of BAD in mitochondrial and cytosolic fractions. The data obtained showed that Aβ- and PrP-treated neurons had higher levels of BAD in mitochondria than control neurons. This increase in mitochondrial BAD levels was matched by a decrease in cytochrome c. FK506 prevented the alterations of mitochondrial BAD and cytochrome c levels induced by Aβ and PrP peptides. Taken together the data suggest that Aβ and PrP increased CaN activity, inducing BAD dephosphorylation and translocation to mitochondria and, subsequently, cytochrome c release that may trigger an apoptotic cascade. Therefore, therapeutic strategies targeting CaN might be valuable for these neurodegenerative disorders.

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1. Introduction

The abnormal accumulation of proteins is a pathological feature in Alzheimer's disease (AD) and prion-related encephalopathies (PRE). The key pathogenic event in AD and PRE is linked to a conformational change of normally expressed proteins: the amyloid-beta (Aβ) and the cellular prion protein (PrP^C). Aβ is a 40–42 amino acid peptide that derives from the proteolytic cleavage of amyloid precursor protein (APP) mediated by β- and γ-secretases (Marks and Berg, 2008). This peptide self-assembles into toxic oligomers and fibrils that accumulate in the brain of AD patients, forming amyloid plaques. The PrP^C is a cell membrane glycoprotein that can undergo disease-associated structural modifications, giving rise to a pathogenic isoform called scrapie prion protein (PrP^{Sc}) that accumulates as amyloid plaques in the brain of patients suffering from PRE (Barnham et al., 2006; Wisniewski and Sigurdsson, 2007). Recently, it was proposed that the C-terminal of α-helices in PrP^C refolds into β-strands, which adopt a parallel alignment (Cobb et al., 2007). Both Aβ and PrP^{Sc} have a high β-sheet content, which renders them insoluble, resistant to proteolysis and neurotoxic (Soto, 1999; Wisniewski and Sigurdsson, 2007). The involvement of these amyloidogenic proteins in disease was validated by the finding that mutations in genes that codified APP and PrP^C result in autosomal-dominant AD and PRE (Carter, 2007; Aguzzi and

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Haass, 2003). These neurodegenerative disorders are characterized by memory and cognitive deficits that occur as a consequence of neuronal dysfunction and death, which are attributable, in part, to the gradual deregulation of neuronal Ca^{2+} homeostasis (LaFerla, 2002; Kawahara, 2004; Demuro et al., 2005). A neuroinflammatory process, characterized by microglia activation, is also involved in AD and PRE pathogenesis (Wisniewski and Sigurdsson, 2007). Currently, the development of anti-Aβ immunotherapeutic approaches that can also modulate microglial activation is a huge challenge for AD treatment (Morgan, 2006).

Calcineurin (CaN) is a serine/threonine phosphatase physiologically activated by Ca^{2+} and calmodulin that exists at high levels in neurons, accounting for 1% of the total protein. This unique Ca^{2+}-activated protein phosphatase, also named as phosphatase 2B, links Ca^{2+} to the dephosphorylation of several targeted molecules, such as proapoptotic factors, transcription factors and ion channels. Moreover, CaN has been shown to play a role in learning and memory processes, as well as in apoptosis of neuronal cells (Mansuy, 2003; Hara and Snyder, 2007). As a consequence of an insult that increases cytosolic Ca^{2+} concentration, such as Aβ or PrP peptides exposure and aging (Agostinho and Oliveira, 2003; Mattson, 2006), the Ca^{2+}-activated CaN can trigger apoptosis through the dephosphorylation of Bcl2-associated death protein (BAD). This proapoptotic protein exists normally in the cytosol bounded to the 14-3-3 protein and phosphorylated on serine residues, mainly serine 122 (S112), S136 and S155 (Springer et al., 2000). After an insult, BAD is dephosphorylated and translocates from the cytosol to mitochondria. In the outer cell membrane of mitochondria, BAD interacts and inhibits the actions of the anti-apoptotic proteins Bcl-$_2$ and Bcl-x$_L$, promoting the release of cytochrome c (cyt c) that leads to the activation of the postmitochondrial caspase apoptotic cascade (Wang et al., 1999; Springer et al., 2000; Kim et al., 2006).

The purpose of this study is to investigate whether the synthetic peptides Aβ$_{25–35}$, Aβ$_{1–40}$ and PrP$_{106–126}$, which mimic the toxic effects of Aβ and PrP^{Sc} (Agostinho and Oliveira, 2003; Ferreiro et al., 2006), change the functional role of CaN. We have observed that Aβ and PrP peptides increased the activity of this phosphatase in rat brain cortical neurons, leading to BAD dephosphorylation and translocation to mitochondria. The data obtained suggest that the neurotoxic effects triggered by Aβ and PrP peptides were partially due to changes in CaN activity and, subsequent, alterations in phosphorylation state of BAD.

2. Experimental procedures

2.1. Reagents

Neurobasal medium and B-27 supplement were purchased from GIBCO (Paisley, UK). Aβ$_{25–35}$, Aβ$_{1–40}$ and PrP$_{106–126}$ were from Bachem (Bubendorf, Switzerland). The mouse anti-calcineurin1 (clone 29) and the mouse anti-BAD (clone 48) antibodies were obtained from BD Biosciences (Erembodegem, Belgium), whereas the phosphospecific antibody against BAD phosphorylated at serine 112 (pS112-BAD) was from Biosource (Nivelles, Belgium). The

monoclonal antibody against the denatured form of cyt c (clone 7H8.2C12) was acquired from PharMingem (San Diego, CA, USA), whereas the anti-actin monoclonal antibody (clone AC-40) was from Sigma (St. Louis, MO, USA). Serine/threonine phosphatase assay kit was obtained from Promega (Madison, WI, USA). Reagents and apparatus used in Western blot assays were obtained from Bio-Rad (Hercules, CA, USA), whereas PVDF membranes, alkaline phosphatase-linked anti-mouse secondary antibody and the enhanced chemifluorescence (ECF) reagent were from Amersham Biosciences (Buckinghamshire, UK). MitoTracker-green, Alexafluor 594 anti-mouse IgG conjugate and Antifade kit were obtained from Molecular Probes (Leiden, Netherlands). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cultures of rat brain cortical neurons

Primary cultures of cortical cells were prepared from 15–16 days embryos of Wistar rats according to the method previously described by Agostinho and Oliveira (2003). Cortical cells were cultured in Neurobasal medium added on with 2 mM L-glutamine, 2% B27 supplement, penicillin (100 U/ml) and streptomycin (100 μg/ml). The neurons were cultured on poly-L-lysine (0.1 mg/ml) coated plates at a density of 0.2×10^6 cells/cm^2, and maintained at 37 °C in a humidified atmosphere of 5% CO$_2$/95% air.

2.3. Cells treatments

Cultured cortical neurons were treated with 25 μM Aβ$_{25-35}$, 1 μM Aβ$_{1–40}$ or 25 μM PrP$_{106–126}$, for 24–48 h. The peptides were added to culture medium at the 4–5th culturing day. FK506 (1 μM) was added just before the peptides.

2.4. Preparation of cell lysates and fractions

Treated cortical cells were lysed with ice-cold isolation buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl$_2$ (pH 7.4), 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail). The cell lysates used to measure the levels of phospho-BAD by Western blot were prepared in the isolation buffer containing 1 mM sodium orthovanadate and 5 mM sodium pyrophosphate, 50 mM sodium fluoride. The cell lysates were rapidly frozen/defrosted three times, and cleared by centrifugation ($20,200 \times g$ for 10 min) before colleting the supernatant. Mitochondrial and cytosolic fractions were prepared, as described previously in Agostinho and Oliveira (2003). Bio-Rad protein dye assay reagent was used to determine protein concentration in the samples.

2.5. Calcineurin activity

The activity of CaN was determined using a serine/threonine phosphatase assay Kit from Promega (V2471), according to the manufacture's instruction. In brief, cell lysates (prepared as described above) were first centrifuged at $100,000 \times g$ for 1 h (4 °C) to remove contaminating components, such as ATP and free endogenous phosphate from samples. After spin columns preparation, accordingly with the instructions of the kit, 250 μl of cell lysates were added on each column. The cell lysates were then centrifuged at $600 \times g$ for 5 min (4 °C) and the samples collected from the bottom of the reservoir. The collected samples (5 μg protein) and standards of free phosphate (0–2000 pmol) were incubated with the phosphopeptide substrate in the presence of a reaction buffer specific for phosphatase 2B (250 mM imidazole, 1 mM EGTA, 50 mM MgCl$_2$, 5 mM NiCl$_2$, 250 μg/ml calmodulin, 0.1% β-mercaptoethanol), during 5 min. Then the reaction was stopped by adding molybdate dye/additive mixture and after 15 min at room temperature, the absorbance was measured at 600 nm in a plate reader.

2.6. Western blot assay

Samples containing an equal amount of total protein were loaded on 15% SDS-polyacrylamide gels. To facilitate the further identification of proteins a pre-stained precision protein standard (Bio-Rad) was used. The proteins of samples were separated by electrophoresis and transferred electrophoretically to PVDF membrane (Agostinho and Oliveira, 2003). These membranes were

blocked using a Tris-buffered solution (150 mM NaCl, 25 mM Tris–HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA) for 1 h at room temperature. Incubation with the primary antibodies (anti-calcineurin 1:250, anti-actin 1:1000, anti-BAD 1:500, anti-pS112-BAD 1:500, anti-cyt c 1:500 in TBS-T 1% BSA) was performed overnight at 4 °C. After extensive washing, the membranes were incubated with phosphatase-linked specific secondary antibodies (1:25000). Bands of immunoreactive proteins were visualized, after membrane incubation with enhanced chemi-fluorescence (ECF) reagent for 5 min, on a Storm 860 Gel. Densities of blot bands were calculated using the Blot Imaging System (Amersham Pharmacia Biotech).

2.7. Immunocytochemistry

Cultured cortical neurons were treated with the peptides in the absence or presence of FK506 and then were processed as described previously by Agostinho and Oliveira (2003). In brief, the cells were incubated with Mito-Tracker green for 45 min at 37 °C, and after being washed with PBS to remove the excess of fluorescence probe, they were fixed with 4% paraformaldehyde for 15 min. Then, the cells were incubated with 20 mM glycine (in PBS) for 15 min and subsequently permeabilized with 0.1% saponin (in PBS) for 30 min at room temperature. Afterwards the cells were incubated with: (i) anti-BAD antibody (1:500) for 2 h and (ii) Alexa Fluor goat anti-mouse IgG antibody (1:100). Finally, the cells were mounted on microscope glass, using Prolong Antifade kit, and examined by confocal microscopy (Bio-Rad MRC 600).

3. Results

In a previous study we have demonstrated that the inhibitor of CaN, FK506, prevents cyt c release and the subsequent caspase-3 dependent apoptotic cell death induced by Aβ and PrP synthetic peptides (Agostinho and Oliveira, 2003), suggesting that CaN is involved in neuronal death caused by these peptides. Therefore, in the present study we investigated the effect of Aβ and PrP peptides in CaN activity and levels, as well as in the phosphorylation state of the CaN target, BAD (Wang et al., 1999). The concentrations and incubation time period used for Aβ and PrP synthetic peptides treatments were chosen based on previous studies made at our lab (Agostinho and Oliveira, 2003; Ferreiro et al., 2006).

The activity of CaN was evaluated in cortical neurons treated with Aβ$_{25–35}$ (25 μM), Aβ$_{1–40}$ (1 μM) or PrP$_{106–126}$ (25 μM) peptides by measuring the formation of free phosphate (PO$_4$), using a specific kit assay (see material and methods section). Fig. 1 shows that the amount of PO$_4$ formed in cells treated with Aβ$_{25–35}$ (791.7 ± 27.8 pmol/μg protein), Aβ$_{1–40}$ (875.0 ± 97.2 pmol/μg protein) or PrP$_{106–126}$ (847.2 ± 55.6 pmol/μg protein) peptides, for 24 h, was significantly higher than in control (untreated) cells (500.0 ± 59.4 pmol/μg protein), which indicates that CaN is upregulated in cells treated with the peptides. This augment in CaN activity was also observed in neurons treated with the peptides for 48 h (data not shown). The reverted sequences Aβ$_{35–25}$ and Aβ$_{40–1}$, as well as the scrambled PrP$_{106–126}$ (PrP$_{Scram}$), did not affect CaN activity comparatively to control cells (Fig. 1). The CaN inhibitor FK506 (Snyder et al., 1998) almost completely prevented PO$_4$ formation in cells untreated or treated with the peptides (Fig. 1B), what reinforces that we were determining the activity of this phosphatase. These data suggest that CaN activity was significantly increased by Aβ$_{25–35}$, Aβ$_{1–40}$ or PrP$_{106–126}$ peptides.
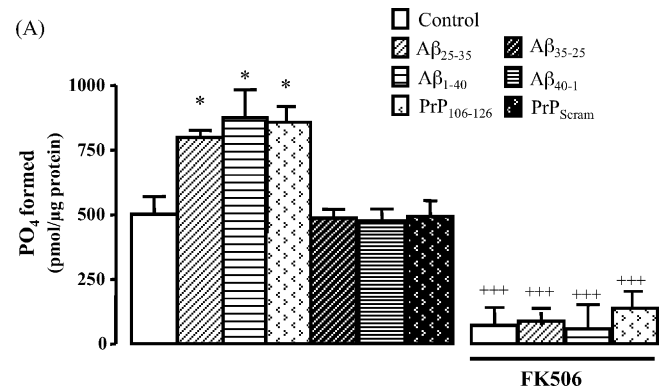

Fig. 1. Effect of amyloid-β (Aβ) and prion (PrP) peptides on calcineurin activity. Cortical neurons were incubated or not (control) with Aβ$_{25–35}$ (25 μM), Aβ$_{1–40}$ (1 μM) or PrP$_{106–126}$ (25 μM) in the presence or absence of FK506 (1 μM) for 24 h. Neurons treated with the reverse Aβ peptides (Aβ$_{35–25}$ and Aβ$_{40–1}$) or the scrambled PrP$_{106–126}$ (PrP$_{Scram}$) were also used as controls. After peptide incubation, CaN activity was assessed by measuring the amount of free phosphate (PO$_4$) formed in each experimental condition. The levels of free PO$_4$ were determined, using a serine/threonine phosphatase assay system (Promega Kit), and expressed as pmol per μg of protein. The data are means ± S.E.M. of four to six independent experiments. *$P < 0.05$ compared with control cells in the absence of FK506, +++$P < 0.001$ compared with the same experimental conditions in the absence of FK506.
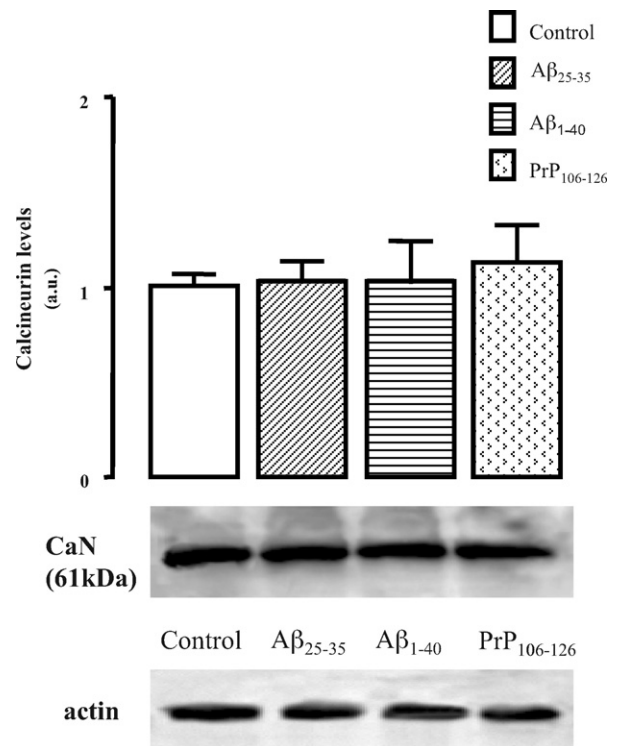

Fig. 2. Effect of Aβ and PrP peptides on calcineurin levels. The cultured cortical neurons were treated with Aβ$_{25–35}$ (25 μM), Aβ$_{1–40}$ (1 μM) and PrP$_{106–126}$ (25 μM) for 24 h. Calcineurin (61 kDa) levels were determined by Western blotting experiments. The blots were re-probed with an antibody against actin (42 kDa) to estimate the total amount of protein loaded in each lane of the gel. Immunoreactive bands were visualized by scanning on a Storm 860. Graphic bars represent the levels of CaN determined using Image Quant analyser. The data were expressed as arbitrary units (a.u.), relatively to control (untreated) cells, and represent the means ± S.E.M. of four independent experiments.

In order to clarify whether the increase in CaN activity, induced by Aβ and PrP peptides, reflects an increase in phosphatase levels, we further determined the levels of this protein by Western blot. The data showed that CaN levels in control cells were similar to those found in cells treated with Aβ$_{25-35}$, Aβ$_{1-40}$ or PrP$_{106-126}$ (Fig. 2), indicating that Aβ and PrP peptides did not affect the levels of this protein phosphatase.

Since BAD, which is normally phosphorylated on serine residues, is one of the substrates of CaN (Springer et al., 2000) we determined the phosphorylation state of this proapoptotic protein in neurons untreated or treated with Aβ and PrP peptides, using a phospho specific antibody that labels BAD phosphorylated at serine 112 (pS112-BAD). Aβ$_{25-35}$, Aβ$_{1-40}$ or PrP$_{106-126}$ peptides significantly ($p < 0.05$) decreased the levels of pS112-BAD by about 30% as compared with control

cells (Fig. 3). FK506 partially prevented the decrease in pS112-BAD levels induced by Aβ and PrP peptides. To ensure that changes in the relative levels of phosho-BAD were not due to differences in the amount of total BAD, we also determined the levels of this protein using an antibody that recognizes BAD independently of its phosphorylation state. The data obtained show that neither Aβ nor PrP peptides affected the total BAD levels in neuronal cell lysates (Fig. 3B).

Dephosphorylated BAD translocates to mitochondria and promotes cytochrome *c* (cyt *c*) release from this organelle (Wang et al., 1999; Kim et al., 2006). Therefore, to provide additional evidence that Aβ and PrP peptides increase CaN and subsequently BAD dephosphorylation, we determined the levels of BAD in the mitochondrial fractions of neurons treated with Aβ and PrP peptides. Since the results obtained with Aβ$_{1-40}$ in CaN activity and cellular BAD levels were
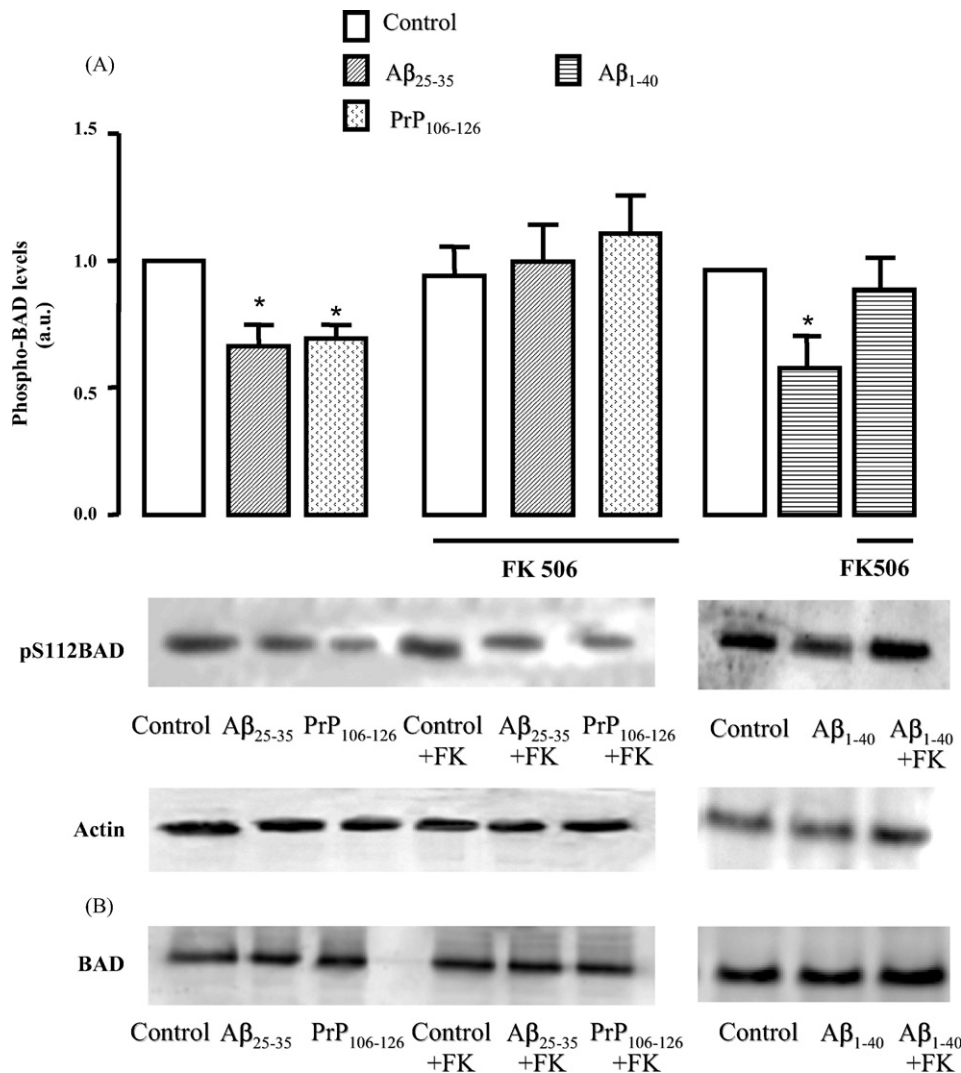

Fig. 3. Alterations in phospho-BAD levels induced by Aβ and PrP peptides. (A) The levels of phospho-BAD at serine 112 (pS122-BAD) in cortical neurons treated or not (control) with Aβ$_{25-35}$ (25 μM), Aβ$_{1-40}$ (1 μM) or PrP$_{106-126}$ (25 μM) in the presence or absence of FK506 (1 μM). After 24 h incubation, the neurons were lysed and the protein levels were determined by Western blotting, using a monoclonal antibody against BAD phosphorylated at serine 112 (23 kDa). An antibody against actin was used to estimate the total amount of protein loaded in each lane of the gel. Graphic bars represent pS122-BAD levels, expressed as arbitrary units relatively to control cells, and are the means ± S.E.M. of four to six independent experiments. (B) The levels of total BAD in neuronal lysates were also determined, using an antibody that recognizes this protein independently of its phosphorylation state (23 kDa). The figure shows a representative blot for BAD, out of four independent experiments. *$P < 0.05$ compared with control cells in the absence of FK506.

similar (qualitatively) to those obtained with $A\beta_{25-35}$, in the following studies we used the smaller peptide. BAD levels in the mitochondria of neurons treated with $A\beta_{25-35}$ (1.24 ± 0.11 a.u.) or $PrP_{106-126}$ (1.27 ± 0.15 a.u.) were significantly ($p < 0.05$) higher than in control cells (0.98 ± 0.06 a.u.). FK506 prevented the increase of mitochondrial BAD levels induced by the peptides (Fig. 4), decreasing the levels of this proapoptotic protein to values slightly lower than those observed in untreated cells. These results indicate that CaN
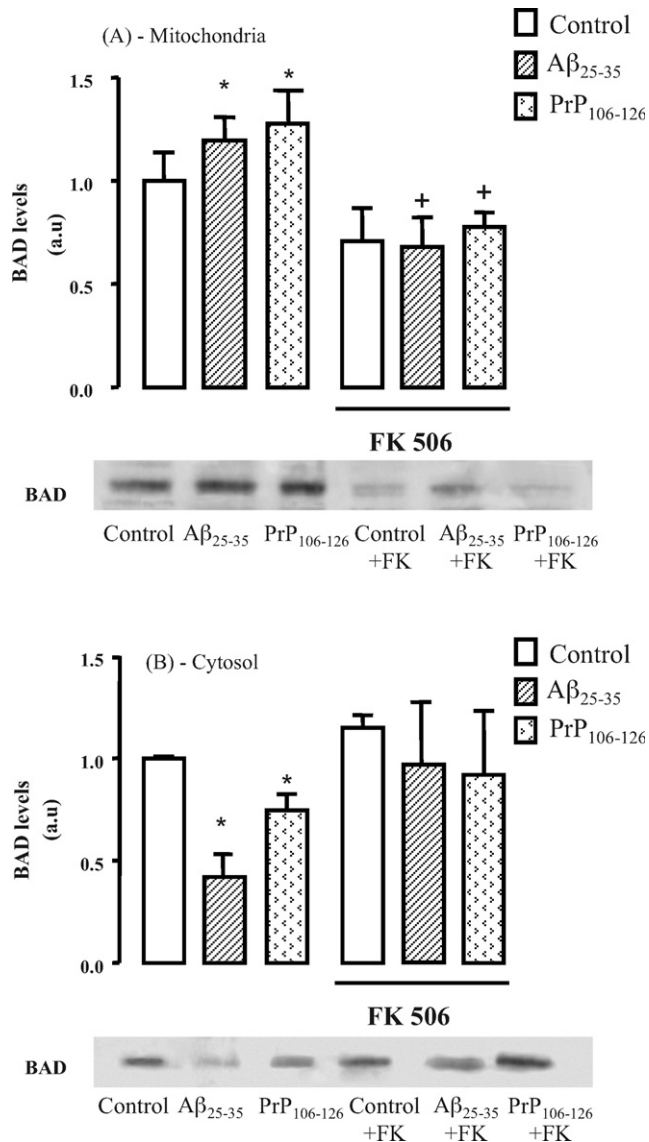

Fig. 4. Levels of total BAD (phosphorylated and nonphosphorylated) in mitochondria (A) and cytosol (B) of neurons exposed to $A\beta$ or PrP. Cultured cortical neurons were treated or not (control) with $A\beta_{25-35}$ ($25~\mu$M) or $PrP_{106-126}$ ($25~\mu$M) in the presence or absence of FK506 ($1~\mu$M), for 24 h. Mitochondrial and cytosolic fractions were obtained after neuronal treatment with peptides and/or FK506. The levels of BAD in these cellular fractions were determined using an antibody that recognizes this propaptotic protein independently of its phosphorylation state (23 kDa). Graphic bars represent BAD levels in mitochondria (A) and in cytosol (B), expressed as arbitrary units, comparing to control cells. The data are means $\pm$ S.E.M. of four independent experiments. *$P < 0.05$ compared with control cells in the absence of FK506, +$P < 0.05$ compared with the same experimental conditions in the absence of FK506.
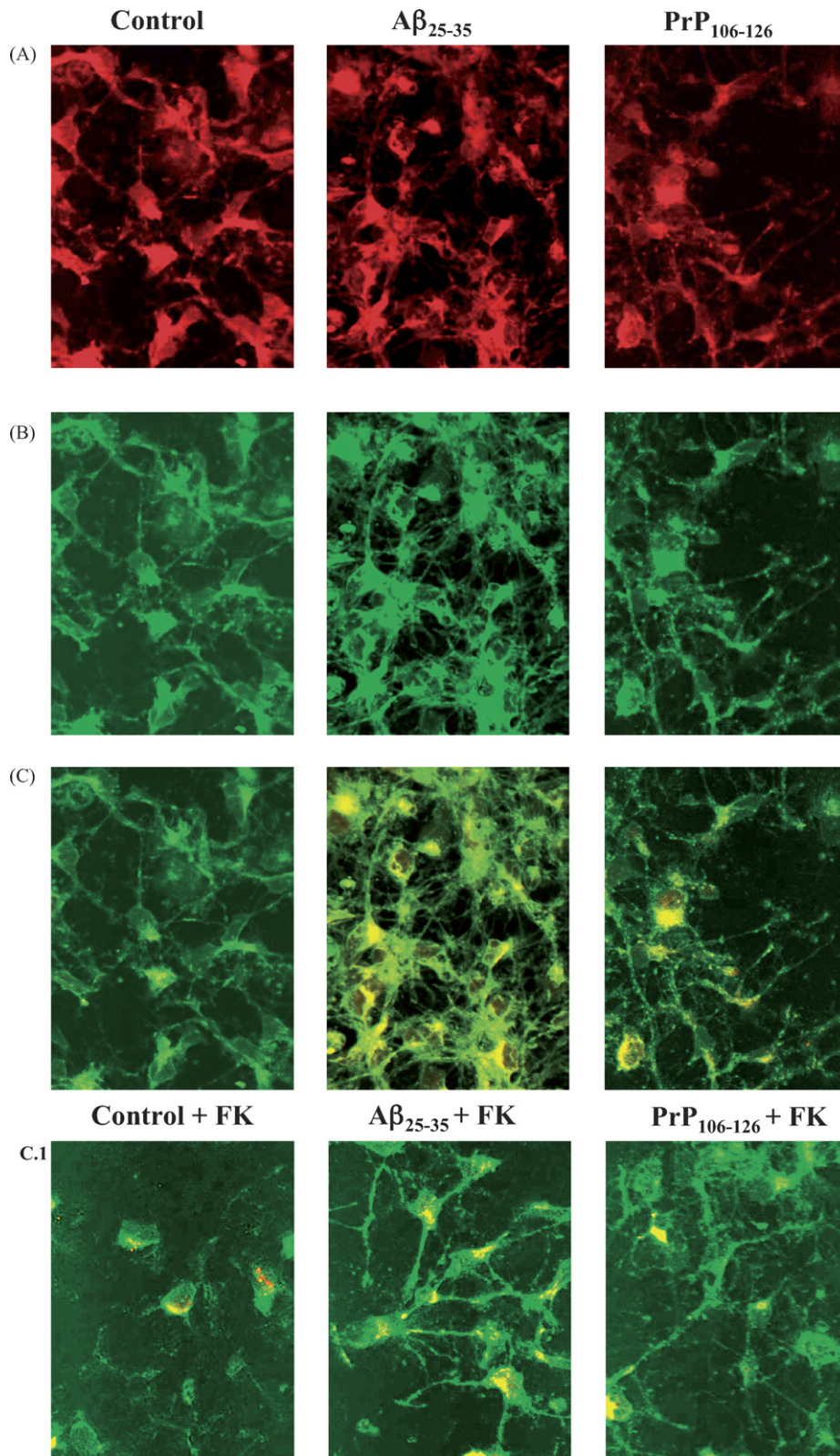
mediates BAD translocation to mitochondria in neurons treated with $A\beta$ or PrP peptides. In control cells it was also observed a slight effect of FK506 in mitochondrial BAD levels, indicating that, even in untreated neurons, CaN was active and mediated BAD translocation to these organelles. Additionally, it was performed immunocytochemistry studies to confirm that in $A\beta$- and PrP-treated neurons the BAD is localized in the mitochondria. As can be seen in Fig. 5 an intense co-localization (yellow label) of anti-BAD and MitoTracker green (mitochondria probe) fluorescence was observed in neurons treated with the peptides as compared with control cells, as well as with cells treated with $A\beta$ or PrP in the presence of FK506 (Fig. 5 C1). Thus, these data support that in our experimental conditions $A\beta$ and PrP peptides caused BAD translocation to mitochondria, and this can be prevented by calcineurin blockade. Fig. 6 shows that the increase of BAD levels in mitochondria of cells treated with $A\beta_{25-35}$ and $PrP_{106-126}$ peptides (Fig. 4A) was associated with a decrease in the cytosolic levels of this proapoptotic protein (Fig. 4B), as well as with a reduction in mitochondrial cyt c levels.

Altogether the data show that $A\beta$ and PrP peptides induce CaN overactivation and subsequent BAD dephosphorylation and translocation to mitochondria, where this proapoptotic protein triggers cyt c release. Therefore, the augment in CaN activity induced by these peptides might be responsible for their neurotoxic effects.

4. Discussion

The involvement of CaN in the neurodegenerative process and memory impairment in AD and PRE remains a controversial issue (Wang et al., 1999; Norris et al., 2005; Biasini et al., 2006; Dineley et al., 2007). In a previous study we demonstrated that $A\beta$ and PrP peptides trigger a caspase-3-dependent apoptotic neuronal death pathway, involving CaN and cyt c release from mitochondria (Agostinho and Oliveira, 2003). However, in that study, we have not addressed whether these peptides affect the activity and/or expression levels of this Ca^{2+}/calmodulin dependent phosphatase 2B.

In the present study we observed that CaN activity was significantly increased in cortical neurons treated with $A\beta$ or PrP peptides for 24 h (Fig. 1) and 48 h (data not shown). This augment in CaN activity was not correlated with alterations in the levels of this phosphatase (Fig. 2), but was probably due to an increase in intracellular Ca^{2+} concentration triggered by $A\beta$ and PrP peptides. In fact, our group has previously shown that $A\beta$ and PrP synthetic peptides cause an early increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cortical neurons, which was in part due to Ca^{2+} release from endoplasmic reticulum (Agostinho and Oliveira, 2003; Ferreiro et al., 2006). Although CaN can modulate the activity of voltage-sensitive Ca^{2+} channels and endoplasmic reticulum-associated Ca^{2+} channels, ryanodine and inositol 1,4,5-triphosphate receptors (Burley and Sihra, 2000; Bultynck et al., 2003), in our previous study we have shown that the augment of $[Ca^{2+}]_i$ caused by $A\beta$ and PrP peptides was not affected by the calcineurin inhibitor, FK506 (Agostinho and Oliveira, 2003). The alterations in Ca^{2+}

Fig. 5. Localization of BAD in neurons treated with Aβ and PrP peptides in the absence and presence of FK506. Representative images obtained by confocal microscopy are shown. Cultured cortical neurons were treated or not (control) with Aβ$_{25-35}$ (25 μM) or PrP$_{106-126}$ (25 μM) in the presence or absence of FK506 (1 μM), for 24 h. Then, the cells were co-labelled with anti-BAD antibody (A, red) and MitoTracker green (B, green). Merged images from A and B gives information about the co-location of BAD in relation to mitochondria (C and C1). Thus, overlay of fluorescence (yellow-orange label) indicates that BAD is within mitochondria. (C1) Merged images obtained, as described above, in neurons treated or not (control) with the peptides in the presence of FK506. Similar patterns of labelling were obtained in four independent experiments.
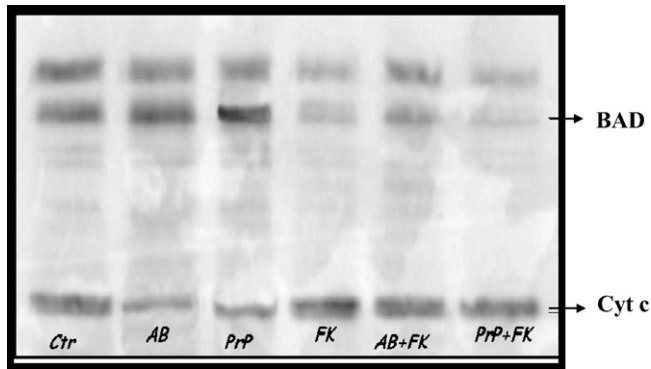
Fig. 6. The increase of BAD levels in mitochondria was associated with a decrease in cytochrome c (cyt c) levels. Mitochondrial fractions of neurons treated or not with $A\beta_{25-35}$ (25 μM) or $PrP_{106-126}$ (25 μM) in the presence or absence of FK506 (1 μM) for 24 h were analyzed by Western blot, using an antibody that recognized total BAD (phospho-independent state) and an antibody that recognized the denatured form of cyt c (15 kDa). The figure shows a representative Western blot for BAD and cyt c, out of 3 independent experiments.

homeostasis caused by Aβ and PrP peptides in cortical neurons were shown to precede reactive oxygen species production (Ferreiro et al., 2006). Several studies have reported that CaN activity is regulated by Ca^{2+}, as well as by oxidative stress conditions (Mansuy, 2003; Celsi et al., 2007), which are two events that are upregulated in AD and PRE (Hur et al., 2002; LaFerla, 2002). However, the manner by which Aβ and PrP peptides affect CaN remains controversial. A recent study showed that CaN activity was augmented in cortex, hippocampus and cerebellum of transgenic AD animal models, and the accumulation of Aβ was coincident with CaN upregulation (Dineley et al., 2007). Moreover, it was demonstrated that Aβ peptides can induce CaN activation, via Ca^{2+} influx through α7 nicotinic acetylcholine receptors, in cortical neurons (Snyder et al., 2005). In the brain of AD patients, CaN was also shown to be upregulated due to its proteolytic cleavage through calpain I activation (Liu et al., 2005). On the other hand, it was reported that Aβ causes downregulation of CaN in a neuronal precursor cell line through the induction of oxidative stress (Celsi et al., 2007). The discrepancies between this study and others (including our present study) show that Aβ peptides upregulate CaN are probably related with differential cell susceptibility and/or peptide exposure (concentration and time period of incubation). Searching the literature, information about the involvement of CaN in PRE falls short. A recent study shows that the activity of this phosphatase was reduced in the cerebellum of a transgenic mouse model of inherited prion disease, but not in hippocampus and cerebral cortex (Biasini et al., 2006).

Two widely used inhibitors of CaN are FK506 and cyclosporin A (Snyder et al., 1998; Pong and Zaleska, 2003). In this study we used FK506 because it can cross the blood-brain barrier, unlike cyclosporin A, which is an advantage when the issue is about brain disorders. Our data show that FK506 reduced CaN activity in neurons treated with Aβ and PrP peptides (Fig. 1). Accordingly it was reported that FK506 reduces the activity of this phosphatase in several brain

regions of an AD mice model, improving the cognitive deficits observed in these animals (Dineley et al., 2007).

In order to get more evidence that Aβ and PrP peptides upregulate CaN activity we explored the phosphorylation state of its downstream target, BAD. After being dephosphorylated, BAD translocates to mitochondria and promotes the release of cyt c, leading to the activation of an apoptotic caspase cascade (Springer et al., 2000; Kim et al., 2006). Studies linking CaN-mediated BAD dephosphorylation to neurodegeneration triggered by Aβ and PrP peptides are lacking. Our data show that cortical neurons treated with Aβ or PrP synthetic peptides exhibited lower levels of phospho-BAD (Fig. 3) and higher levels of BAD in mitochondria than untreated neurons (Figs. 4 and 5), and these effects were prevented by the CaN inhibitor, FK506. Moreover, it was shown that in neurons treated with the peptides the increase of mitochondrial BAD levels was parallel to the decrease of cyt c levels (Fig. 6), indicating that BAD translocation to mitochondria triggers cyt c release that can activate apoptotic neuronal death pathways. These data are in accordance with our previous study showing that FK506 prevents cyt c release from mitochondria, caspase-3 activation and the subsequent apoptotic neuronal death triggered by Aβ and PrP peptides (Agostinho and Oliveira, 2003). Furthermore, it was also demonstrated that CaN activation and BAD dephosphorylation are upstream in premitochondrial signaling events leading to caspase-3 activation in a human teratocarcinoma cell line treated with Aβ peptides (Cardoso and Oliveira, 2005).

Taken together, our data prove that Aβ and PrP peptides induce CaN overactivation and, as a consequence, the proapoptotic protein BAD is dephosphorylated and translocated to mitochondria, triggering cyt c release. Cyt c in the cytosol may be responsible for the activation of neuronal apoptotic pathways. Therefore, we can conclude that altered CaN activity is one of the processes that links Ca^{2+} dyshomeostasis to neurodegeneration in Alzheimer's and Prion's diseases.

References

Agostinho, P., Oliveira, C.R., 2003. Involvement of calcineurin in the neurotoxic effects induced by amyloid-beta and prion peptides. Eur. J. Neurosci. 17, 1189–1196.

Aguzzi, A., Haass, C., 2003. Games played by rogue proteins in prion disorders and Alzheimer's disease. Science 302, 814–818.

Barnham, K.J., Cappai, R., Beyreuther, K., Masters, C.L., Hill, A.F., 2006. Delineating common molecular mechanisms in Alzheimer's and prion diseases. Trends Biochem. Sci. 31, 465–472.

Biasini, E., Massignan, T., Fioriti, L., Rossi, V., Dossena, S., Salmona, M., Forloni, G., Bonetto, V., Chiesa, R., 2006. Analysis of the cerebellar proteome in a transgenic mouse model of inherited prion disease reveals preclinical alteration of calcineurin activity. Proteomics 6, 2823–2834.

Bultynck, G., Vermassen, E., Szlufcik, K., De Smet, P., Fissore, R.A., Callewaert, G., Missiaen, L., De Smedt, H., Parys, J.B., 2003. Calcineurin and intracellular Ca2+-release channels: regulation or association? Biochem. Biophys. Res. Commun. 311, 1181–1193.

Burley, J.R., Sihra, T.S., 2000. A modulatory role for protein phosphatase 2B (calcineurin) in the regulation of Ca2+ entry. Eur. J. Neurosci. 12, 2881–2891.

Cardoso, S.M., Oliveira, C.R., 2005. The role of calcineurin in amyloid-beta-peptides-mediated cell death. Brain Res. 1050, 1–7.

Carter, C.J., 2007. Convergence of genes implicated in Alzheimer's disease on the cerebral cholesterol shuttle: APP, cholesterol, lipoproteins, and atherosclerosis. Neurochem. Int. 50, 12–38.

Celsi, F., Svedberg, M., Unger, C., Cotman, C.W., Carri, M.T., Ottersen, O.P., Nordberg, A., Torp, R., 2007. Beta-amyloid causes downregulation of calcineurin in neurons through induction of oxidative stress. Neurobiol. Dis. 26, 342–352.

Cobb, N.J., Sönnichsen, F.D., McHaourab, H., Surewicz, W.K., 2007. Molecular architecture of human prion protein amyloid: a parallel, in-register beta-structure. Proc. Natl. Acad. Sci. U.S.A. 104, 8946–8951.

Demuro, A., Mina, E., Kayed, R., Milton, S.C., Parker, I., Glabe, C.G., 2005. Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. J. Biol. Chem. 280, 17294–17300.

Dineley, K.T., Hogan, D., Zhang, W.R., Taglialatela, G., 2007. Acute inhibition of calcineurin restores associative learning and memory in Tg2576 APP transgenic mice. Neurobiol. Learn. Mem. 88, 217–224.

Ferreiro, E., Resende, R., Costa, R., Oliveira, C.R., Pereira, C.M., 2006. An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. Neurobiol. Dis. 23, 669–678.

Hara, M.R., Snyder, S.H., 2007. Cell signaling and neuronal death. Annu. Rev. Pharmacol. Toxicol. 47, 117–141.

Hur, K., Kim, J.I., Choi, S.I., Choi, E.K., Carp, R.I., Kim, Y.S., 2002. The pathogenic mechanisms of prion diseases. Mech. Ageing Dev. 123, 1637–1647.

Kawahara, M., 2004. Disruption of calcium homeostasis in the pathogenesis of Alzheimer's disease and other conformational diseases. Curr. Alzheimer Res. 1, 87–95.

Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J., Cheng, E.H., 2006. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat. Cell Biol. 8, 1348–1358.

LaFerla, F.M., 2002. Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. Nat. Rev. Neurosci. 3, 862–872.

Liu, F., Grundke-Iqbal, I., Iqbal, K., Oda, Y., Tomizawa, K., Gong, C.X., 2005. Truncation and activation of calcineurin A by calpain I in Alzheimer disease brain. J. Biol. Chem. 280, 37755–37762.

Mansuy, I.M., 2003. Calcineurin in memory and bidirectional plasticity. Biochem. Biophys. Res. Commun. 311, 1195–1208.

Marks, N., Berg, M.J., 2008. Neurosecretases provide strategies to treat sporadic and familial Alzheimer disorders. Neurochem. Int. 52, 184–215.

Mattson, M.P., 2006. Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders. Antioxid. Redox. Signal. 8, 1997–2006.

Morgan, D., 2006. Modulation of microglial activation state following passive immunization in amyloid depositing transgenic mice. Neurochem. Int. 49, 190–194.

Norris, C.M., Kadish, I., Blalock, E.M., Chen, K.C., Thibault, V., Porter, N.M., Landfield, P.W., Kraner, S.D., 2005. Calcineurin triggers reactive/inflammatory processes in astrocytes and is upregulated in aging and Alzheimer's models. J. Neurosci. 25, 4649–4658.

Pong, K., Zaleska, M.M., 2003. Therapeutic implications for immunophilin ligands in the treatment of neurodegenerative diseases. Curr. Drug Targets CNS Neurol. Disord. 2, 349–356.

Snyder, S.H., Lai, M.M., Burnett, P.E., 1998. Immunophilins in the nervous system. Neuron 21, 283–294.

Snyder, E.M., Nong, Y., Almeida, C.G., Paul, S., Moran, T., Choi, E.Y., Nairn, A.C., Salter, M.W., Lombroso, P.J., Gouras, G.K., Greengard, P., 2005. Regulation of NMDA receptor trafficking by amyloid-beta. Nat. Neurosci. 8, 1051–1058.

Soto, C., 1999. Alzheimer's and prion disease as disorders of protein conformation: implications for the design of novel therapeutic approaches. J. Mol. Med. 77, 412–418.

Springer, J.E., Azbill, R.D., Nottingham, S.A., Kennedy, S.E., 2000. Calcineurin-mediated BAD dephosphorylation activates the caspase-3 apoptotic cascade in traumatic spinal cord injury. J. Neurosci. 20, 7246–7251.

Wang, H.G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F., Reed, J.C., 1999. Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284, 339–343.

Wisniewski, T., Sigurdsson, E.M., 2007. Therapeutic approaches for prion and Alzheimer's diseases. FEBS J. 274, 3784–3798.