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Calpain activates caspase-8 in neuron-like differentiated PC12 cells via the amyloid-β-peptide and CD95 pathways

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ABSTRACT

The neurotoxic amyloid-β-peptide (Aβ) is important in the pathogenesis of Alzheimer's disease (AD). Calpain (Ca^{2+}-dependent protease) and caspase-8 (the initiating caspase for the extrinsic, receptor-mediated apoptosis pathway) have been implicated in AD/Aβ toxicity. We previously found that Aβ promoted degradation of calpastatin (the specific endogenous calpain inhibitor); calpastatin degradation was prevented by inhibitors of either calpain or caspase-8. The results implied a cross-talk between the two proteases and suggested that one protease was responsible for the activity of the other one. We now report on the previously unrecognized caspase-8 activation by calpain. In neuron-like differentiated PC12 cells, calpain promotes active caspase-8 formation from procaspase-8 via the Aβ and CD95 pathways, along with degradation of the procaspase-8 processing inhibitor caspase-8 (FLICE)-like inhibitory protein, short isoform ($FLIP_S$). Inhibition of calpain (by pharmacological inhibitors and by overexpression of calpastatin) prevents the cleavage of procaspase-8 to mature, active caspase-8, and inhibits $FLIP_S$ degradation in the Aβ-treated and CD95-triggered cells. Increased cellular Ca^{2+} per se results in calpain activation but does not lead to caspase-8 activation or $FLIP_S$ degradation. The results suggest that procaspase-8 and $FLIP_S$ association with cell membrane receptor complexes is required for calpain-induced caspase-8 activation. The results presented here add to the understanding of the roles of calpain, caspase-8, and CD95 pathway in AD/Aβ toxicity. Calpain-promoted activation of caspase-8 may have implications for other types of CD95-induced cell damage, and for nonapoptotic functions of caspase-8. Inhibition of calpain may be useful for modulating certain caspase-8-dependent processes.

1. Introduction

Alzheimer's disease (AD) is clinically manifested by progressive dementia. The neuropathological alterations include the accumulation of amyloid-β-peptide (Aβ), a neurotoxic peptide derived from the amyloid precursor protein (APP), extracellular neuritic plaques containing Aβ, intracellular neurofibrillary tangles (NFT) that are composed mainly of the protein tau, and neuronal cell loss. Aβ plays a major role in the pathogenesis of AD and is toxic to neurons (Hardy and Selkoe, 2002). Aβ leads to increased cellular Ca^{2+}, mitochondrial dysfunction, enhanced generation of reactive oxygen species, and may cause neuronal cell death by apoptosis or necrosis (LaFerla, 2002; Fifre et al., 2006).

The intracellular Ca^{2+}-dependent protease calpain and its specific endogenous inhibitor calpastatin have been implicated in the pathogenesis of AD. Activated forms of μ-calpain and m-calpain (activated by μM Ca^{2+} and mM Ca^{2+}, respectively) and decreased levels of calpastatin have been found in some regions in the brains of AD patients (Nixon et al., 1994; Veeranna et al., 2004), and in Tg2576 mice (transgenic for a human APP mutant) in brain regions that exhibit accumulation of Aβ (Vaisid et al., 2007). Caspases have also been implicated in AD. Partial activation of the caspase pathways may occur early in AD, whereas full activation of caspase-cascades and apoptosis may occur in the more advanced stages of the disease (Roth, 2001). Activated caspase-8, caspase-9 and caspase-3 were shown to be associated with neuritic plaques and NFT (Rohn et al., 2002).

Abbreviations: AD, Alzheimer's disease; α-CD95, anti-CD95 antibody; Aβ, amyloid-β-peptide; APP, amyloid precursor protein; BAPTA, BAPTA-AM (1,2-Bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraacetoxymethyl ester); DISC, death-inducing signaling complex; DMEM, Dulbecco's modified Eagle's medium; DM, differentiation medium; ERK, extracellular signal-regulated kinase; FAC, focal adhesion complex; FADD, Fas-associated death domain; FLIP, caspase-8 (FLICE)-like inhibitory protein; GM, growth medium; H.S., horse serum; IB, immunoblotting; NFT, neurofibrillary tangles; NGF, nerve growth factor; Pc, control plasmid; Pst, calpastatin plasmid; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sAβ, Aβ25-35; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; ZIET, Z-IETD-FMK.

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The involvement of the caspase and/or calpain systems in Aβ-toxicity is also indicated by results of in vitro studies. In some cases, caspase activity (caspase-8, -3) was shown to be associated with Aβ-induced apoptosis of cultured neuronal cells (Cantarella et al., 2003; Ivins et al., 1999; Lu et al., 2003; Morishima et al., 2001). In other cases, calpain (Kelly and Ferreira, 2006), or calpain and caspase-12 (Nakagawa and Yuan, 2000), or calpain, caspase-9 and caspase-3 (Fifre et al., 2006), were shown to be involved in Aβ-induced proteolysis and cytotoxicity.

We previously found that in differentiated, neuron-like PC12 cells, Aβ induced the activation of calpain and caspase-8, and promoted degradation of calpastatin. The Aβ-promoted calpastatin degradation was prevented by inhibitors of calpain and of caspase-8 (Vaisid et al., 2008a). Calpastatin is a caspase substrate (including caspase-1, caspase-3 and caspase-8) (Altznauer et al., 2004; Wang et al., 1998). Under certain conditions, calpastatin is also degraded by calpain (Mellgren et al., 1986; Nakamura et al., 1989). Assuming independent activation and activities of calpain and caspase-8, inhibition of one protease should still allow calpastatin degradation by the other protease. The fact that pharmacological inhibitors of either protease inhibited calpastatin diminution suggested a cross-talk between the two proteases (Vaisid et al., 2008a), and pointed to the possibility that one protease was responsible for the activity of the other one.

Caspase-8 is the initiator caspase of the extrinsic, receptor-induced apoptosis pathway. The activation of caspase-8 via this pathway requires association with certain membrane receptors, such as CD95. CD95 (also called APO-1 and Fas) is a member of the tumor necrosis factor (TNF) superfamily of receptors. Binding of natural ligand or agonistic antibodies to CD95 initiates trimerization of CD95, recruitment of the adapter molecule Fas-associated death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC). The recruitment of procaspase-8 leads to its activation (Chang et al., 2003; Medema et al., 1997). Caspase-8 activation is blocked by the association of the inhibitor proteins $FLIP_S$ (short) and $FLIP_L$ (long) with the DISC (Krueger et al., 2001).

To probe the interaction between calpain and caspase-8, we studied calpain and caspase-8 activation in differentiated PC12 cells exposed to Aβ, to anti-CD95 antibody (α-CD95) and to high external Ca^{2+}. In the present study, we show for the first time that calpain is involved in caspase-8 activation via the Aβ and the CD95 pathways. Inhibition of calpain (by a pharmacological inhibitor and by overexpression of calpastatin) prevents the conversion of the procaspase-8 to active caspase-8 and inhibits $FLIP_S$ degradation in cells exposed to Aβ and to α-CD95. Exposing the cells to high external Ca^{2+} results in calpain activation but not caspase-8 activation, nor $FLIP_S$ degradation. The results suggest that the association of procaspase-8 and $FLIP_S$ with cell membrane receptor complexes is required for calpain-induced caspase-8 activation.

2. Materials and methods

2.1. PC12 cell cultures and differentiation

PC12 were grown in Petri dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented by 8% fetal calf serum (F.C.S.), 8% horse serum (H.S.), 2 mM glutamine and 100 Units/ml penicillin + streptomycin (growth medium, GM). Cells were induced to differentiate, by plating 2.5×10^6 cells on collagen-coated 90 mm petri dishes, according to published procedures (Vaisid et al., 2008a), and cultured in DMEM, supplemented by 1% H.S., 2 mM glutamine, 50 Units/ml of penicillin + streptomycin, and 50 ng/ml nerve growth factor (NGF) (Alomone Labs, Jerusalem, Israel) (differentiation medium, DM). The time of cell replating in DM is defined as 0 h. The DM was replaced every 48 h.

2.2. Calpastatin overexpression in differentiated PC12 cells

Stable PC12 cell lines overexpressing calpastatin were generated in PC12 cells cultured in GM medium. Cells were transfected with a plasmid containing the full length human calpastatin cDNA (calpastatin plasmid, Pst), or with the empty plasmid (control plasmid, Pc), as previously described (Vaisid et al., 2008b). The cells were transfected using Lipofectamine 2000 (Invitrogen). The transfected PC12 cells were grown for 4 weeks in the presence of 800 μg/ml of G418 (Gibco), added to the medium every 48 h. The cells that survived the 4 weeks selection were defined as stably transfected cells. G418-resistant clonal cell colonies were cultured in GM in the presence of 400 μg/ml of G418. For differentiation, cells were transferred to DM, as described above.

2.3. Treatment of differentiated PC12 cells with sAβ, anti-CD95 antibody, Ca^{2+}, and with protease inhibitors

Amyloid-β-peptide 25–35 (sAβ) (purchased from BioSight Ltd., Karmiel, Israel and from Bachem, Bubendorf, Switzerland) was suspended in sterile double distilled water (DDW) at a concentration of 1.0 mM, and preincubated for 48 h prior to addition to the cell cultures (Morishima et al., 2001). The sAβ suspension was added to the cultures at 96 h of differentiation to a final concentration of 50 μM. Control and sAβ-treated cells were cultured in DM for additional 24 h. $CaCl_2$ (Sigma, St Louis, MI, USA) was dissolved in DDW (100 mM stock solution), added to the DM at 96 h of differentiation at a final concentration of 5 mM, and cultures continued for additional 24 h. Anti-CD95 antibody (α-CD95) (Upstate cell signaling, Lake Placid, NY, USA, clone CH11) was added to the DM at 120 h of differentiation at a final concentration of 50 ng/ml, and cultures continued for additional 3 h.

To study effects of protease inhibition on the treated cells, the cell permeable selective calpain inhibitor calpeptin (Calbiochem, La Jolla, CA, USA), was added to the DM at 72 h and 96 h of differentiation at a final concentration of 50 μM. The cell membrane permeable Ca^{2+} chelator BAPTA-AM (Sigma, St Louis, MI, USA) was added at 96 h of differentiation at a final concentration of 3.5 μM. To study effects of calpastatin overexpression, differentiated Pc cells and Pst cells were treated with sAβ or α-CD95, as described above.

2.4. Preparation of cell extracts for SDS-PAGE, and immunoblotting analyses

PC12 cell lysates were prepared using 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2.5 mM EGTA, and 1:200 protease inhibitors cocktail set III (Calbiochem). Lysates were kept on ice for 30 min and centrifuged. Protein concentration in the supernatants was determined (Vaisid et al., 2008a). Aliquots of supernatants were mixed with Laemmli sample buffer for SDS-PAGE.

SDS-PAGE was carried out according to standard procedures (using 15% acrylamide for caspase-8 and FLIP, 10% acrylamide for calpain). Samples containing 20–40 μg of PC12 cell proteins were electrophoresed and then transferred to nitrocellulose membranes (Schleicher & Schuell, Maidstone, UK). Immunoblotting was carried out as previously described (Vaisid et al., 2008a), using polyclonal anti-caspase-8 antibody p18 (H-134): Sc-7890 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500); monoclonal anti-μ-calpain antibody (1:1000) (Vaisid et al., 2008a); polyclonal anti-$FLIP_{S/L}$ antibody (H-202): Sc-8347 (Santa Cruz (1:500); monoclonal anti-β tubulin antibody (Sigma, St Louis, MO, USA); The appropriate peroxidase-conjugated secondary antibodies were used, and detection of bands carried out with ECL (KPL, Gaithersburg, MD, USA), as previously described (Vaisid et al., 2008a). Membranes were stripped off and reprobed with monoclonal anti-β tubulin

antibody (Santa Cruz Biotechnology) (1:2000) for estimation of loading. Bands were quantified by densitometry.

2.5. Measurement of calpain and caspase activities in PC12 cell extracts

For estimation of calpain activity, PC12 cells were differentiated for 72 h, and cultures continued for 48 h without and with added calpeptin. Cells were then cultured for additional 3 h in the absence or presence of α-CD95, as described above. The measurement of calpain activity was carried out in cell extracts, prepared as previously described (Vaisid et al., 2008a). Calpain activity was estimated by using FITC-casein (Sigma) as a substrate, according to published procedure (Cottin et al., 2000), as previously described (Vaisid et al., 2008a). m-Calpain was isolated from a PC12 cell extract by column chromatography, according to published methods (Cottin et al., 2000) and served as a positive control.

For estimation of caspase activity, Pc and Pst cells were differentiated for 120 h, then cultures continued for 3 h in the presence and absence of α-CD95, as described above. The cells were harvested, washed with PBS, and centrifuged at 700 × *g*. Cells were lysed in 10 mM Tris–HCl, pH 7.4, 130 mM NaCl, 10 mM Na pyrophosphate, 1% Triton X-100, 10 mM NaH_2PO4/Na_2HPO4, pH 7.4. Lysates were kept on ice for 30 min, centrifuged and the protein concentration in the supernatants measured (Bradford, 1976). Supernatants were kept at −20 °C prior to the estimation of caspase activity. Caspase activity in the supernatants was estimated according to published methods (Talanian et al., 1997) in 20 mM Hepes, pH 7.4, 2 mM dithiothreitol (DTT), 10% glycerol, using the caspase-8 fluorogenic substrate Ac-IETD-AMC (Alexis Biochemicals, Lausen, Switzerland), in the absence and presence of the selective caspase-8 inhibitor Z-IETD-FMK (ZIET). Fluorescence levels of the released AMC were estimated by spectrofluorimetry (excitation at 380 nm, emission at 460 nm).

2.6. Statistical analysis

Data are expressed as mean ± SEM. For the comparison of means of two groups, *t-test* for independent samples was performed; when control was considered as 100%, one sample *t-test* was performed (where the test value = 100). All comparisons were two tailed; $p < 0.05$ was considered as significant. Data were analyzed with SPSS 15.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Activation of caspase-8 in differentiated PC12 cells exposed to sAβ is mediated by calpain

Caspase-8 in control and in sAβ-treated cells was analyzed by immunoblotting (IB), as described in Materials and methods. A band of 18 kDa, indicative of activation of the procaspase (~55 kDa band), was observed in control cells, with significantly increased levels of the 18 kDa band seen in sAβ-treated cells (Fig. 1A and B). A significant inhibition in caspase-8 activation was observed in the cells treated with the selective calpain inhibitor calpeptin and exposed to sAβ, as compared to cells treated only with sAβ (Fig. 1A and B). The inhibition of caspase-8 activation by calpeptin indicated that calpain was responsible for the appearance of the 18 kDa caspase-8. In order to substantiate this conclusion, we used PC12 cells overexpressing the calpain specific inhibitor, calpastatin (Pst cells). The Pst cells used expressed about 140–220% of calpastatin levels as compared to levels in control cells transfected with empty plasmid (Pc cells) (Vaisid et al., 2008b). Caspase-8 activation was significantly inhibited in sAβ-treated calpastatin-overexpressing cells (Fig. 1C and D). Thus the results show that calpain is involved in caspase-8 activation in the sAβ-treated PC12 cell.

3.2. Activation and activity of calpain and caspase-8 in differentiated PC12 cells treated with anti-CD95 antibody

In order to further examine the relationship between calpain and caspase-8, we used an agonist antibody (α-CD95) as a ligand for triggering the receptor CD95. We assessed calpain and caspase-8 activation in cells exposed to α-CD95, and examined the effects of calpeptin and calpastatin overexpression on the proteases in the α-CD95-treated cells. Calpain and caspase-8 activation was assessed by immunoblotting (IB) and activity estimated in cell extracts, using the appropriate fluorogenic substrates, as described in Materials and methods. IB analysis of μ-calpain showed that the ratio of the 76 kDa band to the original 80 kDa band (calpain autolysis) was significantly greater in the α-CD95-treated cells than in the control; the increased calpain autolysis is indicative of enhanced μ-calpain activation in the treated cells. The α-CD95-promoted calpain activation was inhibited by calpeptin (Fig. 2A and B). Calpain activity (using FITC-casein as a substrate) was significantly increased in extracts made of α-CD95-treated cells as compared with the control cells; calpain activity was inhibited in cells treated with both calpeptin and α-CD95 as compared with the α-CD95-treated cells (Fig. 2C). The results are consistent with the results on calpain activation presented in Fig. 2A and B, and indicate that calpain activity is enhanced by α-CD95. Calpain was activated in α-CD95-treated Pc cells, and inhibited in α-CD95-treated Pst cells (Fig. 2D), further substantiating the conclusion that calpain is activated by triggering the CD-95.

Caspase-8 is known to be activated at the CD95 death-inducing signaling complex upon triggering of the cell CD95 (Krueger et al., 2001; Medema et al., 1997). As shown in Fig. 3, caspase-8 was activated in the cells exposed to α-CD95, as indicated by the significant increase in the 18 kDa band in the treated cells. Caspase-8 activation was inhibited by calpeptin (Fig. 3A and B), and by calpastatin overexpression (Fig. 3C and D). Caspase-8 activity (using Ac-IETD-AMC as a substrate) was significantly increased in extracts made of α-CD95-treated Pc cells as compared with the control Pc cells. In contrast, caspase-8 activity in extracts of the α-CD95-treated Pst cells was not different from that of the control cells (Fig. 3E). These results indicated that overexpression of calpastatin inhibited the α-CD95-promoted caspase-8 activity, supporting the results of the IB analysis (Fig. 3C and D). Overall, the results indicated that calpain was activated by stimulation of CD95, and that calpain was involved in caspase-8 activation via the CD95 signaling pathway.

3.3. Intracellular Ca^{2+} involvement in calpain and caspase-8 activation in α-CD95 and sAβ-treated PC12 cells

Calpain activation implies an increase in intracellular Ca^{2+}. We previously found that calpain was activated in differentiated PC12 cells exposed to sAβ (Vaisid et al., 2008a). In the present study, μ-calpain was analyzed in cells treated with the cell membrane permeable Ca^{2+} chelator BAPTA-AM (BAPTA) and exposed to Aβ and to α-CD95. As shown in Fig. 4, a decrease in the ratio of the 76 kDa band to the 80 kDa band was observed in BAPTA-treated cells, as compared with BAPTA-untreated cells exposed to Aβ or to α-CD95 (Fig. 4A–C). The results indicated that BAPTA inhibited calpain activation induced by Aβ and by α-CD95. Under the same conditions there was also less caspase-8 activation in BAPTA-treated cells than in cells without BAPTA, when exposed to sAβ or to α-CD95 (Fig. 4D and E). These results are consistent with the results described above, showing that in both the Aβ-treated and α-CD95-treated cells, Ca^{2+}-promoted calpain activation leads to caspase-8 activation.

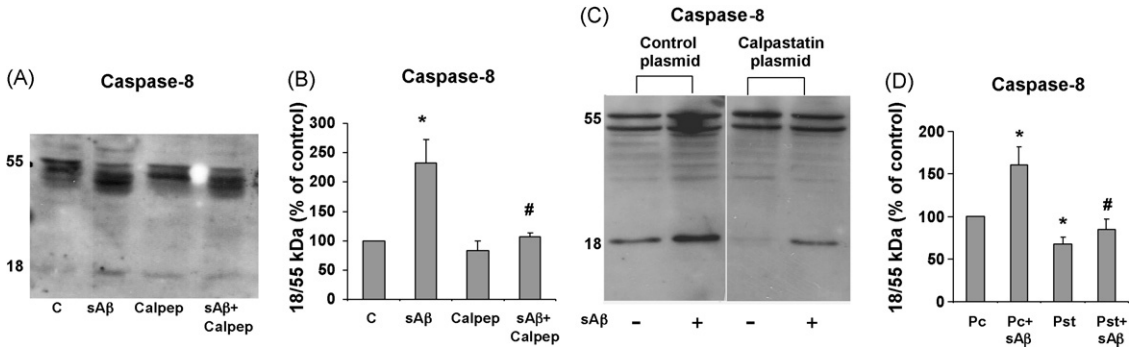

Fig. 1. Caspase-8 activation is enhanced in Aβ-treated differentiated PC12 cells and activation is inhibited by calpeptin and by calpastatin overexpression. (A) and (B) Caspase-8 in control and sAβ-treated cells in the absence and presence of calpeptin. C, control; Calpep, calpeptin. (A) Immunoblot of caspase-8. (B) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means ± SEM ($n = 4$). $^{*}p < 0.05$ vs. control (considered 100%); $^{\#}p < 0.05$ vs. sAβ. (C) and (D) Caspase-8 in control and sAβ-treated cells transfected with control plasmid (Pc) and with calpastatin plasmid (Pst). (C) Immunoblot of caspase-8. (D) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means ± SEM ($n = 4$). $^{*}p < 0.05$ vs. Pc (considered 100%); $^{\#}p < 0.05$ vs. Pc + sAβ.

3.4. Increased cellular Ca^{2+} per se leads to calpain activation but not to caspase-8 activation

Differentiated cells were placed in Ca^{2+}-rich medium. Calpain was activated under these conditions; calpain activation was prevented when BAPTA was added to the cells (Fig. 5A and B). Ca^{2+}-induced calpain activation was also inhibited by calpeptin (not shown). In contrast, caspase-8 was not activated in cells cultured in Ca^{2+}-rich medium (Fig. 5C and D). Caspase-8 activity, using Ac-IETD-AMC as a substrate, was not enhanced in cells cultured in Ca^{2+}-rich medium (data not shown). The results indicate that calpain activation leads to enhanced caspase-8 activation under conditions where caspase-8 is recruited to the membrane (i.e., upon binding of Aβ or of α-CD95 to the cell membrane), but not when caspase-8 is free in the cytoplasm.

3.5. $FLIP_S$ is degraded in differentiated PC12 cells exposed to sAβ and to α-CD95, but not when exposed to high Ca^{2+}

When recruited to the DISC in CD95-stimulated cells, $FLIP_S$ prevents caspase-8 activation by inhibiting the initial cleavage step of procaspase-8, whereas $FLIP_L$ allows the initial cleavage step of procaspase-8 but blocks further processing to caspase-8 (Krueger et al., 2001). As shown in Fig. 6, $FLIP_S$ levels were decreased in sAβ-treated cells; calpeptin inhibited the sAβ-induced decrease in

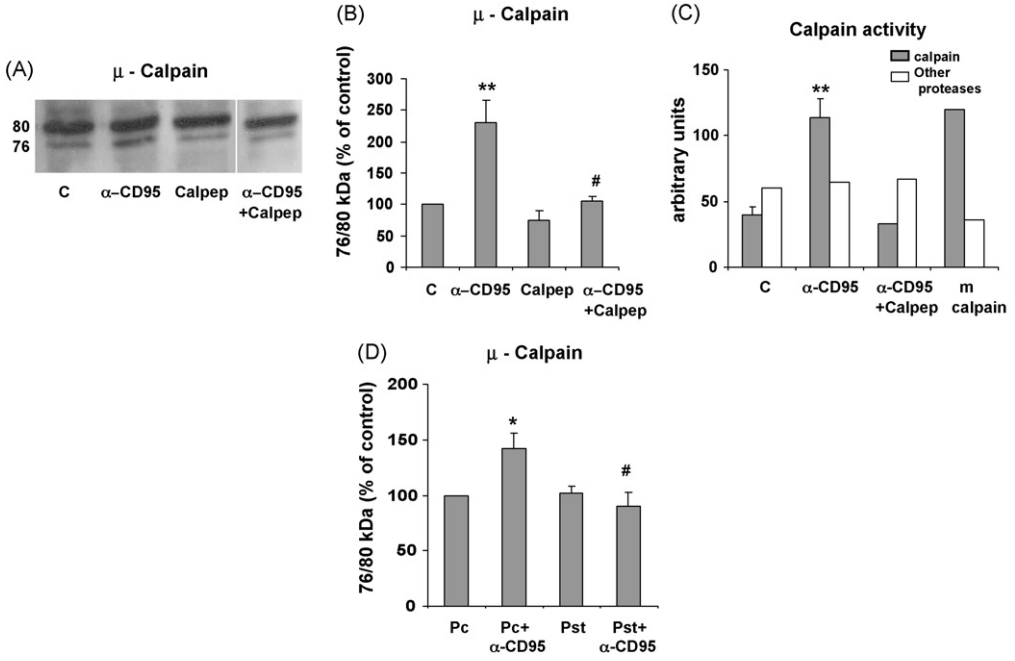

Fig. 2. μ-Calpain activation is enhanced in α-CD95-treated differentiated PC12 cells, and activation is inhibited by calpeptin and by calpastatin overexpression. (A)–(C) μ-Calpain in control and α-CD95-treated cells in the absence and presence of calpeptin. C, control; Calpep, calpeptin. (A) Immunoblot of μ-calpain. (B) Ratios of μ-calpain 76 kDa/80 kDa. Graph represents means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. control (considered 100%); $^{\#}p < 0.05$ vs. α-CD95. (C) Calpain activity in control and in α-CD95-treated cells cultured in the absence and presence of calpeptin. Activity of calpain was estimated in cell extracts, using FITC-casein as a substrate, without and with calpeptin (0.2 mM) added to the reaction mixture samples. The activity exhibited by the cell extract reaction mixtures containing calpeptin represents cleavage of FITC-casein in the extracts by proteases other than calpain. The activity in the reaction mixtures containing calpeptin was subtracted from the total activity measured without calpeptin addition to the reaction mixture, to obtain values for calpain activity. Calpain activity was normalized for equal protein content in the cell extracts. For control and α-CD95-treated cells, the graph represents means ± SEM ($n = 3$). $^{**}p < 0.01$ vs. control. For α-CD95-treated cells cultured in the presence of calpeptin (α-CD95 + calpep), average of two experiments is shown. (D) Ratios of μ-calpain 76 kDa/80 kDa in control and α-CD95-treated cells transfected with control plasmid (Pc) and with calpastatin plasmid (Pst). Graph represents means ± SEM ($n = 3$). $^{*}p < 0.05$ vs. Pc (considered 100%); $^{\#}p < 0.05$ vs. Pc + α-CD95.

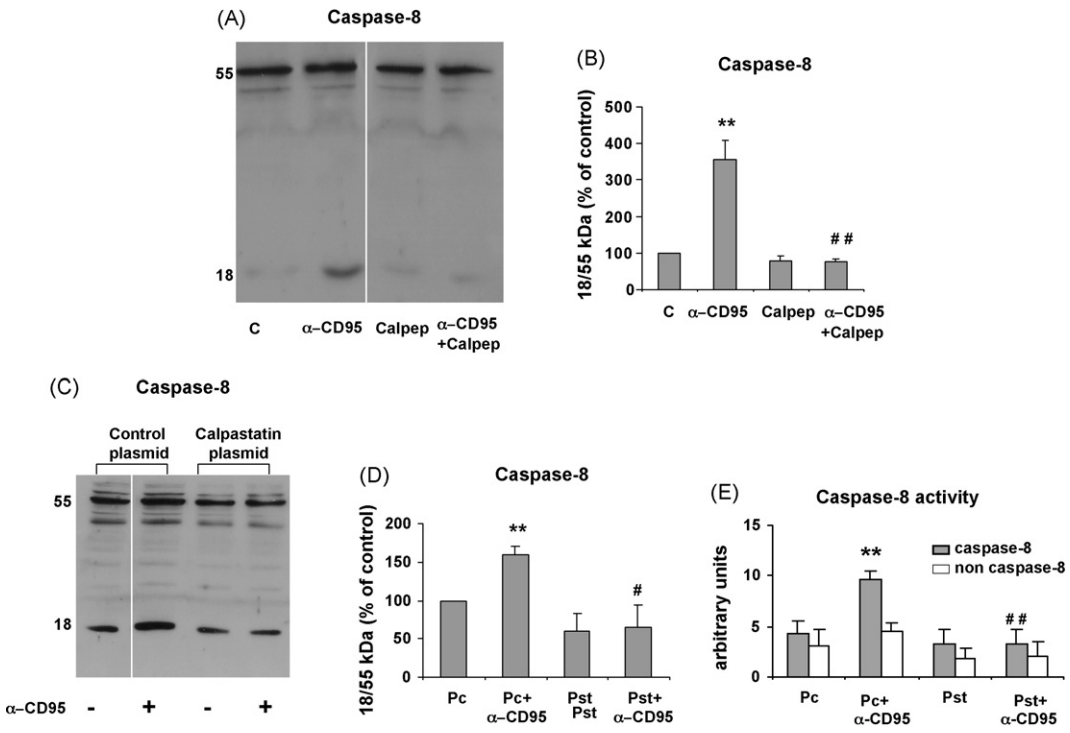

Fig. 3. Caspase-8 activation in α-CD95-treated differentiated PC12 cells is inhibited by calpeptin and by calpastatin overexpression. (A) and (B) Caspase-8 in control and α-CD95-treated cells in the absence and presence of calpeptin. C, control; Calpep, calpeptin. (A) Immunoblot of caspase-8. (B) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. control (considered 100%); $^{\#\#}p < 0.01$ vs. α-CD95. (C) and (D) Caspase-8 in control and α-CD95-treated cells transfected with control plasmid (Pc) and with calpastatin plasmid (Pst). (C) Immunoblot of caspase-8. (D) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. Pc (considered 100%); $^{\#}p < 0.05$ vs. Pc + α-CD95. (E) Activity of caspase-8 in extracts made of α-CD95-treated Pc and Pst cells, using Ac-IETD-AMC as a substrate, in the absence and presence of the caspase-8 inhibitor Z-IETD-FMK (ZIET) added to the reaction mixture samples. The activity exhibited by the samples containing ZIET represents cleavage of Ac-IETD-AMC by proteases other than caspase-8. The activity in the samples containing ZIET was subtracted from the total activity measured in the absence of ZIET to obtain values for caspase-8 activity. Caspase-8 activity was normalized for equal protein content in the cell extracts. Graph represents means ± SEM ($n = 3$). $^{**}p < 0.01$ vs. Pc; $^{\#\#}p < 0.01$ vs. Pc + α-CD95.

$FLIP_S$ levels (Fig. 6A and B). α-CD95 treatment also led to decreased $FLIP_S$ levels and calpeptin inhibited this decrease (Fig. 6C and D). In contrast, increased Ca^{2+} per se had no effect on the levels of $FLIP_S$ (Fig. 6E). The lack of effect of high Ca^{2+} in the medium on $FLIP_S$ suggests that increasing cellular Ca^{2+} per se, though it activates calpain, does not affect $FLIP_S$, similar to the lack of effect on caspase-8. No changes were seen in $FLIP_L$ under any of the treatments described here (data not shown).

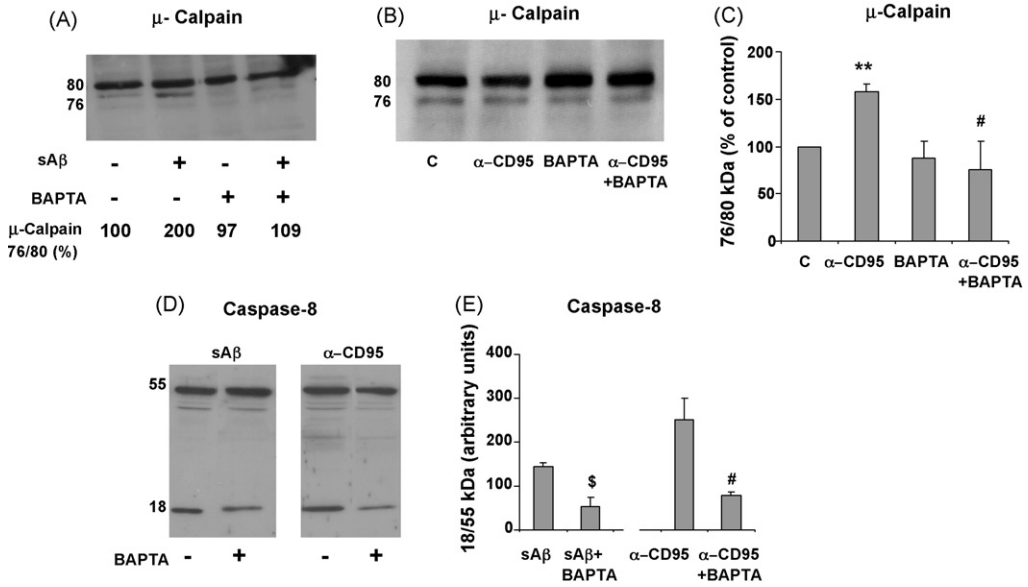

Fig. 4. BAPTA-AM inhibits activation of μ-calpain and of caspase-8 in cells exposed to sAβ, and to α-CD95. (A) μ-calpain in cells exposed to Aβ. Immunoblot, representative of two experiments. (B) and (C) μ-calpain in cells exposed to α-CD95. (B) Immunoblot of μ-calpain. (C) Ratios of μ-calpain 76 kDa/80 kDa. Graph representing means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. control (considered 100%); $^{\#}p < 0.05$ vs. α-CD95. (D) and (E) Caspase-8 in cells exposed to sAβ, and to α-CD95. (D) Caspase-8 immunoblots. (E) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means ± SEM ($n = 4$). $^{\$}p < 0.02$; $^{\#}p < 0.05$ vs. Aβ-treated and α-CD95-treated cells, respectively.

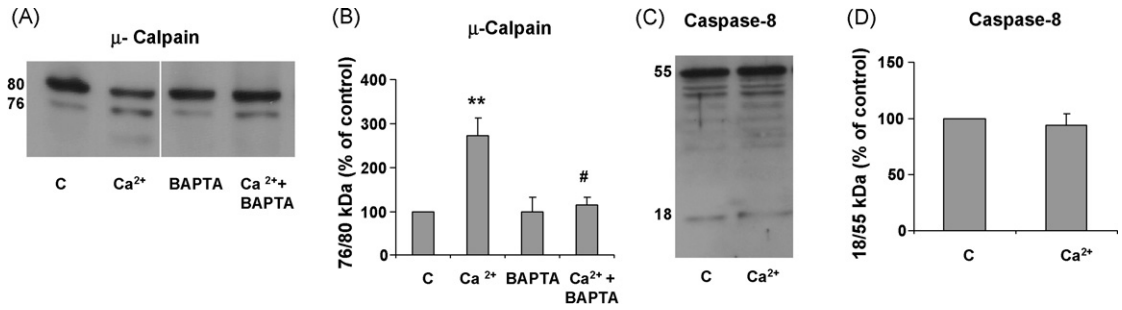

Fig. 5. μ-Calpain and caspase-8 in cells exposed to high Ca^{2+} in the medium. (A) and (B) μ-Calpain in cells exposed to high Ca^{2+} in the presence and absence of BAPTA-AM (BAPTA). (A) Immunoblot of μ-calpain. (B) Ratios of μ-calpain 76 kDa/80 kDa. Graph represents means ± SEM ($n = 4$). $^{**}p < 0.01$ vs. control (considered 100%); $^{\#}p < 0.05$ vs. Ca^{2+}. (C) and (D) Caspase-8 in cells exposed to high Ca^{2+}. (C) Immunoblot of caspase-8. (D) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means ± SEM ($n = 6$) of Ca^{2+}-treated cells vs. control (considered 100%).

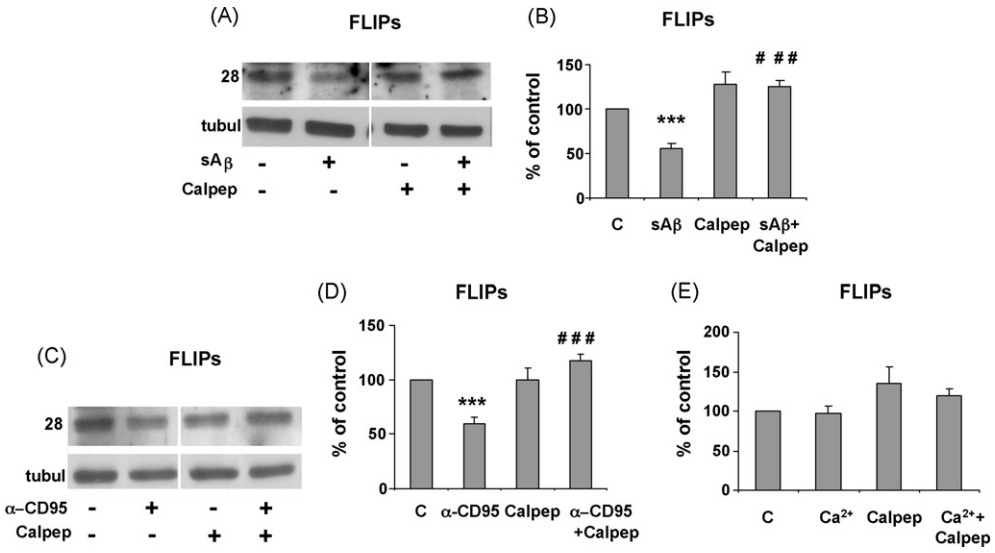

Fig. 6. $FLIP_S$ levels in cells exposed to sAβ, α-CD95 and to high Ca^{2+} in the medium, in the presence and absence of calpeptin. C, control; Calpep, calpeptin; Tubul, Tubulin. (A) and (B) Cells treated with sAβ. (A) Immunoblot of $FLIP_S$. (B) Graph represents means ± SEM ($n = 4$). $^{***}p < 0.001$ vs. control (considered 100%); $^{\#\#\#}p < 0.001$ vs. sAβ. (C) and (D) Cells treated with α-CD95. (C) Immunoblot of $FLIP_S$. (D) Graph represents means ± SEM ($n = 4$). $^{***}p < 0.001$ vs. control (considered 100%); $^{\#\#\#}p < 0.001$ vs. α-CD95. (E) $FLIP_S$ levels in cells exposed to high Ca^{2+} in the medium. Graph represents means ± SEM ($n = 4$).

4. Discussion

Cross-talk between caspases and the calpain-calpastatin system has been described in processes leading to cell damage under conditions of high Ca^{2+}. Active calpain promotes the cleavage of several procaspases, resulting in either inactivation or activation of the caspases (Orrenius et al., 2003). Calpain cleaves and inactivates caspase-9 in cell lysate and in intact cells, and may decrease caspase-3 by cleavage of the active caspase-3 (Bizat et al., 2003; Chua et al., 2000). Calpain promotes the activation of the endoplasmic reticulum-associated caspase-12 (Nakagawa and Yuan, 2000), and has been implicated in the activation of caspase-7 (Ruiz-Vela et al., 1999) and caspase-3 during certain insults (Blomgren et al., 2001). Caspase-8 has been shown to be cleaved by calpain in cell lysate, but the functional consequences are not known and the effects of calpain on caspase-8 in intact cells have not been studied (Chua et al., 2000). Here we demonstrate that in differentiated PC12 cells, calpain is involved in caspase-8 activation via the Aβ-induced and the CD95-triggered pathways.

Calpain is known to be activated in several types of neuronal cells exposed to Aβ (Fifre et al., 2006; Kelly and Ferreira, 2006; Nakagawa and Yuan, 2000; Vaisid et al., 2008a,b), whereas calpain activation via the CD95 pathway has not been previously described. We now show that calpain is activated upon binding of α-CD95 to differentiated PC12 cells, and that the α-CD95-induced activation is inhibited in cells treated with calpeptin, with Ca^{2+} chelator, and in cells overexpressing calpastatin. Caspase-8 is known to be activated in cells treated with α-CD95 (Krueger et al., 2001; Medema et al., 1997) and with Aβ (Cantarella et al., 2003; Vaisid et al., 2008a). Unexpectedly, the enhanced caspase activation via both the Aβ and CD95 pathways was inhibited under conditions of calpain inhibition and Ca^{2+} chelation. These results, based on the use of pharmacological inhibitors, indicated that calpain activity led to caspase-8 activation. To further probe the effect of calpain on caspase-8, we studied differentiated PC12 cells overexpressing calpastatin. The fact that overexpression of calpastatin inhibits Aβ and α-CD95-induced caspase-8 activation substantiates the conclusion that calpain is involved in the caspase-8 activation in both pathways.

FLIP has been identified as a protein capable of blocking CD95-mediated apoptosis (Krueger et al., 2001; Medema et al., 1997). FLIP associates with procaspase-8 in the DISC; when present in excess to procaspase-8, FLIP inhibits the processing of the procaspase to active caspase-8. Both FLIP isoforms, $FLIP_S$ (short) and $FLIP_L$ (long) inhibit the generation of active caspase-8. $FLIP_S$ prevents the initial cleavage step of procaspase-8, so that the full length of procaspase-8 is detected at the DISC. In contrast, $FLIP_L$ allows the initial cleavage step but blocks further processing to active caspase-8 (Krueger et al., 2001). FLIP can be degraded by caspase (Fischer et al., 2003), but there is no information on whether it is a substrate for calpain. We

show here that $FLIP_S$ (but not $FLIP_L$) is degraded in cells exposed to Aβ or to α-CD95, and that the degradation is inhibited by inhibition of calpain. These results indicate that calpain is responsible for $FLIP_S$ degradation under the conditions used here.

Overall, the results are consistent with published data on the activation of procaspase-8 at the cell membrane (Krueger et al., 2001). In the case of the CD95 pathway, it has generally been assumed that when associated with the CD95-death-inducing signaling complex (DISC), procasopase-8 is dimerized. Subsequently, two sequential cleavage events generate mature stable active caspase-8 that is released into the cytosol, where it can activate a cascade of other caspases (Chang et al., 2003; Doseff, 2004; Krueger et al., 2001; Maelfait and Beyaert, 2008; Medema et al., 1997). The results presented here indicate that calpain promotes the proteolysis of the α-CD95-induced, DISC-associated, dimerized procaspase-8. In the case of Aβ, binding of the peptide to the cell membrane appears to be required for its toxicity (Simakova and Arispe, 2007). Aβ binds to membrane lipids and to a variety of membrane proteins (Laurén et al., 2009; Verdier et al., 2004). Aβ binding has been shown to lead to the formation of Aβ-APP complexes, and to the recruitment and activation of caspase-8 (Lu et al., 2003). Thus, though the exact identity of the membrane receptors and membrane-associated proteins involved in the Aβ-induced toxicity and caspase-8 activation is not known at present, the overall mechanism responsible for the Aβ-induced caspase-8 activation may be similar to that involved in the CD95 pathway, i.e., Aβ-binding to the membrane triggers the recruitment of procaspase-8 to the membrane, followed by activation to caspase-8.

It is of interest to note that the CD95 pathway and FLIP have been implicated in AD, and that Aβ toxicity may involve the CD95 pathway. Increased levels of the death receptor CD95 have been observed in postmortem AD brain (De La Monte et al., 1997), and FLIP has been found to be decreased in AD as compared to age-matched control brains (Zhao et al., 2003). Aβ-mediated neuronal death is prevented by caspase-8 inhibition, or in dominant negative FADD-cells (Ivins et al., 1999). Aβ may also participate in apoptosis of neurons via the activation of c-Jun and induction of CD95 ligand, leading to activation of the CD95 pathway (Morishima et al., 2001). In addition, a tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) contributes to Aβ-toxicity; caspase-8 inhibition and FADD deficiency prevent the TRAIL and Aβ-induced neurotoxicity (Cantarella et al., 2003). Thus Aβ may, either directly or via forming protein complexes upon binding to other membrane sites, interact with the CD95 pathway, leading to calpain activation and calpain-dependent caspase-8 activation. It should be noted that caspase-8 can be activated by interchain proteolysis via other triggered apoptotic pathways, such as the mitochondrial pathway (Murphy et al., 2004; Sohn et al., 2005). A possible role of calpain in these pathways remains to be studied.

Ca^{2+} is increased in cells exposed to Aβ (LaFerla, 2002), and Aβ plaques impair Ca^{2+} homeostasis in vivo, resulting in disruption of neuronal networks (Kuchibhotla et al., 2008). Aβ-induced calpain activation is inhibited by Ca^{2+}-chelation, as documented in published studies (Fifre et al., 2006; Kelly and Ferreira, 2006), and confirmed in the present study. Ca^{2+} is also increased in cells exposed to CD95 ligands (Rovere et al., 1996). In addition, it has been shown that Ca^{2+} mobilization is impaired in cells deficient in FADD (Hueber et al., 2000). Since increased cellular Ca^{2+} is required for promoting calpain activation, we studied calpain and caspase-8 activation under conditions of increased cellular Ca^{2+} per se (i.e., by placing differentiated PC12 cell cultures in medium containing high Ca^{2+}). The fact that under these conditions, calpain activation is promoted whereas caspase-8 activation is not, indicates that calpain leads to enhanced caspase-8 activation when procaspase-8 is associated with the membrane protein complexes formed by α-CD95 or by Aβ binding. It is of interest to note that in a study on rat retinal ganglion cells, binding of interferon-gamma to the cells-induced caspase-8 activation (inhibited by calpeptin), whereas increased Ca^{2+} by ionophore did not lead to caspase-8 activation (Das et al., 2006). The results in the present study may cast light on the published results, i.e., that in the intact cell calpain is involved in caspase-8 activation when a membrane site is triggered, whereas free caspase-8 is not activated by ionophore-induced Ca^{2+} increase.

As shown here, $FLIP_S$ is also not affected by active calpain in cells exposed to high Ca^{2+} per se. The fact that in the absence of membrane binding ligands (i.e., in the absence of Aβ or CD95 triggering) $FLIP_S$ is not degraded by calpain is consistent with the notion that $FLIP_S$ is degraded when associated with procaspase-8 at the cell membrane. It is not clear at present whether the dimerization of caspase-8 at the DISC promotes its cleavage by calpain, or whether the calpain-promoted degradation of $FLIP_S$ allows autoproteolysis of procaspase-8. Additional study is necessary to further clarify this point. Involvement of additional factors (e.g., binding to phospholipids, extracellular signal-regulated kinase (ERK) activation) (Lüschen et al., 2005; Wells et al., 2005) remains to be studied.

Caspase-8, in addition to being an initiator of receptor-induced apoptosis (Doseff, 2004), plays a role in nonapoptotic processes (Lamkanfi et al., 2007; Maelfait and Beyaert, 2008). In some processes, caspase-8 activity is required. Specific examples of participation of active caspase-8 in nonapoptotic procesess include NF-kB activation in lymphocytes (Su et al., 2005), placental trophoblast differentiation and fusion (Black et al., 2004), T-cell activation (Koenig et al., 2008). In such cases, calpain may be involved in promoting caspase-8 activation. In other nonapoptotic functions, caspase-8 may play a role independently of its proteolytic activity. Inactive procaspase-8 protein or its death effector domains (DED) are involved in cell motility, via association with the integrin-dependent focal adhesion complex (FAC) proteins, including calpain/calpastatin (Barbero et al., 2009; Finlay and Vuori, 2007; Helfer et al., 2006). The FAC-associated inactive caspase-8 appears to promote calpastatin dissociation from calpain, thereby facilitating calpain activation (Barbero et al., 2009). Calpain cleaves certain FAC components and plays a role in the regulation of cell adhesion and motility (Wells et al., 2005). Other nonapoptotic processes involving caspase-8 include embryonic development (Kang et al., 2004) and transformation suppression (Krelin et al., 2008); however, it is not yet clear whether the presence of the protein suffices or active caspase-8 is required in these processes. It is possible that in all cases, membrane protein complex-associated caspase-8 protein promotes dissociation of calpain from its inhibitor calpastatin, allowing calpain activation. In some cases, calpain helps to transform procaspase-8 to active caspase-8, resulting in caspase and calpain-induced protein degradation. In cases where caspase-8 formation is precluded, calpain-promoted protein degradation still occurs.

A scheme, contrasting the effects of calpain on caspase-8 and $FLIP_S$ via CD-95 and Aβ-associated membrane complexes with lack of effects of calpain on free caspase-8 and $FLIP_S$, is presented in Fig. 7. In addition, a possible procaspase-promoted dissociation of calpain/calpastatin is indicated, as proposed by Barbero et al. (2009) for FAC-recruited caspase-8.

In sum, calpain is shown here to be involved in the processing of procaspase-8 to caspase-8. The results suggest that recruitment of procaspase-8 to the membrane is required for calpain-promoted activation to caspase-8. The results presented here add to the understanding of the roles of calpain, caspase-8 and the CD95 pathway in AD and in Aβ-toxicity. Calpain-promoted activation of caspase-8 may have implications for other types of CD95-induced cell damage, and for certain nonapoptotic functions of caspase-8. Increased expression of calpastatin or FLIP and/or decrease in calpain and caspase-8 may serve as means for the prevention of Aβ-toxicity and CD95-induced cell death.

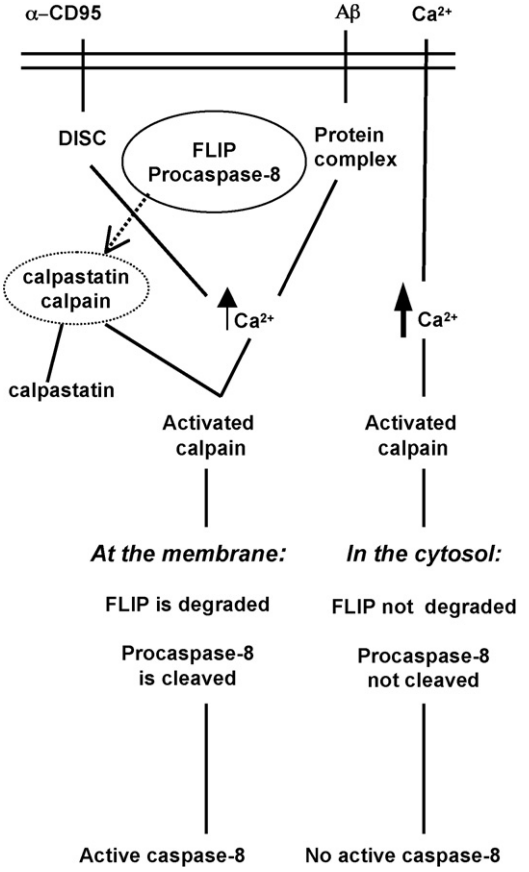

Fig. 7. Schematic representation of the proposed pathways involved in calpain-induced caspase-8 activation. Triggering of the CD95 receptors leads to DISC formation (including FADD, procaspase-8 and FLIP). Similarly, binding of Aβ to the cell membrane leads to the formation of membrane-associated protein complex, incorporating procaspase-8/FLIP. In both cases, cellular Ca^{2+} is increased; the membrane-associated procaspase-8 may enhance calpastatin dissociation from calpain (proposed by Barbero et al. (2009) for the focal adhesion complex-associated caspase-8). As a result, calpain activation is facilitated in the CD95-triggered and in Aβ-treated cells; in turn, calpain activates caspase-8 (by direct cleavage of procaspase-8, or possibly allowing procaspase-8 autoproteolysis by degradation of $FLIP_S$). A significant increase in Ca^{2+} per se leads to calpain activation without effect on procaspase-8/$FLIP_S$. For details, see text.

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