Specific Increase in Amyloid β-Protein 42 Secretion Ratio by Calpain Inhibition

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ABSTRACT: Cerebral deposition of amyloid β-protein (Aβ) as senile plaques is a pathological hallmark of Alzheimer's disease (AD). Aβ falls into two major subspecies defined by their C-termini, Aβ40 and Aβ42, ending in Val-40 and Ala-42, respectively. Although Aβ42 accounts for only ∼10% of secreted Aβ, Aβ42 is the predominant species accumulated in senile plaques in AD brain and appears to be the initially deposited species. Its secretion level has recently been reported to be increased in the plasma or culture media of fibroblasts from patients affected by any of early-onset familial AD (FAD). Thus, inhibition of Aβ42 production would be one of the therapeutic targets for AD. However, there is little information about the cleavage mechanism via which Aβ40 and Aβ42 are generated and its relationship to intracellular protease activity. Here, we examined by well-characterized enzyme immunoassay the effects of calpain and proteasome inhibitors on the levels of Aβ40 and Aβ42 secretion by cultured cells. A calpastatin peptide homologous to the inhibitory domain of calpastatin, an endogenous calpain specific inhibitor, induced a specific increase in secreted Aβ42 relative to the total secreted Aβ level, a characteristic of the cultured cells transfected with FAD-linked mutated genes, while a proteasome specific inhibitor, lactacystin, showed no such effect. These findings suggest that the Aβ42 secretion ratio is modulated by the calpain−calpastatin system and may point to the possibility of exploring particular compounds that inhibit Aβ42 secretion through this pathway.

Cerebral deposition of amyloid β-protein (Aβ)[1] as senile plaques is the earliest immunocytochemically detectable lesion in the brain affected by Alzheimer's disease (AD). Aβ is a 39−43 residue protein which is cleaved from a much larger precursor, β-amyloid precursor protein (βAPP), by as yet unidentified enzymes, so-called β- and γ-secretases (Haass & Selkoe, 1993). Currently, there exists growing evidence that Aβ42, ending in Ala-42, plays a key role in the β-amyloidogenesis in both leptomeningeal vessels and brain parenchyma. First, Aβ42 is the major component of both vascular amyloid (Roher et al., 1993a; Shinkai et al., 1995) and senile plaques (Roher et al., 1993b; Iwatsubo et al., 1994). Second, Aβ42 deposition appears to be the initial event in vascular amyloid formation in normal aging brains (Shinkai et al., 1995) and in senile plaque formation in AD (Iwatsubo et al., 1994), Down's syndrome (Iwatsubo et al., 1995), and normal aging brains (Fukumoto et al., 1996). Third, *in vitro* experiments have revealed that Aβ42 has a much higher aggregation potential than Aβ40, thus accelerating the formation of Aβ fibrils by a nucleation-dependent mechanism (Jarrett et al., 1993). Finally, all three early-onset familial AD (FAD) genes identified to date, βAPP717 mutations and presenilin 1 and presenilin 2 mutations, have been shown to be invariably associated with an increased level of Aβ42 secretion (Suzuki et al., 1994; Scheuner et al., 1996; Tomita et al., 1997). The secretion and thus production of Aβ42 therefore appears to be one of prime targets for therapeutic intervention in AD. To this end, identification of a particular perturbation, which differentially affects Aβ40 and Aβ42 secretion, is essential. Thus far, there have been a few studies about the effects of protease inhibitors on Aβ secretion (Klafki et al., 1995; Higaki et al., 1995), but the levels of secreted Aβ40 and Aβ42 were not separately determined in these studies. Thus, we have examined the effects of several protease inhibitors on the secretion of Aβ40 and Aβ42, using a well-characterized enzyme immunoassay (EIA) and highly specific intracellular protease inhibitors. Here, we report that the increased level of Aβ42 secretion relative to the total Aβ secretion level (Aβ42 ratio) of the cells carrying early-onset FAD genes can be simulated by use of calpain inhibitors even for cells harboring a wild-type βAPP gene.

MATERIALS AND METHODS

Cell Culture. All transfected cell lines described in this report carry derivatives of pCMV695, a plasmid carrying

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[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; βAPP, β-amyloid precursor protein; CAA, cerebral amyloid angiopathy; cBz, carbobenzoxyl; CMV, cytomegalovirus; CS peptide, calpastatin peptide; DMSO, dimethyl sulfoxide; EIA, enzyme immunoassay; FAD, familial Alzheimer's disease; FBS, fetal bovine serum; nLeu, norleucine; PVDF, polyvinylidenefluoride.

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the βAPP695 gene under the control of the cytomegalovirus (CMV) promoter (Selkoe et al., 1988). K269 cells are human embryonic kidney 293 cells stably transfected with pCMV695. K695sw cells are 293 cells stably transfected with a construct carrying the Swedish mutation, K595N/M596L (Citron et al., 1992). K695717G cells are 293 cells stably transfected with βAPP695 carrying the mutation V717G (βAPP770 numbering; Chartier-Harlin et al., 1991). SH-SY5Y cells derived from human neuroblastoma cells secrete detectable levels of Aβ without βAPP gene transfection (Fuller et al., 1995). All the above cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS).

*EIA for Aβ*40 and 42. A well-characterized EIA for Aβ40 and Aβ42 quantitation was used as described in detail elsewhere (Suzuki et al., 1994). BNT77, a monoclonal antibody generated against synthetic Aβ11−28 (Asami-Odaka et al., 1994), was used as a capture antibody so that, in the EIA, Aβ N-terminally truncated up to position 11 (Tamaoka et al., 1994) as well as full-length Aβ could be detected. However, in this EIA, p3 is not detected because the epitope of BNT77 is located in Aβ11−16 (unpublished data). The specificities of the detecting antibodies, BA27 and BC05, have been previously shown (Suzuki et al., 1994). This EIA system is highly sensitive and can be used to detect Aβ40 and Aβ42 at pM levels. EIA values for culture media were normalized against the βAPP level in the figures except Figure 1A. The data were statistically analyzed using a two-tailed *t*-test. All the experiments were performed in triplicate at least three times, and the results were found to be reproducible.

Drug Treatment. Protease inhibitors, calpeptin (benzyl-oxycarbonyl-leucyl-leucyl-norleucynal; Tocris Cookson), ALLNal (*N*-acetyl-leucyl-leucyl-norleucynal; Boehringer Mannheim, Inc.), and MG132 (carbobenzoxyl-leucyl-leucyl-leucynal; Peptide Institute) were dissolved in dimethyl sulfoxide (DMSO). A23187 was purchased from Sigma. As a highly specific inhibitor of calpain, a 27-mer synthetic calpastatin peptide (CS peptide, acetyl-DPMSSTYIEEL-GKREVTIPPKYRELLA-NH_2) homologous to the minimal inhibitory domain I of human calpastatin (Maki et al., 1989; Kawasaki et al., 1989) and its corresponding scrambled peptide for control (acetyl-APRLEIVPTMYIYKLSPTG-SEKLEDER-NH_2; Eto et al., 1995) were used. Lactacystin was dissolved in DMSO at concentrations of 1−100 mM. Confluent 6 cm dishes were conditioned in 4 mL of medium containing 10% FBS and the above inhibitors at various concentrations for 5 h. Control cells were treated with vehicle alone. Each stock solution for the inhibitors was added to culture medium at a dilution of >1/1000 (v/v). At these concentrations, no vehicle effects on Aβ secretion were observed.

Quantitation of βAPP by Western Blotting. Cells were grown to confluence in 6 cm dishes and treated with the drugs listed above. Conditioned media and 1% Triton X-100-extracted cell lysates were run on 7.5% SDS−polyacrylamide gels for detection of truncated βAPP and full-length βAPP, respectively. After the separated proteins were transferred onto polyvinylidenefluoride (PVDF) membranes, the membranes were incubated with 22C11(Boehringer Mannheim, Inc.) or C4 (polyclonal antibodies to βAPP666−695; Takio et al., 1989). These experiments were performed in triplicate and all the results were quantitated with a densitometer (Model GS-700 imaging densitometer, Bio-Rad).

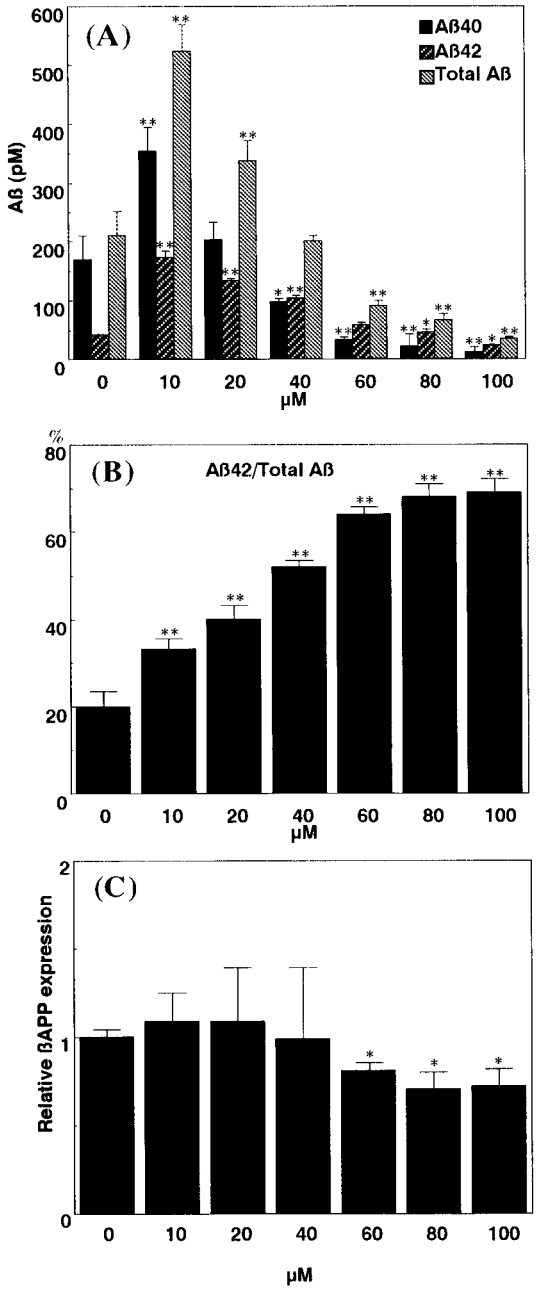

FIGURE 1: Effect of calpeptin on Aβ secretion by and βAPP expression in cultured K269 cells. The cells were cultured in the presence of calpeptin or vehicle alone for 5 h. Conditioned media were measured for Aβ40 and Aβ42 by EIA. The levels of Aβ40 and Aβ42 and total Aβ are given in panel A and the Aβ42 ratio (Aβ42/total Aβ) in panel B. The levels of intracellular full-length βAPP were determined by Western blotting with C4 and a densitometry (C). The mean control values for βAPP were arbitrarily assigned the value 1.00, and other values were normalized accordingly. Error bars indicate standard deviation. Each value was statistically compared to the control (vehicle alone value). (*) $p < 0.05$, (**) $p < 0.01$ (two-tailed *t*-test).

Detection of Calpain-Catalyzed Fodrin Degradation Product in the Cells. To confirm the effect of the CS peptide on intracellular calpain, we examined the levels of a 150 kDa degradation product of fodrin α subunit that is known to be generated by calpain decrease in the cell lysate. For the detection of the degradation product on the blot, an end-specific antibody that recognizes its amino-terminal end was used (Saido at al., 1993).

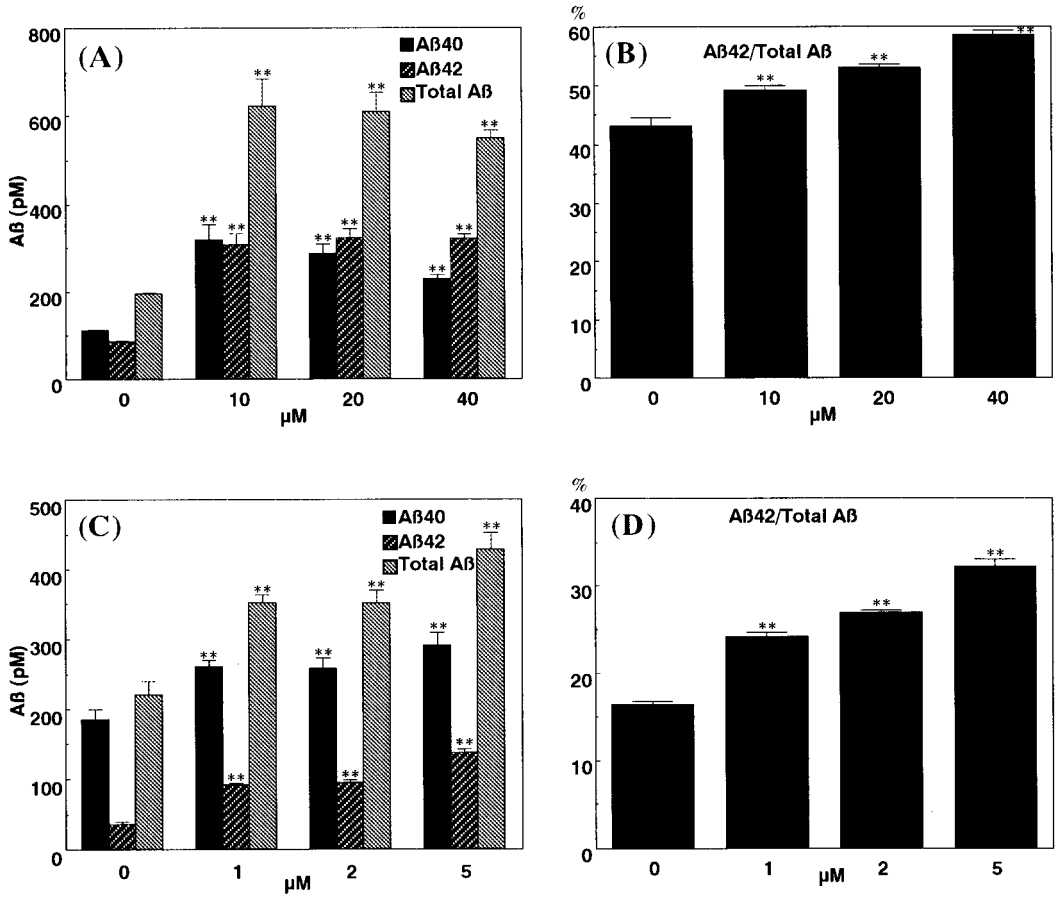

FIGURE 2: Effect of synthetic protease inhibitors on Aβ secretion by intact cultured cells. K695717G (A and B) and K269 (C and D) cells were cultured in the presence of calpeptin (A and B) or MG132 (C and D) for 5 h. The levels of Aβ40 and Aβ42 in conditioned media are presented in panels A and C, and the Aβ42 ratio (Aβ42/total Aβ) is given in panels B and D. Error bars indicate standard deviation. Each value was statistically compared to the control (vehicle alone value). (**) $p < 0.01$ (two-tailed *t*-test).

RESULTS

Synthetic Calpain and Proteasome Inhibitors Affect Aβ Secretion and Aβ42 Ratio. There are several reports that synthetic calpain inhibitors reduce the total Aβ secretion level (Klafki et al., 1995; Higaki et al., 1995). In particular, Higaki et al. (1995) showed that MDL28170 suppresses both total Aβ and p3 secretion and causes intracellular accumulation of corresponding 12 kDa and 10 kDa C-terminal fragments of βAPP that are left in cells, an observation which suggests direct inhibition of γ-secretase by MDL28170 (Higaki et al., 1995). Thus, we first treated K296 cells with a similar peptidyl aldehyde inhibitor, calpeptin (cBz-Leu-nLeu-H). The treatment, at a concentration of 10 μM for 5 h, led to an increase in the level of both Aβ40 and Aβ42 secretion (Figure 1A). At the same time, the Aβ42 ratio profoundly increased ($p = 0.000\,01$; Figure 1B). With increasing calpeptin concentration above 20 μM, the levels of Aβ40 and Aβ42 decreased, and at 60 μM calpeptin the total Aβ level was lower than the control level (Figure 1A). In contrast, the Aβ42 ratio increased continuously with increasing calpeptin concentration up to 100 μM. These results suggest that calpeptin affects Aβ42 and Aβ40 secretion differentially: at less than 20 μM calpeptin Aβ42 secretion is more enhanced than that of Aβ40 and at greater than 40 μM calpeptin Aβ40 secretion is affected to a greater extent than that of Aβ42. The results obtained at concentrations of calpeptin higher than 60 μM are consistent with the reported finding that MD28170 at 200 μM suppresses γ-secretase cleavage at the carboxyl side of Val-40 to a greater extent than that at the carboxyl side of Ala-42 (Citron et al., 1996). However, in our hands, at calpeptin concentrations higher than 40 μM, the treated cells underwent morphological changes, strongly suggesting that such high concentrations of calpeptin cause generalized metabolic derangements leading to nonspecific alterations of Aβ production and/or secretion. Furthermore, the intracellular level of mature βAPP, which is considered to be a direct precursor of Aβ, gradually decreased at over 60 μM calpeptin, as shown by Western blotting (Figure 1C). Considering all of the above findings, we have reservations about the claim that the reduction in the level of Aβ secretion is a specific effect of calpain inhibition.

Thus, to learn about specific effects of calpeptin, we used lower concentrations of calpeptin where the cell morphology and βAPP expression level were not altered (Figure 1C). At calpeptin concentration of 20 μM or less, both the total Aβ secretion level and the Aβ42 ratio increased. The same effects of calpeptin were observed with K695717G cells (Figure 2, panels A and B), K695sw cells (data not shown), and SH-SY5Y cells of an untransfected human neuroblastoma cell line (data not shown). We also treated the cells with another peptidyl aldehyde calpain inhibitor ALLNal (Ac-Leu-Leu-nLeu-H). This inhibitor at 20 μM caused a similar increase in the levels of Aβ40 and Aβ42 secretion and an increase in the Aβ42 ratio for K269 cells (data not shown).

Because proteasome is another major cytosolic protease, the cells were treated with a synthetic proteasome inhibitor,

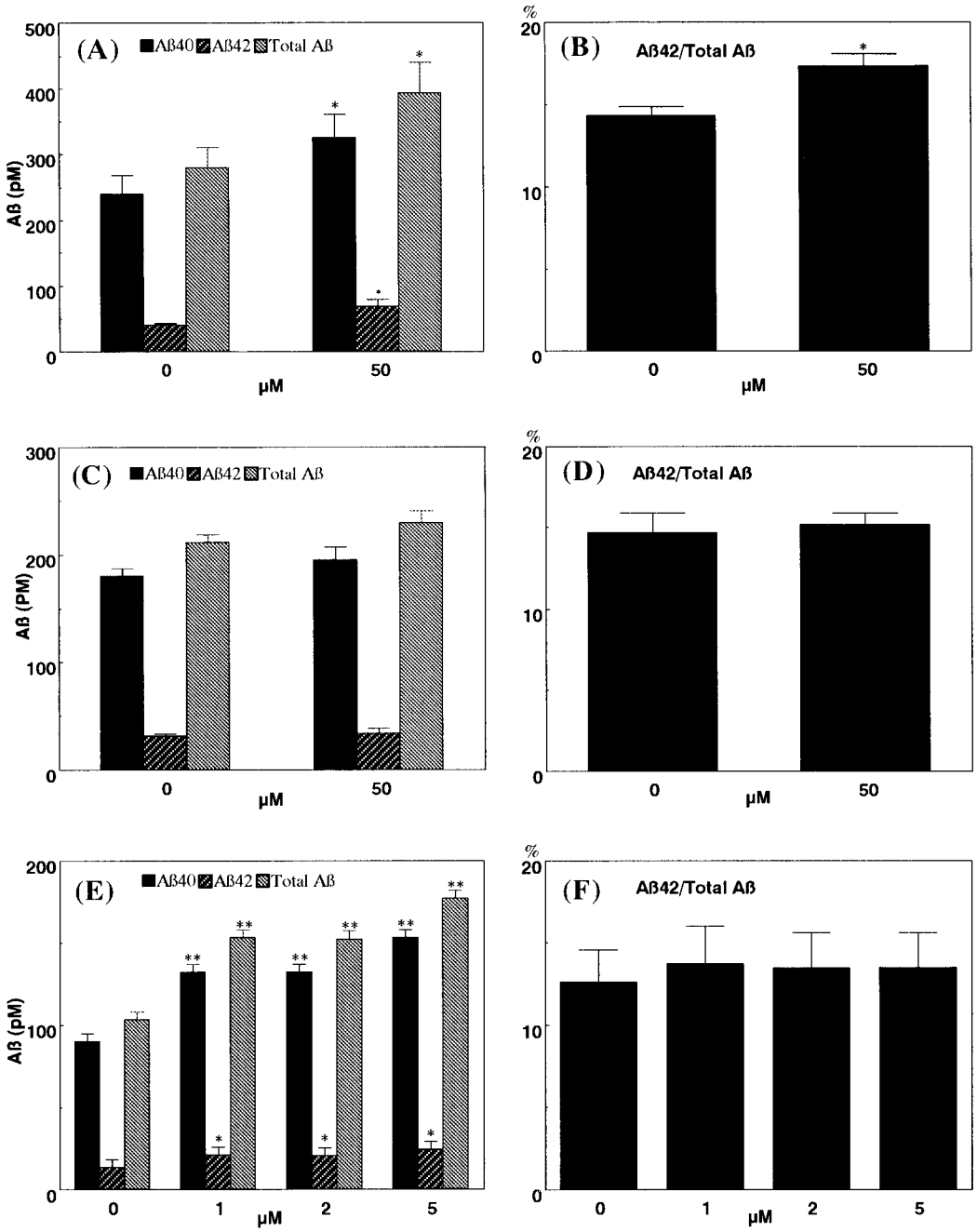

FIGURE 3: Effect of specific protease inhibitors on Aβ secretion by cultured cells. K269 (A−D) and SH-SY5Y (E and F) cells were treated with the CS peptide (A and B), its scrambled peptide (C and D) or lactacystin (E and F) for 5 h. The levels of Aβ species in conditioned media measured by EIA are shown in panels A, C, and E, and the Aβ42 ratio (Aβ42/total Aβ) is given in panels B, D, and F. Error bars indicate standard deviation. Each value was statistically compared to the control (vehicle alone value). (*) $p < 0.05$; (**) $p < 0.01$ (two-tailed *t*-test).

MG132 (cBz-Leu-Leu-Leu-H). Unexpectedly, as shown in Figure 2, panels C and D, this inhibitor also affected Aβ secretion, causing an increase in the levels of Aβ40 and Aβ42 secretion and an increase in the Aβ42 ratio. Interestingly, these effects were observed at MG132 concentrations that are 10-fold lower than the concentrations of calpeptin or ALLNal.

Calpastatin Peptide but Not Lactacystin Induced Increase in Aβ42 Ratio. Widely used peptidyl aldehyde inhibitors of calpain could also inhibit proteasome, although much higher concentrations are required for inhibition of the latter than the former. Calpeptin has been reported to be much more specific for calpain than are ALLNal and calpain inhibitor 2, which inhibit proteasome to a significant extent (Figueiredo-Pereira et al., 1994). Nevertheless, we cannot completely exclude the possibility that calpeptin affects proteasome or other cysteine proteases to a slight extent, but enough to cause a large effect on Aβ secretion. To further distinguish the effect on the Aβ42 ratio of calpain inhibition from that of proteasome or other cysteine proteases (for example, cathepsin B) inhibition in the cultured cells, we employed the synthetic calpastatin (CS) peptide, which is homologous to the inhibitory domain of calpastatin, an endogenous inhibitor which is highly specific for calpain, and does not inhibit other proteases (Dayton et al., 1976; Eto et al., 1995). Although high concentrations of the peptide were required for it to penetrate into the cells, this peptide brought about increases in the levels of Aβ40 and

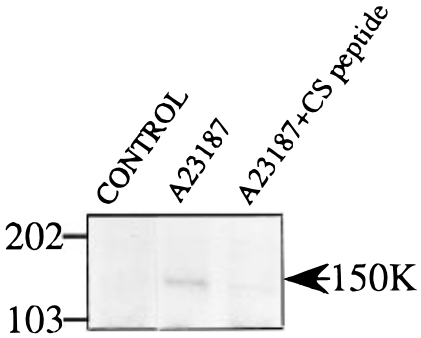

FIGURE 4: Effect of CS peptide on degradation of fodrin α subunit in cultured cells. Confluent K269 cells were untreated (control) or treated with 0.5 μM of A23187 (A23187) or with 0.5 μM of A23187 and 50 μM of CS peptide (A23187 + CS peptide) for 5 h and subsequently analyzed for a calpain-catalyzed 150 kDa product of fodrin α subunit by Western blotting using an end-specific antibody (Saido et al., 1993). An increased calpain activity induced by the A23187 treatment is demonstrated by the increased levels of the degradation product (arrow), but this enhancement is blocked by the addition of CS peptide. The positions of molecular mass standards (kilodaltons) are indicated in the left.

Aβ42 secretion and the Aβ42 ratio (Figure 3, panels A and B). The penetration of the CS peptide into the cells was confirmed by use of the rhodamine-labeled CS peptide under fluorescence microscopy (data not shown) and by Western blotting using an antibody specific for a 150 kDa form of fodrin α subunit, a calpain-catalyzed degradation product (Saido et al., 1993). As shown in Figure 4, the treatment of the cells with the calcium ionophore A23187 induced the 150 kDa proteolytic product, while the treatment with A23187 in the presence of CS peptide caused the band barely visible, strongly suggesting that CS peptide indeed inhibits the calpain activity in the cells.

In contrast, its corresponding scrambled peptide consisting of a randomized sequence with an identical amino acid composition (Eto et al., 1995), at the same concentrations as those of the CS peptide, caused no significant increase in the Aβ42 ratio ($p = 0.51$; Figure 3, panels C and D). The CS peptide at apparently nontoxic concentrations induced similar increases in the Aβ42 ratio for all the cell lines used in this study (data not shown).

Although the synthetic proteasome inhibitor MG132 showed a profound effect on Aβ42 secretion (see above), it is now known that MG132 can also inhibit calpain *in vitro* (Tsubuki et al., 1996). To learn more about the effects of proteasome on Aβ secretion, we used lactacystin (Omura et al., 1991), which is the most selective proteasome inhibitor presently known and has no effects on the cysteine proteases including calpain and cathepsin B (Coux et al., 1996; Fenteany, et al., 1995; Tanaka, 1995). Although lactacystin enhanced both Aβ40 and Aβ42 secretion, it induced no increase in the Aβ42 ratio (Figure 3, panels E and F). Taken together, these results indicate that the Aβ42 ratio is modulated by the calpain−calpastatin system but not by proteasome. Lactacystin had the same effects on all the cell lines used in this study.

Incubation of intact cells with the calcium ionophore A23187 in the presence of calcium results in activation of calpain in the cells, as demonstrated in Figure 4 and by autolysis of μ-calpain (Nixon, 1986). Thus, we treated the cells with A23187 to examine whether increased calpain activity inversely affects the level of Aβ42 secretion. However, A23187 did not alter the Aβ42 ratio (Figure 5, panels A and B), although it induced an increase in the total Aβ secretion level, consistent with the previous report (Querfurth & Selkoe, 1994). This may suggest that calpain is not directly involved in determination of the Aβ42 ratio.

DISCUSSION

During the last three years, several lines of evidence indicating that Aβ42 plays the central role in the senile plaque formation not only in FAD but also in sporadic AD and normal aging brains have been accumulated. Furthermore, increased secretion of Aβ42 has been found to be common to all three types of early-onset FAD with βAPP717, presenilin 1, or presenilin 2 mutation. Thus, a possible scenario is that an increased concentration of Aβ42 in the extracellular space caused by increased secretion of Aβ42 or its decreased clearance induces β-amyloidogenesis after middle age, eventually followed by neurofibrillary tangle formation and neuronal death. According to this scenario, an increased Aβ42 ratio should be an important determinant of AD pathogenesis, and the calpain inhibition shown here provides the fourth condition where a specific increase in the Aβ42 ratio occurs. Presumably, the mechanisms underlying an increase in the Aβ42 ratio may differ among these four conditions, and in this context it should be noted that this effect of calpain inhibition can be observed in all the cells used here, even in the cells harboring a βAPP717 mutation. This strongly suggests that this effect occurs

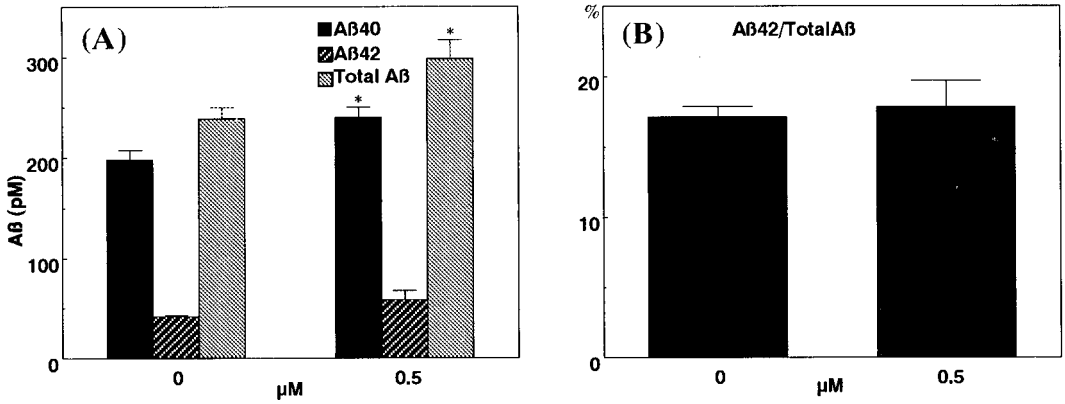

FIGURE 5: Effect of calcium ionophore, A23187, on Aβ secretion by K269 cells. Cells were treated with A23187 in the presence of calcium for 5 h and conditioned media were subjected to EIA. The levels of Aβ species are shown in panel A, and the Aβ42 ratio (Aβ42/total Aβ) is given in panel B. Error bars indicate standard deviation. Each value was statistically compared to the control (vehicle alone value). (*) $p < 0.05$ (two-tailed *t*-test).

through a pathway different from that in case of βAPP717 mutation. In other words, the present observation may point to the possibility of development of a particular reagent which reduces the Aβ42 ratio in any condition.

Synthetic cell-penetrating calpain inhibitors are useful for examining the role of calpain in intact cells. However, those peptidyl aldehyde inhibitors may not be specific enough for calpain (Figueiredo-Pereira et al., 1994; Mehdi, 1991; Tsubuki et al., 1996). For example, calpeptin and MDL28170 also inhibit cathepsin B, α-chymotrypsin, trypsin (Sasaki et al., 1990), and proteasome (Figueiredo-Pereira et al., 1994; Tsubuki et al., 1996). Thus, we should interpret very carefully the results obtained using those inhibitors alone. In the present study, we used the CS peptide and lactacystin, both of which are the most specific inhibitors thus far known for calpain and proteasome, respectively. One may note that the effect of the CS peptide was not so remarkable as that of other calpain inhibitors, raising a question about its effectiveness for calpain inhibition. But if its size (27-mer oligopeptide) is taken into account, it would not be unreasonable to assume that a small effect is due to its limited ability to penetrate the cell membrane. However, we do not exclude the possibility that other proteases, in particular cysteine proteases other than calpain, are also involved in increase in the Aβ42 ratio. It is possible that the calpain−calpastatin system causes the effect via indirect routes since enhanced calpain activity induced by the calcium ionophore did not affect the Aβ42 ratio.

In the present study, calpeptin and ALLNal (data not shown) clearly affected the secretion of Aβ40 and Aβ42 differentially; at the lower concentrations, Aβ42 secretion was more enhanced than that of Aβ40, while at higher concentrations Aβ40 secretion was more affected than that of Aβ42. These observations support the present hypothesis that distinct γ-secretases mediate the generation of Aβ40 and Aβ42 (Citron et al., 1996; Klafki et al., 1996).

Interestingly, all of the calpain inhibitors used here, as well as proteasome inhibitors and A23187, enhanced both Aβ42 and Aβ40 secretion. In particular, the CS peptide and lactacystin caused the same effect. This strongly suggests that increased total Aβ secretion is a nonspecific phenomenon which is caused probably by many kinds of perturbation. There is an important implication of this observation: Aβ secretion may be enhanced by many nonspecific unfavorable conditions where the general metabolism of cells is significantly affected. Such enhancement may underlie rapid accumulation of Aβ after head injury (Roberts et al., 1991, 1994) and steep increase in the concentration of Aβ in the leptomeninges (Shinkai et al., 1995) and brain parenchyma (unpublished observation) during aging in the general population. It should be noted that upregulation of βAPP as previously claimed for leptomeningeal vascular smooth muscle cells treated with Aβ (Davis-Salinas et al., 1995; Davis-Salinas & Van Nostrand, 1995) is not associated with this increased secretion of Aβ. Presumably, the sorting of βAPP into Aβ production pathways is controlled independently of the level of full-length βAPP.

Currently, we have no suitable explanation for parallel enhancement of Aβ40 and Aβ42 secretion by the cells in the presence of various inhibitors. If we assume that Aβ secretion directly reflects its intracellular production, one possible explanation would be that Aβ42 is a direct precursor of Aβ40. The inhibitors used in the present study would enhance production of Aβ42, which would be converted to Aβ40 by the action of a carboxyl dipeptidase, resulting in increased Aβ40 secretion. If the activity of the postulated peptidase is partially (and indirectly) blocked by calpain inhibitors, the proportion of secreted Aβ42 with respect to total Aβ would be increased. In the case of lactacystin the converting enzyme would not be affected, resulting in an unaltered Aβ42 ratio despite an increased amount of Aβ42.

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