We have studied the effects of peptide aldehyde protease inhibitors on the secretion of β-amloid peptide 1–40 (Aβ(1–40)) and Aβ(1–42) by HEK 293 and COS-1 cells expressing β-amloid precursor protein with the Swedish double mutation. A multiphasic SDS-polyacrylamide gel electrophoresis system was used for the discrimination of Aβ(1–40) and Aβ(1–42). Calpain inhibitor I, carbenzoxyl-Leu-Leu-leucinal, and calpentin were found to reduce the amount of Aβ(1–40) released into the medium in a dose-dependent manner. The reduction of Aβ(1–40) after treatment with 50 μM calpain inhibitor I or 5 μM carbenzoxyl-Leu-Leu-leucinal was accompanied by a slight increase of Aβ(1–42) released into the medium. These observations suggest that the cleavages at residues 40 and 42 are accomplished by different enzye activities.

Characteristic pathological findings in the brains of Alzheimer’s disease (AD) patients include amyloid deposits containing the β-amloid peptide (Aβ) as a major component. Aβ is a proteolytic fragment of the β-amloid precursor protein (APP), a large transmembrane glycoprotein with a single membrane-spanning region that exists in different isoforms (1). Proteolytic cleavage of APP just outside the transmembrane region within the Aβ sequence (2) by an unknown enzyme called α-secretase releases the soluble ectodomain of APP (3). This cleavage precludes Aβ production. Soluble Aβ peptides are generated by an alternative proteolytic processing pathway during normal metabolism of cells expressing APP (4–6). They can also be detected in cerebrospinal fluid (7). A double mutation within the APP gene (NL670/671) found in a Swedish early onset AD family (8) increases Aβ production (9–12). The enzymes cleaving APP to produce the amino and carboxyl termini of Aβ are referred to as β- and γ-secretases, respectively (13). None of these secretases has yet been identified.

Analyses of the Aβ peptides in cerebrospinal fluid (14), in conditioned media from HEK 293 cells transfected with an APP cDNA containing the Swedish double mutation (15) and from IMR 32 cells (16) revealed a series of Aβ peptides of various lengths. A minor portion of these has a longer carboxyl terminus and ends at amino acid residue 42 or 43 of the Aβ sequence. Point mutations located in codon 717 of the APP gene (APP770 numbering) found in some familial forms of AD increase the proportion of the longer Aβ peptides generated by transfected cells (17, 18). The longer peptides aggregate more rapidly (19) and were reported to be deposited preferentially in amyloid plaques (20–22) and at an early stage (23). Taken together, these findings strongly suggest that the peptides ending at residue 42 or 43 are critical for amyloidogenesis in AD.

Recent findings indicate that Aβ production can be reduced by inhibition of γ-secretase cleavage of APP. Higaki et al. (24) observed inhibition of Aβ formation with carbenzoxyl-Val-Phe-alaninal in Chinese hamster ovary cells transfected with a wild type APP cDNA. We previously reported the reduction of Aβ secretion from HEK 293 cells expressing APP with the Swedish double mutation after treatment with N-acyt-Leu-Leu-norleucinal (calpain inhibitor I) (25). Since no discrimination between Aβ(1–40) and Aβ(1–42) was accomplished in these studies, the observations presumably reflect the effects of the peptide aldehydes on the major Aβ species. In this study we investigated the influence of three related peptide aldehydes, calpain inhibitor I, carbenzoxyl-Leu-Leu-leucinal (MG 132) and carbenzoxyl-Leu-norleucinal (calpentin) on the secretion of Aβ(1–40) and Aβ(1–42) from HEK 293 and COS-1 cells expressing APP with the Swedish double mutation. For the discrimination of Aβ(1–42) from Aβ(1–40), we used Bicine/Tris SDS-polyacrylamide gels containing 8 M urea (26) and an antisera specific for the carboxyl terminus of Aβ(1–42).

**EXPERIMENTAL PROCEDURES**

Construction of the Expression Vector pCMVAPP771(SWE)—The Swedish double mutation NL670/671 (APP770 numbering) was introduced into wild type APP751 cDNA, and an optimal Kozak consensus sequence (GCC GCC ATG G) (27) was generated at the initiation codon as described (28). The APP751(SWE) cDNA was subsequently cloned into an expression vector with the cytomegalovirus promoter.

**Antibodies**—The rabbit antiserum NT11 and the mouse monoclonal antibody β1 were raised against synthetic Aβ(1–40) (29). The rabbit antiserum 42–14 was raised against synthetic Aβ36–42 coupled to maleimide-activated keyhole limpet hemocyanin and preabsorbed with solid phase coupled Aβ17–40 (Amino link plus kit, Pierce).

**Cell Culture, Transfections, and Pulse-Phase Experiments**—HEK 293 cells were routinely grown in 10-cm plates in minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Prior to experiments, penicillin and streptomycin were added to final concentrations of 100 IU/ml and 100 μg/ml, respectively. HEK 293 cells were transiently transfected with calcium phosphate DNA precipitates formed in BES (30). In most experiments, the cells were replated 16–20 h after addition of the DNA into 60-mm dishes. COS-1 cells were transfected with Transfectam (Promega) according to the manufacturer’s instructions. 40–44 h after transfection the cells were pulse labeled for 60 min at 37°C with 0.1 mCi/ml [35S]methionine in methionine-free Dulbecco’s modified Eagle’s medium (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 μM HEPES, pH 7.4, and 2% dialyzed fetal calf serum and chased for 3 h in the presence of the indicated compounds with medium containing 10% fetal calf serum, penicillin, streptomycin, and an excess of cold methionine. Immunoprecipitations were performed as

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1 The abbreviations used are: AD, Alzheimer’s disease; Aβ, β-amloid peptide; APP, β-amloid precursor protein; MG 132, carbenzoxyl-Leu-Leu-leucinal; Bicine, N,N-bis(2-hydroxyethyl)glycine; BES, 2-bis(2-hydroxyethyl)aminolethanesulfonic acid; DMF, N,N-dimethylformamide; SWE, Swedish double mutation NL670/671.
Different γ-Secretases Generate Aβ(1–40) and Aβ(1–42)

**RESULTS**

**Characterization of the Antibodies—** HEK 293 cells were transiently transfected with APP751(SWE) and metabolically labeled overnight with 0.1 mCi/ml [35S]methionine. Aβ-related peptides were immunoprecipitated from conditioned media with 4 μl of the NT11 antisera (raised against Aβ(1–40); lane 1), 5 μg of the monoclonal antibody β1 (raised against Aβ(1–40)); lane 2) or 8 μl of the antisera 42–14 (raised against Aβ(36–42) and preabsorbed with immobilized Aβ(17–40); lane 3). Samples were separated on a 15% T, 5% C gel containing 8 M urea or 17.4% glycerol (26). Gels were fixed, stained with Coomassie Blue G-250, impregnated with Amplify fluorographic reagent (Amersham Corp.), dried, and analyzed by fluorography using Kodak Biomax MR films. Quantification of the signals was performed with a computerized image analysis system (Microcomputer Imaging Device, Imaging Research Inc., St. Catherines, Ontario, Canada). After digitalization, areas of interest were manually outlined and calculated into relative absorbance values. The experiment shown in Fig. 2 was also quantified on a PhosphorImager (Molecular Dynamics). This was not possible for the other experiments, since the sensitivity of the PhosphorImager was not sufficient for the quantification of Aβ(1–42).

Calpain inhibitor I was obtained from Boehringer Mannheim, MG 132 (31) and calpeptin (32) were synthesized by the following sequence. First the corresponding N-benzoxycarbonyl-protected di- and tripeptide methylesters were synthesized by standard coupling techniques, and then these were reduced at the carboxyl terminus to the corresponding aldehydes using 1.2 eq lithium chloride/1.2 eq sodium borohydride in a mixture of ethanol/tetrahydrofuran (4:3) at room temperature. In the last step the corresponding alcohols were oxidized to the corresponding aldehydes with 1.5 eq of 1-hydroxy-1,3-dioxo-3H-benzo[d][1,2]iodoxol-3-one in dimethylsulfoxide at room temperature. Stock solutions (25 mM) were prepared in dimethylformamide (DMF) and stored at −20 °C.

**Different Synthetic Peptide Aldehydes Reduce Aβ secretion from HEK 293 cells expressing APP751(SWE).** Transiently transfected cells were pulse labeled for 1 h in the absence and chased for 3 h in the presence of the indicated substances and 0.2% DMF. Aβ-related peptides were immunoprecipitated from the conditioned media with the NT11 antisera and analyzed on a 15% T, 5% C Bicine/Tris gel containing 17.4% glycerol. A, effect of calpain inhibitor I on Aβ secretion. Lanes 1 and 2, control (0.2% DMF); lanes 3 and 4, 5 μM calpain inhibitor I; lanes 5 and 6, 25 μM calpain inhibitor I; lanes 7 and 8, 50 μM calpain inhibitor I; lane 9, immunoprecipitate from the conditioned medium of cells transfected with the vector lacking an insert, pulse labeled for 1 h and chased for 3 h in the presence of 0.2% DMF. B, effects of calpeptin and MG 132 on Aβ secretion. Lanes 1 and 5, control (0.2% DMF); lane 2, 2.5 μM calpeptin; lane 3, 25 μM calpeptin; lane 4, 50 μM calpeptin; lane 6, 5 μM MG 132; lane 7, 25 μM MG 132; lane 8, 50 μM MG 132. Arrows, positions of synthetic Aβ(1–40).
p3 secretion in a dose-dependent manner (Fig. 2A). In agreement with our previous observations (25), quantification of the experiment shown on a PhosphorImager indicated that 50 μM calpain inhibitor I reduced the amount of Aβ released into the medium by approximately 45% (Fig. 2A). MG 132 and calpeptin were more potent (Fig. 2B). At concentrations of 5, 25, and 50 μM, calpeptin reduced Aβ secretion by approximately 45, 65, and 84%, respectively. MG 132 decreased the amount of radiolabeled Aβ by approximately 39% at a concentration of 5 μM and by approximately 87% at a concentration of 25 μM and almost completely blocked Aβ secretion at 50 μM. Similar values were obtained by quantification of fluorographs corresponding to Fig. 2, A and B, with a computerized image analysis system (Table I). When Aβ secretion was studied with stock solutions of calpeptin or MG 132 that had been stored for a prolonged time at −20°C, comparable but smaller effects were observed, indicating that the peptide aldehydes are not stable under these conditions (data not shown).

The Secretion of Aβ(1–40) and Aβ(1–42) Is Affected Differently by the Peptide Aldehydes Calpain Inhibitor I and MG 132—To study the effects of the peptide aldehydes in more detail, secreted Aβ peptides were analyzed on 15% T, 5% C Bicine/Tris gels containing 8 M urea (Fig. 3). Aβ(1–40) and Aβ(1–42) can be separated under these conditions. We observed a reduction of Aβ(1–40) after treatment with 5–50 μM calpain inhibitor I but a simultaneous increase of Aβ(1–42) (Fig. 3A and Table I). In a similar fashion, incubation with 5 μM MG 132 caused a marked decrease of Aβ(1–40), accompanied by an increase of Aβ(1–42). (Fig. 3B and Table I). Higher concentrations (25–50 μM) of MG132 reduced the amount of this faster moving peptide band. Calpeptin also decreased the amount of Aβ(1–40) but had less clear effects on Aβ(1–42). An increase of Aβ(1–42) after treatment with 50 μM calpain inhibitor I was observed in five independent experiments. The effect of 5 μM MG 132 was studied in four experiments. An increase of Aβ(1–42) accompanying the reduction of Aβ(1–40) was found in three experiments; in one case Aβ(1–40) was decreased, whereas Aβ(1–42) appeared to be unaffected, which presumably was due to experimental variations.

To confirm that the peptide species we assigned as Aβ(1–42) contains the longer carboxyl terminus, transfected cells were pulse labeled and chased for 3 h in the presence of calpain inhibitor I or MG 132 in 100-mm dishes. The conditioned media were split into two aliquots, and these were subjected to immunoprecipitation with 5 μg of the monoclonal antibody β1 and 10 μg of the preabsorbed antiserum 42–14, respectively (Fig. 4). Again, immunoprecipitation with β1 revealed that 50 μM calpain inhibitor I and 5 μM MG132 decreased the amount of Aβ(1–40) and simultaneously increased a peptide comigrating with Aβ(1–42) (Fig. 4A and Table I). Following immunoprecipitation with the 42–14 antiserum only this particular peptide comigrating with synthetic Aβ(1–42) was detected (Fig. 4B), indicating that it contains the longer carboxyl terminus. We also tested the effect of MG 132 on cells transfected with a Swedish mutant APP695 cDNA, which encodes APP lacking the Kunitz-type protease inhibitor domain (1). Similar results were obtained with this APP isoform (data not shown).

Comparable effects were observed when radiolabeled Aβ peptides were precipitated with the β1 antibody from conditioned media of COS-1 cells expressing APP751(SWE) (Fig. 5 and Table I). Aβ(1–40) secretion was reduced by 50 μM calpain inhibitor I, 5–25 μM MG 132, and 25 μM calpeptin. Again, 50 μM calpain inhibitor I and 5 μM MG 132 decreased the main Aβ species, whereas they simultaneously increased a peptide species moving slightly faster on Bicine/Tris/urea gels.

**DISCUSSION**

One proposed potential therapeutic strategy for preventing or slowing the pathogenetic mechanism in AD involves the reduction of Aβ generation by partial inhibition of β- and/or γ-secretase (1). Previous results from our group (25) and from others (24) showed that Aβ secretion from transfected cells can be reduced with certain cell-penetrating peptide aldehydes that were found to inhibit γ-secretase activity. In the present study, we confirmed the reduction of Aβ and p3 secretion from transfected HEK293 cells overexpressing APP carrying the Swedish double mutation by calpain inhibitor I. In addition, we present two related peptide aldehydes, MG 132 and calpeptin, that are
more potent. These observations about the secretion of total Aβ, however, primarily reflect the influences of the inhibitors on the generation of the main Aβ species, which is Aβ(1–40) in the case of HEK 293 cells expressing the Swedish mutant APP (15).

Several lines of evidence suggest that Aβ peptides ending at residue 42 or 43 are of particular importance in AD: (i) missense mutations in codon 717 of the APP gene responsible for rare familial forms of the disease increase the proportion of the longer peptides (17, 18); (ii) in vitro experiments indicate a faster rate of polymerization for peptides including the critical additional residues Ile-41 and Ala-42 (19); and (iii) the longer Aβ peptides were reported to present a major component of senile plaques (20–22) and were shown to be initially deposited (23).

In view of these observations we investigated whether selected peptide aldehyde inhibitors affect the secretion of Aβ peptides (1–40) and (1–42) in the same way. Aβ peptides generated by HEK 293 cells expressing the Swedish mutant APP primarily start with Asp-1 (11, 15). To discriminate between

FIG. 3. Calpain inhibitor I and MG 132 reduce Aβ(1–40) and simultaneously increase Aβ(1–42). The same immunoprecipitates as in Fig. 2 were reanalyzed on a 15% T, 5% C Bicine/Tris gel containing 8 M urea and therefore allowing for the separation of Aβ(1–40) and Aβ(1–42). A, effect of calpain inhibitor I on the secretion of Aβ(1–40) and Aβ(1–42). Lanes 1 and 2, control (0.2% DMF); lanes 3 and 4, 5 μM calpain inhibitor I; lanes 5 and 6, 25 μM calpain inhibitor I; lanes 7 and 8, 50 μM calpain inhibitor I; lane 9, immunoprecipitate from the conditioned medium of cells transfected with the vector lacking an insert, effects of calpain inhibitor I; lanes 5, 6, and 8, 50 μM calpain inhibitor I; lanes 7 and 8, 50 μM MG 132; lane 9, immunoprecipitate from the conditioned medium of cells transfected with the vector lacking an insert, effects of calpain inhibitor I and MG 132. We conclude that the secretion of Aβ(1–42) is differentially affected by calpain inhibitor I. Although 50 μM calpain inhibitor I clearly reduced the amount of immunoprecipitable Aβ(1–40) released into the medium, Aβ(1–42) was increased. A different peptide aldehyde, MG 132, had similar effects but was more potent. At 5 μM, MG 132 clearly decreased Aβ(1–40) and simultaneously increased Aβ(1–42) (or at least did not alter the amount of Aβ(1–42)). At higher concentrations (25–50 μM), MG 132 reduced both Aβ(1–40) and Aβ(1–42). Comparable findings were obtained when radiolabeled Aβ peptides secreted from COS-1 cells and immunoprecipitated with the β1 antibody were analyzed. Taken together, our results indicate that the cleavages of APP at Aβ residues 40 and 42 exhibit different sensitivities toward calpain inhibitor I and MG 132. We conclude that the carboxyl termini of Aβ(1–40) and Aβ(1–42) are generated by at least two different proteolytic activities, a γ(40)-secretase and a γ(42)-secretase. Future experiments could be directed toward
Among the enzymes involved in Aβ formation, we consider this secretase activity the most attractive target for the development of inhibitors that are specific for the γ-secretase.

FIG. 5. Effect of synthetic peptide aldehydes on the release of Aβ peptides from COS-1 cells. COS-1 cells were transiently transfected with pCMVAPP751(SWE) and pulse labeled for 1 h in the absence and chased for 3 h in the presence of the indicated substances. Radiolabeled Aβ peptides were immunoprecipitated from the conditioned media with the β1 antibody and separated on a 15% T, 5% C/8 M urea Bicine/Tris gel. Lane 1, control (0.2% DMF); lane 2, 50 μM calpain inhibitor I; lane 3, 5 μM MG 132; lane 4, 25 μM MG 132; lane 5, 25 μM calpeptin. Arrows, positions of synthetic Aβ(1–40) and Aβ(1–42).

Acknowledgments—We thank K.-H. Wiederhold for help with the quantification of the fluorographs.

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