Chloroquine inhibits intracellular degradation but not secretion of Alzheimer β/A4 amyloid precursor protein

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ABSTRACT The metabolic fate of the Alzheimer β/A4 amyloid precursor protein (APP) includes intram amyloid proteolysis that leads to the production of secreted N-terminal and cell-associated C-terminal fragments. The cellular sites at which this processing occurs are not known. We have examined the route of APP processing in metabolically labeled PC12 cells. The lysosomotropic drug chloroquine exerted inhibitory effects on the degradation of mature APP holoprotein. In addition, recovery of a C-terminal fragment resulting from normal intramyloid cleavage was significantly increased in the presence of chloroquine, suggesting that further degradation of the C-terminal fragment was inhibited. Chloroquine had virtually no effect on APP maturation (N- and O-glycosylation and tyrosine sulfation) or secretion. Treatment with either monensin (which inhibits distal Golgi function) or brefeldin A (which causes resorption of the Golgi into the endoplasmic reticulum and fusion of the trans-Golgi network with the endosomal system) prevented normal APP maturation and abolished APP secretion and recovery of C-terminal fragments, indicating that intact Golgi function is necessary for APP maturation and processing. Our results suggest that a substantial proportion of APP is degraded in an intracellular acidic compartment but that the coupled APP cleavage/secre tion event occurs in a chloroquine-insensitive compartment. The observations are consistent with the existence of multiple cellular routes for the trafficking and proteolysis of APP.

The leading cause of dementia and the fourth leading cause of death in the developed world is Alzheimer disease (AD), which affects an estimated 10% of the population over 65 years of age in the United States (1). The disease manifests itself as insidious memory loss, cognitive decline, and personality changes that result in loss of functional ability over the course of a decade. In their debilitated state, patients usually retain only vegetative neurologic function and succumb to secondary infections (2).

AD is characterized by certain neuropathological lesions, including intracellular neurofibrillary tangles and extracellular parenchymal and cerebrovascular amyloid (3). The principal component of the amyloid deposits is a protein designated β/A4 amyloid (4, 5), an ~4-kDa polypeptide arising from cleavage of the amyloid precursor protein (APP) (6–9). APP exists as three major transmembrane isoforms (APP751, APP770, and APP695) that result from alternative splicing of a single primary transcript (Fig. 1A) (7, 10–12). Secretory processing of APP (13) leads to cleavage within the β/A4 domain (“normal” cleavage) and precludes amyloidogenesis (14, 15). The biochemical defect responsible for amyloid production in AD might, therefore, involve either a deficiency in this proteolytic pathway or excessive activity of an alternative pathway. It is noteworthy that two types of inherited cerebral amyloidoses—hereditary cerebral hemorrhage with amyloidosis (Dutch type) and familial early-onset AD—are associated with mutations in the coding sequence of APP near the β/A4-amylloid domain (16–19).

Although the events leading to abnormal APP processing and amyloidosis are not known, elucidation of normal processing pathways may provide insights into AD pathogenesis. Cultured mammalian cells provide a readily accessible and well-characterized system for studies of protein trafficking. In the present investigation, we have examined the intracellular trafficking and processing of APP by [35S]methionine pulse-labeling of PC12 neuroendocrine cells in the presence of pharmacologic agents known to affect the function of specific intracellular organelles.

MATERIALS AND METHODS

Chloroquine was purchased from Sigma, monensin was purchased from Calbiochem, and brefeldin A was purchased from Epicentre Technologies (Madison, WI). Asceis fluid from mice injected intraperitoneally with hybridoma cells producing the anti-N-terminal APP monoclonal antibody 22C11 (13) was the kind gift of T. V. Ramahhadran (our laboratory) and S. S. Sisodia (The Johns Hopkins University School of Medicine, Baltimore). Affinity-purified rabbit anti-C-terminal APP antibody, here termed 369A, has been described (20). Agarose-coupled anti-mouse and anti-rabbit secondary antibodies were purchased from HyClone. Protein A-Sepharose CL-4B was obtained from Pharmacia LKB. Undifferentiated PC12 cells were grown to confluency on three 10-cm culture dishes in Dulbecco's modified Eagle's medium containing 10% (vol/vol) heat-inactivated fetal bovine serum, 5% (vol/vol) heat-inactivated horse serum, and antibiotic–antimycotic solution (GIBCO) with 5% CO2/95% air at 37°C (all subsequent incubations up until cell lysis were performed at this temperature). Cells were washed twice with Hepes-buffered saline (HBS = 10 mM Hepes, pH 7.4/110 mM NaCl/5 mM KCl/2 mM CaCl2/1 mM MgSO4) suspended in HBS, and pelleted by brief centrifugation. The cells were resuspended in 1 ml of methionine-free Eagle's modified minimal essential medium (MEM) containing 25 mM Hepes (pH 7.4). After a 45-min precubation, cells were pulse-labeled for 20 min by the addition of 1 mCi of [35S]methionine (1000 mCi/mmol; 1 Ci = 37 GBq; NEN Research Products). The chase period was initiated by the addition of 5 ml of MEM containing excess unlabelled methionine (200 μM) and 25 mM Hepes (pH 7.4). Portions (200 μl) of samples were incubated in microcentrifuge tubes.

When chloroquine (50 μM) or ammonium chloride (50 mM) was used, drug was added at the start of the chase period. When the effects of monensin (10 μM) or brefeldin A (10 μM) were examined, drugs were added at 2-h intervals. Values presented were the means ± SEM from at least three separate experiments.

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Abbreviations: APP, amyloid precursor protein; KPI, Kunitz serine protease inhibitor; AD, Alzheimer disease. †To whom reprint requests should be addressed.
APP\textsubscript{231} and APP\textsubscript{770} isoforms could not be resolved by densitometry, they were reported together as immature APP\textsubscript{KPI}, where KPI is Kunitz serine protease inhibitor, a domain common to both APP\textsubscript{231} and APP\textsubscript{770}. Values were corrected for length of exposure time, signal decay, and the number of methionine residues in individual APP species and normalized to the total APP holoprotein present in untreated cells at the start of the chase period (100 relative units). It should be noted that synthesis of labeled protein continued after the start of the chase and, therefore, that the values for the recovery of mature APP, secreted APP, and the C-terminal APP fragment are overestimated in absolute terms but accurate in relative terms.

RESULTS

Identification of APP Species. Immunoprecipitates from metabolically labeled PC12 cells using an antibody directed against the C terminus of APP contained six protein bands of molecular masses 106, 112, 116, 125, 139, and 146 kDa (Fig. 1B). The three lower bands have previously been designated as immature APP\textsubscript{695}, APP\textsubscript{721}, and APP\textsubscript{770} holoprotein, respectively, and the three higher bands have been shown to correspond to mature APP isoforms, in which the proteins are fully N- and O-glycosylated and sulfated (13, 20). Using agents that inhibit conversion of immature to mature APP (see below) and a monoclonal antibody directed against the KPI domain of isoforms APP\textsubscript{231} and APP\textsubscript{770} (21), and based upon the lengths of the individual isoforms, we confirmed the protein band assignment.

Also present in the immunoprecipitated cell lysates was a protein of molecular mass 16.3 kDa\textsuperscript{5} (Fig. 1C). This truncated APP fragment represents the C-terminal product with a predicted molecular mass of 9.2 kDa resulting from the normal intraamyloid cleavage of APP (15), as determined by its protein sequencing (our unpublished observations), and was previously referred to as a 15-kDa peptide (20). Immunoprecipitation of the seven APP species with the C-terminal antibody could be abolished by preincubation of antibody with peptide corresponding to APP (645–694) (APP\textsubscript{695} numbering system).

Immunoprecipitates of culture medium with antibody directed against the N terminus of APP contained three protein bands of molecular masses 109, 123, and 129 kDa (Fig. 1D). The two higher bands could also be immunoprecipitated with the antibody directed against the KPI domain insert. These proteins were not immunoprecipitated with the anti-C-terminal antibody. Since the difference in masses between the mature APP holoproteins (from which the secreted forms are believed to arise; see below) and the APP C-terminal fragment closely agree with the masses of the secreted forms, we conclude that they are the secreted fragments of APP\textsubscript{695}, APP\textsubscript{721}, and APP\textsubscript{770}. No secreted forms were recovered from cell lysates when supernatants from immunoprecipitations with anti-C-terminal antibody were reincubated with anti-N-terminal antibody.

Effect of Chloroquine on Acidic Organelles. Since some evidence for lysosomal APP processing has been reported (22, 23), we examined the effects of chloroquine on APP metabolism. Chloroquine is a weak base that is taken up by cells where it is concentrated in and neutralizes acidic organelles such as lysosomes (24, 25). The elevated pH of

\textsuperscript{5}This APP species comigrated with the \(\alpha\)-lactalbumin standard marker that has a reported molecular mass of 14.2 kDa. However, when the molecular mass of the APP species was determined by linear regression analysis using a series of protein standards, as was done for the APP holoproteins, the molecular mass was calculated to be 16.3 kDa. For consistency we will report the molecular mass as 16.3 kDa.
these organelles results in the inhibition of their acid-dependent hydrolases. Chloroquine neutralization of acidic organelles in the PC12 cells was confirmed by fluorescence microscopy after acridine orange treatment (26, 27) in the absence and presence of chloroquine (data not shown).

**Effect of Chloroquine on APP Maturation.** At the start of the chase period nearly all of the labeled APP was in the form of immature holoprotein (Fig. 1B). Within ~15 min, half of the immature APP was converted to mature APP (Fig. 2). No difference in the rate of maturation was found between APP_{695} and APP_{KPI}. Chloroquine had virtually no effect on APP maturation (Fig. 2).

**Effect of Chloroquine on Mature APP Holoprotein.** In untreated cells, the level of mature APP holoprotein rose to a maximum by 30 min, corresponding to conversion of immature APP to its fully glycosylated and sulfated forms (Fig. 3) (13). The amount of mature APP isoforms then decreased with a half-life of ~90 min. At 8 h of chase, the levels of mature APP isoforms had returned to their starting levels. We attribute the decrease of APP levels with time to conversion of mature APP to secreted forms, as well as to proteolytic degradation unassociated with secretion.

When cells were treated with chloroquine, a significant effect on turnover of mature APP was observed (Fig. 3). The levels of mature APP holoprotein peaked 30 min later than in control samples. APP isoforms were present at approximately twice the levels found in untreated cells, from 1 to 8 h of chase. The magnitude of the chloroquine effect was the same for the different APP isoforms.

**Effect of Chloroquine on APP Secretion.** In untreated cells, after a brief lag, levels of secreted APP isoforms rose linearly from 0 to 4 h of chase (Fig. 4). Little or no further increase in APP secretion was observed up to 8 h of chase. Maximal levels of secreted APP represented ~14% of total APP present at the start of chase.

**Fig. 3.** Recovery of mature APP in the absence (open circles) or presence (solid diamonds) of chloroquine. (A) Mature APP_{695}, (B) Mature APP_{751}, (C) Mature APP_{770}. Results are the mean ± SEM of seven experiments (n = 3 to 7 for individual time points). Statistical significance between untreated and treated samples for individual time points was determined by Student’s unpaired t test (*, P < 0.05).

When cells were treated with chloroquine, virtually no change in the rate of APP secretion was found for any APP isoform (Fig. 4). The maximal level of APP secretion was approximately the same for control and chloroquine-treated samples at 4 h. At 6 and 8 h of chase, there tended to be a small decrease in recovery of secreted APP. This decrease was attributable to proteolytic degradation in the medium, since recovery of secreted APP in 4-h chase medium incubated in the absence of cells decreased with time. Chloroquine was found to promote the proteolysis in the medium to an extent comparable to that shown in Fig. 4 (data not shown).

**Effect of Chloroquine on the APP C-Terminal Fragment.** In untreated cells, the level of the APP C-terminal fragment reached a maximum at 1 h of chase and decreased slowly thereafter (Fig. 5). Since mature APP holoprotein was still being degraded and secreted APP was still being produced at this time, we conclude that the C-terminal APP fragment is subject to further proteolytic degradation.

Chloroquine had a marked effect on recovery of the C-terminal fragment. At all time points from 2 to 8 h, recovery of the 16.3-kDa APP fragment was significantly greater from chloroquine-treated than from untreated cells. These data are consistent with the proposal that the APP C-terminal frag-
APP maturation (Fig. 6A). Furthermore, no secreted APP fragment (Fig. 6B) or 16.3-kDa APP fragment (data not shown) was recovered.

The data obtained with monensin and brefeldin A suggest that partially glycosylated APP (13) passes through the cis- and medial-Golgi and then reaches the trans-Golgi, where final glycosylation and sulfation (31) occur. Both monensin and brefeldin A inhibited complete APP maturation and prevent APP proteolysis and secretion. This indicates that proteolysis and secretory cleavage occur in the distal Golgi or a subsequent compartment. These results appear to exclude the possibility that APP secretory cleavage might occur in the endoplasmic reticulum or proximal Golgi.
DISCUSSION

We have demonstrated that the lysosomotropic agent chloroquine significantly affects metabolism of mature APP and an APP C-terminal fragment but has virtually no effect on APP maturation or secretion. In the present study, the APP fragments released into the medium represented ~14% of the APP holoprotein present at the start of the chase. Recovery of the APP C-terminal fragment in the presence of chloroquine represented ~13% of total APP holoprotein but 92% of total secreted APP. The quantitative agreement between the production of secreted APP fragments and the C-terminal APP fragment suggests that the 16.3-kDa fragment arising by intraamyloid cleavage results almost entirely from the secretory pathway. The C-terminal fragment would then be sorted for further proteolysis in acidic organelles such as lysosomes. In support of this possibility, chloroquine caused increased recovery of the 16.3-kDa peptide; since secretion was unchanged by chloroquine, the increased recovery of the C-terminal fragment is unlikely to be due to enhanced production but rather to decreased degradation.

The chloroquine-sensitive proteolytic turnover of APP presumably takes place in an acidic compartment such as endosomes or lysosomes. The specific cleavage of APP to produce a secreted species, on the other hand, was not sensitive to chloroquine, suggesting that this event occurs at a different cellular location. Quantitatively, our results suggest that intraamyloid cleavage of APP and the associated secretion of N-terminal APP fragments represent a minor portion of total APP metabolism and that most APP molecules are degraded along a separate subcellular route of trafficking. The identification of the cellular locations of the enzymes responsible for these distinct proteolytic pathways should facilitate the development of drug therapies directed at altering APP metabolism and treating AD.

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