Intracellular Production of βA4 Amyloid of Alzheimer's Disease: Modulation by Phosphoramidon and Lack of Coupling to the Secretion of the Amyloid Precursor Protein†

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Received November 21, 1994; Revised Manuscript Received March 16, 1995⊗

ABSTRACT: The amyloid precursor protein (APP) undergoes abnormal metabolism in Alzheimer's disease, resulting in the accumulation of βA4 amyloid in the brain. Normal APP metabolism includes the release of a truncated form (sAPP) which has been cleaved at the α-secretase site within the βA4 amyloidogenic domain. However, intact forms of βA4 protein may also be generated by the β- and γ-secretases. Soluble forms of βA4 have been detected in various cell lines and in cerebrospinal fluid. Previous studies of protein kinase C activation have suggested a reciprocal relationship between sAPP secretion and βA4 production and release. We find that phorbol ester activation of protein kinase C in untransfected SH-SY5Y neuroblastoma cells increases the release of sAPP without affecting βA4 secretion. We provide further evidence for intracellular βA4 production. Treatment of SY5Y cells with the protease inhibitor phosphoramidon results in a 2-fold increase in βA4 secretion and an increase in the amount of βA4 recovered from cell lysates, yet it does not affect sAPP secretion. The protease inhibitors thiorphan and *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine had no effect on βA4 or sAPP secretion. The lysosomotropic agents chloroquine and NH_4Cl decreased βA4 secretion, providing additional evidence for the involvement of intracellular acidic compartments in the production of βA4. Our results therefore demonstrate a double dissociation between the secretion of sAPP and βA4 in the SH-SY5Y cell line. The effect of phosphoramidon supports previous studies which show that metalloproteases are involved in the biogenesis of βA4.

Alzheimer's disease (AD)[1] is characterized by the abnormal accumulation of an amyloid protein in the brain. The βA4 amyloid peptide is a 39−43 residue proteolytic cleavage product of the much larger amyloid precursor protein (APP) (Glenner & Wong, 1984; Masters et al., 1985; Kang et al., 1987). Full-length APP (APP_{FL}) has a receptor-like structure with a large ectodomain, a single membrane-spanning region, and a short cytoplasmic tail (Figure 1). Different APP isoforms result from alternative splicing of a single gene (Tanzi et al., 1988; Kitaguchi et al., 1988; König et al., 1992).

In the APP secretory pathway APP_{FL} is cleaved within the βA4 region by α-secretase, releasing sAPP (secreted APP) and thereby preventing the formation of the βA4 fragment. In the amyloidogenic pathway, APP_{FL} is cleaved at the NH_2-terminus of the βA4 sequence by APP β-secretase, releasing a shorter form of sAPP and producing the transmembrane βA4/C-terminus fragment of APP (βA4CT). Further cleavage of this βA4CT fragment by γ-secretase releases the COOH-terminus of the βA4 peptide. Another secreted cleavage product of APP is the p3 peptide, produced by cleavage of APP at the α- and γ-secretase sites. The APP α-, β-, and γ-secretases have not yet been fully characterized (for a review, see Evin et al., 1994).

Soluble βA4, the p3 fragment, and sAPP (Figure 1) are all secreted by cell lines as part of normal APP metabolism. βA4 is detectable in cerebrospinal fluid and plasma (Haass et al., 1992; Dovey et al., 1992; Shoji et al., 1992) and can be extracted from human brain (Q. X. Li and S. J. Fuller, unpublished observation).

The involvement of APP in the pathogenesis of AD has been confirmed by the study of families in which early-onset familial Alzheimer's disease (FAD) has been linked to point mutations close to or within the βA4 sequence (Goate et al.,

† Supported in part by grants from the Victorian Health Promotion Foundation, the National Health and Medical Research Council (NH&MRC) of Australia, and the Aluminium Development Council. A.I.S. is supported by the NH&MRC. K.B. is supported by the Deutsche Forschungsgemeinschaft and the Bundenministerium für Forschung und Technologie.

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

[1] Abbreviations: AD, Alzheimer's disease; APP_{FL}, full-length form of the amyloid precursor protein; APP_{770}, full-length amyloid precursor protein consisting of 770 amino acids; βA4, the 39−43 amino acid peptide produced by cleavage of APP; βA4CT, APP fragment starting at the N−terminus of the βA4 sequence spanning through to the C-terminus (cytoplasmic tail) of APP; CPPL, *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F12 medium; ECE, endothelin converting enzyme; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; mAb, monoclonal antibody; NEP, neutral endopeptidase 24:11; PAD, phosphoramidon; Pdbu, phorbol 12,13-dibutyrate, phorbol ester; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); sAPP, secreted APP; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane.

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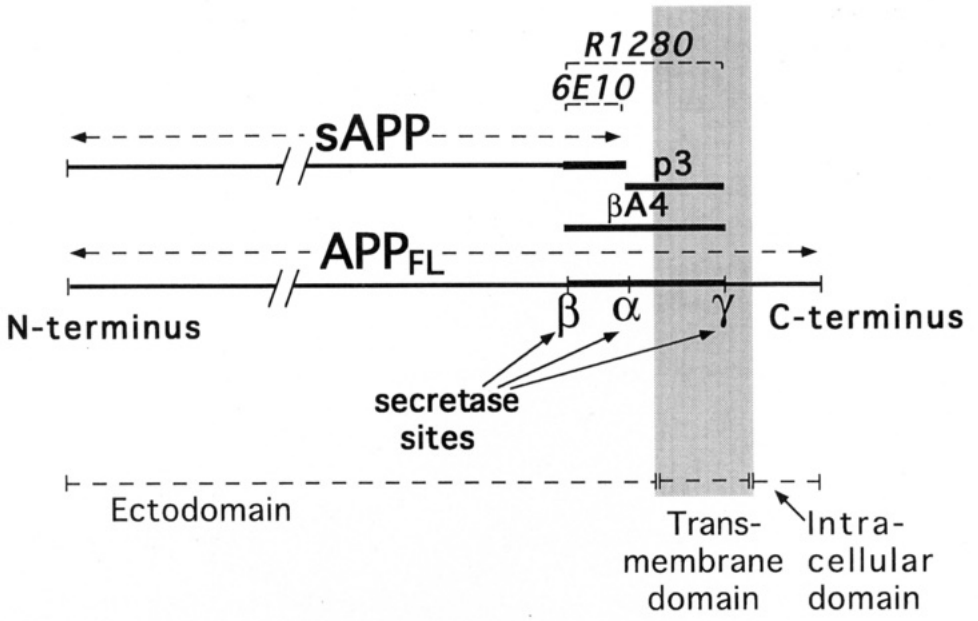

FIGURE 1: Schematic diagram of the amyloid precursor protein (APP). Full-length APP (APP_{FL}) is a transmembrane protein with a large glycosylated extracellular region, a single membrane-spanning region, and a short cytoplasmic tail. The APP molecule can be cleaved at the APP α-, β-, and γ-secretase sites, and some byproducts of cleavage include the βA4, p3, and sAPP molecules. The antibody recognition sites for mAb 6E10 and the rabbit polyclonal R1280 are also indicated.

1991; Mullan et al., 1992; Chartier-Harlin et al., 1991). Human cells transfected with constructs containing the codons 670 and 671 Swedish mutation near the β-secretase site of APP (Felsenstein et al., 1994; Citron et al., 1992; Cai et al., 1993) or the A to G substitution at codon 692 (Haass et al., 1994) have shown a severalfold increase in βA4 secretion. Cells transfected with APP with substitutions at codon 717 produce similar amounts of βA4 compared to controls (Cai et al., 1993); however, they produce a significantly larger proportion of $\beta A4_{1-42}$ (Suzuki et al., 1994), which is known to be more amyloidogenic than $\beta A4_{1-40}$.

There are conflicting reports concerning the processing events required to produce soluble βA4. A separate APP secretory pathway (Busciglio et al., 1993) and the lysosomal and/or endosomal degradation pathways (Shoji et al., 1992; Koo et al., 1994) have all been implicated as possible sources. The activation of protein kinase C by phorbol esters in cell culture causes an increases in sAPP and p3 secretion. Using cultures of APP-transfected cell lines, this effect was shown to be accompanied by a decrease in βA4 secretion, suggesting that the APP secretory pathway and the amyloidogenic breakdown pathway are coupled so that increasing the flow of APP through one pathway results in a decrease through the other (Buxbaum et al., 1993; Jacobsen et al., 1994; Hung et al., 1993). Recently, Dyrks et al. (1994) found that phorbol ester treatment resulted in an increase in βA4 secretion from cells transfected with the A4CT region of APP, and no change in βA4 secretion from APP_{FL}-transfected cells. In this report, we show that the pathway responsible for βA4 production and the APP secretory pathway are not coupled in the untransfected neuroblastoma SH-SY5Y cell line. We chose the SY5Y cell line as it is a human cell line with neuronal properties (Perez-Polo et al., 1979), and because βA4 secretion levels from untransfected SY5Y cells were readily detectable. Using the protease inhibitor phosphoramidon, we provide further evidence that metalloproteases are involved in βA4 metabolism. We also show that βA4 is produced intracellularly, and our results support the concept that an acidic compartment is involved in the βA4 secretion process.

MATERIALS AND METHODS

Materials. Methionine-free Ham's F-12 and Dulbecco's modified Eagle's medium (full medium and methionine- and cysteine-free medium) were obtained from Gibco BRL. Trans ^{35}S label ([^{35}S]methionine and [^{35}S]cysteine) was purchased from ICN Biomedicals, Australasia; fetal calf serum (FCS) was obtained from Commonwealth Serum Laboratories, Australia; insulin was from Eli Lilly, France. Other cell culture reagents and the protease inhibitors phosphoramidon, thiorphan, and *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine were purchased from Sigma, St. Louis, MO. Protein A−Sepharose was from Pharmacia LKB, Sweden. Rabbit anti-mouse serum was from Dakopatts, Denmark. The R1280 antiserum was kindly donated by Dr. D. J. Selkoe, Harvard Medical School, Boston, MA; mAb 6E10 was purchased from the Institute for Basic Research in Developmental Disabilities, New York, NY; mAb anti-neurofilament 68 was obtained from Sigma, St. Louis, MO. PVDF membranes (0.2 μm) were obtained from ICN Biomedicals, Australasia. All other reagents were of AR quality.

Cell Culture. SY5Y cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12, 1:1 mixture) supplemented with nonessential amino acids (NEAA, Gibco BRL) and 10% (v/v) heat inactivated fetal calf serum (FCS), penicillin (50 units/mL), and streptomycin (50 μg/mL). Cells were grown on culture dishes coated with collagen Type I.

Metabolic Labeling. Cells were subcultured into collagen-coated plates at least 48 h prior to each experiment. Equal aliquots of cells from a single cell suspension were dispensed into individual wells of a 12-well plate. Cells were labeled on reaching 90% confluence (approximately 1.5×10^6 cells/well). Labeling was performed in methionine-free DMEM/

F12 with insulin (1 unit/mL), progesterone (10 ng/mL), sodium selenite (40 ng/mL), prostaglandin $F_{2\alpha}$ (250 ng/mL), iron-saturated transferrin (50 μg/mL), hydrocortisone (50 nM), putrescine (100 μM), 1% NEAA (v/v), and 1% FCS (v/v). The culture medium with the supplements as listed above was adapted from formulae recommended for primary neuronal cultures (Bottenstein, 1985; Butler, 1992) and was used in preference to unsupplemented DMEM medium, as SY5Y cells will not divide or survive for much more than 24 h in DMEM alone.

Cells were incubated in the above medium for 30 min at 37 °C to help clear residual methionine and cysteine: this was then replaced with fresh methionine-free medium (300 μL/well), containing 0.5−1.0 mCi of [^{35}S]methionine and [^{35}S]cysteine/mL. Following labeling, washes (2 min × 1) and chase periods (20 min−3 h) were carried out using 300 μL/well methionine-enriched DMEM/F12 (20 mg/L methionine) with the addition of all the supplements mentioned above except for FCS.

Immunoprecipitation. Each immunoprecipitation was carried out on all the medium or all the cells from an individual well. After harvesting, media samples were centrifuged for 1 min at 1000*g* to remove nonadherent cells and then centrifuged at 15000*g* for 10 min to remove other cell debris. Samples were diluted 1:1 with STEN buffer (STEN: 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.2% (v/v) Nonidet P-40) containing 0.1% (w/v) SDS and 0.1 mM β-mercaptoethanol, 2 mM PMSF, and leupeptin (2 μg/mL). Cell samples were harvested in STE buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM PMSF, leupeptin (2 μg/mL), and 0.1 mM β-mercaptoethanol). Cells were microfuged for 1 min at 1000*g*, resuspended in fresh STE buffer, and sonicated for 10 short bursts on a Branson sonifier (50% pulse, setting on 1), after which Triton X-100 was added to a final concentration of 1% (v/v). After 15 min incubation on ice, the cell lysates were microfuged at 12000*g* for 15 min at 4 °C, and the resulting supernatants were used for immunoprecipitation.

Immunoprecipitation was performed with mAb 6E10 at a dilution of 1/600 (this antibody recognizes an epitope between residues 1 and 17 of the βA4 sequence; Kim et al., 1992), or R1280 at a dilution of 1/300 (rabbit polyclonal antibody raised to synthetic βA4 1−40; Tamaoka et al., 1992). MAb α-neurofilament 68 was used as a negative control. Protein A−Sepharose (Pr-A-Seph), 12 mg/sample, already coupled to rabbit anti-mouse IgG at 1 μL/mg of Pr-A-Seph and washed extensively, was added to the samples, which were then incubated overnight at 4 °C. Pr-A-Seph−antibody−antigen complexes were washed for 4 × 20 min in STEN buffer containing 0.1% SDS, followed by 3 washes in STEN buffer containing 0.5 M NaCl, and finally one wash in STEN buffer. Pr-A-Seph pellets were resuspended in 2× sample buffer containing 10% β-mercaptoethanol and 4% SDS, and boiled for 10 min. Labeled proteins were then separated on 10/15% Tris/Tricine gels (Schagger & von Jaggow, 1987) and transferred to 0.2 μm PVDF membranes (150 mA for 14 h followed by 600 mA for 1 h). Each gel lane contained the immunoprecipitated material from the medium or cell lysate from one whole well of a 12-well culture plate.

Quantitation of Results. Scanning and quantitation of data were performed using a Fujix BAS 1000 phosphorimager. The PVDF membranes were placed against a phosphorimaging screen overnight. From the scanned image of the screen, βA4 and sAPP levels were quantitated following densitometric analysis of the radioactivity in the 4 kDa and 100−120 kDa bands, respectively, using the McBas program (Fuji), after first subtracting the background intensity readings of the appropriate bands from the α-neurofilament 68 immunoprecipitation lanes. Assay parameters had been established previously and resulted in a linear relationship between the possible concentrations of βA4 and sAPP and the corresponding band intensities as determined by the phosphorimage analysis.

RESULTS

Phorbol Ester Treatment Does Not Reduce βA4 Secretion in SY5Y Cells. To examine the relationship between the α- and β-secretase pathways in SY5Y cells, the cells were treated with 1 μM phorbol 12,13-dibutyrate (Pdbu) for 40 min (our standard chase period) or for 2 h (as described previously by Buxbaum et al. (1993) and Jacobsen et al., (1994)) after a 3 h [^{35}S]methionine and [^{35}S]cysteine labeling period. The short (40 min) chase period was chosen so that the observed results would be more likely to reflect a change in protein metabolism rather than effects on transcription. The media samples from these cells were subjected to immunoprecipitation with mAb 6E10 followed by SDS/PAGE on Tris/Tricine gels, and transfer to PVDF membranes. Labeled sAPP and βA4 were then quantitated by phosphorimage analysis. Results from both chase periods showed that sAPP secretion was stimulated approximately 4-fold compared to control sAPP secretion levels (Figure 2). However, the βA4 secretion levels did not change upon treatment with Pdbu. These results suggest that the α-secretase and β-secretase pathways are not coupled in untransfected SY5Y cells.

Phosphoramidon Increases βA4 Secretion from SY5Y Cells. The proteases involved in APP metabolism have not yet been identified or characterized, but there is increasing evidence that the APP α- and β-secretases may be metalloproteases (for a review, see Evin et al., 1994). To study this possibility in cell culture, SH-SY5Y neuroblastoma cells were treated with various protease inhibitors that are known to inhibit metalloproteases. SY5Y cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, and then inhibitors were added to the cells in fresh medium for a 40 min chase period. Treatment with the inhibitor phosphoramidon (PAD; Powers & Harper, 1986) increased βA4 secretion 2-fold, but had no significant effect on sAPP secretion (Figure 3A,B). The high-specificity neutral endopeptidase inhibitors thiorphan at 4 and 40 μM (Olins et al., 1989) and *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine (CPPL) at 10 μM (Fournié-Zaluski et al., 1982) produced no effect. SY5Y cells were labeled for 4 h in the presence or absence of PAD, and immunoprecipitation of the conditioned labeling medium was carried out with mAb 6E10 and the rabbit polyclonal antibody R1280 (R1280 detects the p3 peptide as well as βA4) (Figure 3C). The polyclonal R1280 immunoprecipitation was carried out to confirm the identity of the βA4 band; it also demonstrated that PAD did not appear to affect the levels of secreted p3 peptide (quantitation not done). The enzyme inhibited by PAD in this experiment is yet to be identified; however, the thiorphan and CPPL data suggest it is not neutral endopeptidase (EC 3.4.24.11). These results

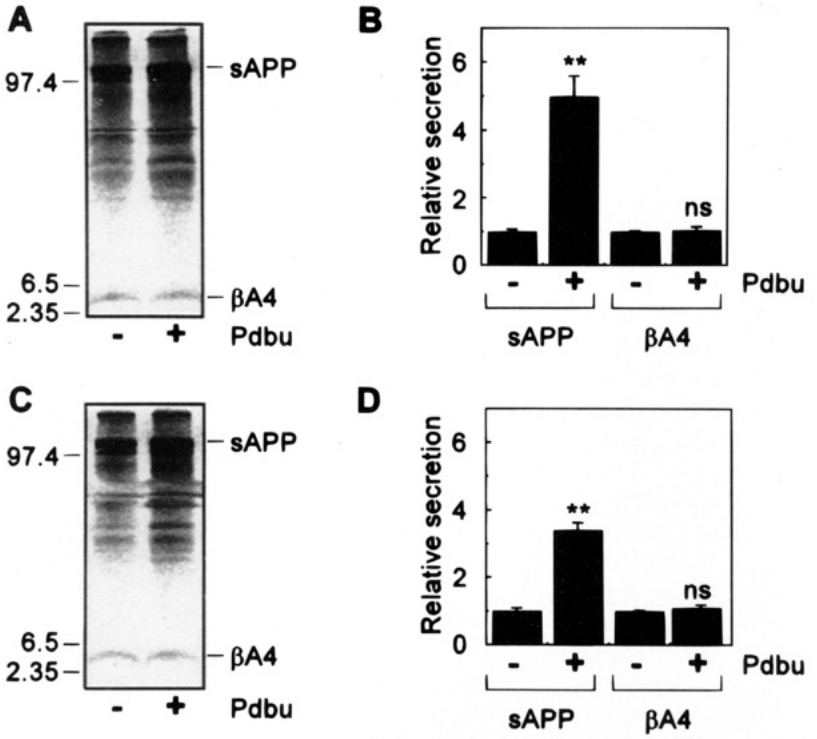

FIGURE 2: Effect of phorbol 12,13-dibutyrate (Pdbu) on the secretion of sAPP and βA4 from SY5Y cells. SY5Y cells were labeled for 3 h with 0.5 mCi/mL [^{35}S]methionine and [^{35}S]cysteine in methionine-free medium (see Materials and Methods). Cells were then chased for either a 40 min (blot A and graph B) or a 2 h period (blot C and graph D) in the presence or absence of 1 μM Pdbu in fresh medium containing an excess of unlabeled methionine. The chase medium samples from these cells were then immunoprecipitated with mAb 6E10 (recognizes βA4 and sAPP) and analyzed on 10/15% Tris/Tricine gels. The positions of molecular size markers are as indicated (in kDa) on the left side of the figures. The results of four separate experiments are summarized in the bar graphs. The mean control values for sAPP and βA4 production were arbitrarily assigned the value 1.00, and other values were normalized accordingly. Error bars indicate standard error. "**" indicates $p \leq 0.01$; "ns" indicates results not significantly different from control (Wilcoxon's Rank Sum test).

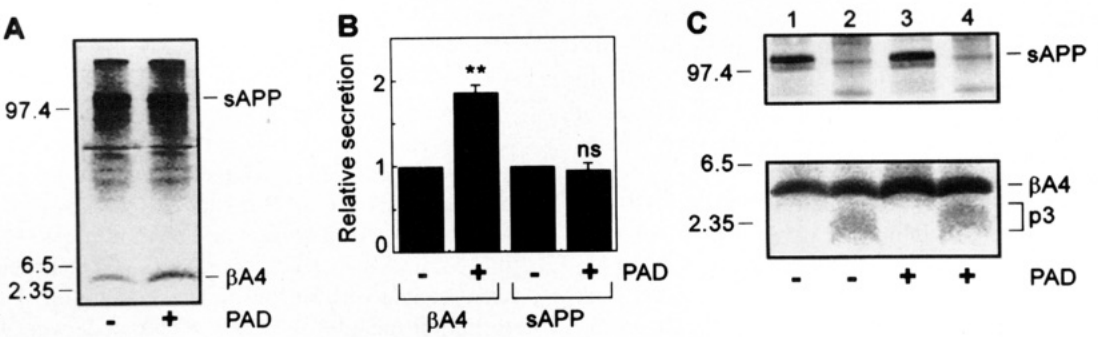

FIGURE 3: Effect of phosphoramidon on the secretion of βA4 and sAPP from SY5Y cells. (A) SY5Y cells were labeled for 3 h with 0.5 mCi/mL [^{35}S]methionine and [^{35}S]cysteine and chased in cold methionine-enriched medium for 40 min in the presence (+) or absence (−) of 20 μM phosphoramidon (PAD). Chase medium samples were immunoprecipitated with mAb 6E10 and then run on 10/15% Tris/Tricine gels and transferred to PVDF membranes. The positions of molecular size markers are as indicated (in kDa) on the left side of the figure. (B) The results of five separate experiments were quantitated and pooled. The mean control values for sAPP and βA4 production were arbitrarily assigned the value 1.00, and other values were normalized accordingly. Each bar represents the mean of a minimum of 8 samples, and error bars indicate standard error. "**" indicates $p \leq 0.01$; "ns" indicates results not significantly different from control (Wilcoxon's Rank Sum test). (C) SY5Y cells were labeled with 1 mCi/mL [^{35}S]methionine and [^{35}S]cysteine in the presence (+) or absence (−) of 20 μM PAD. The conditioned medium samples were harvested after the 4 h label period and immunoprecipitated with mAb 6E10 (lanes 1 and 3) or rabbit polyclonal antibody R1280 (lanes 2 and 4). Note that R1280 does not effectively detect sAPP but does detect the p3 secreted product.

also provide further evidence that the APP secretory pathway and the βA4 biogenesis pathway are not coupled in the SY5Y cell line.

The βA4 Peptide Is Produced Intracellularly and Then Secreted. The cellular site of βA4 production is still under debate. Studies have suggested that it may be produced either intracellularly or at the cell surface (Wertkin et al., 1993; Dyrks et al., 1993; Haass et al., 1993). To clarify this issue, we labeled SY5Y cells for 3−4 h periods, and on analysis of the cell lysates by immunoprecipitation with mAb 6E10, some intracellular βA4 was detected (results not shown). To confirm that βA4 detected in the cell lysates was intracellular and not due to extracellular nonspecific binding or uptake of secreted βA4, SY5Y cells were labeled for a 3 h period with 1 mCi/mL [^{35}S]methionine and [^{35}S]-cysteine and chased for various times after labeling. If the amount of βA4 measured in the cell lysate samples were to increase during the chase period, this would indicate nonspecific extracellular binding or reuptake of secreted βA4, whereas a decrease over time in the amount of βA4 recovered

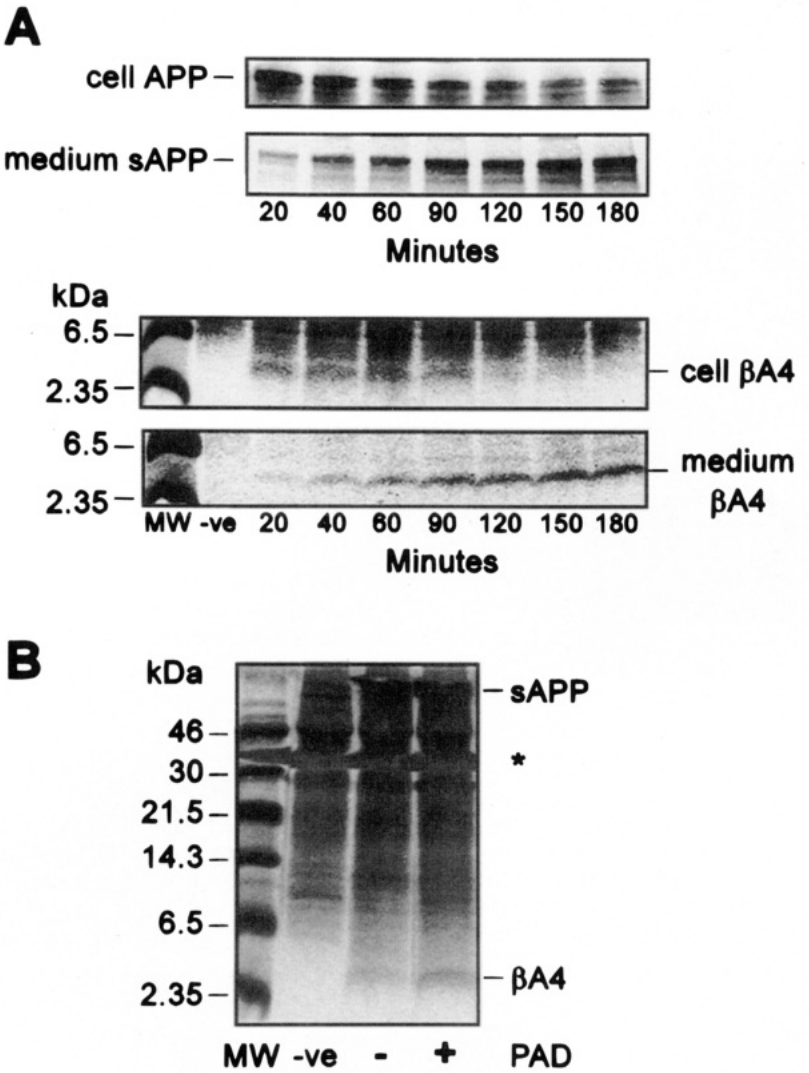

FIGURE 4: Time course of βA4 production and secretion from SY5Y cells and the effect of phosphoramidon on intracellular βA4 peptide. (A) Cells were labeled for 4 h in the presence of 1 mCi/mL [^{35}S]methionine and [^{35}S]cysteine and then chased in medium enriched with unlabeled methionine for time intervals up to 3 h. Cell and media samples from each time interval were then immunoprecipitated with mAb 6E10 and applied to 10/15% Tris/Tricine gels. The transferred gels depict the labeled sAPP and βA4 recovered from the media and cell lysate samples. The βA4 blots include some low molecular weight markers (MW) and the negative control lanes for which anti-neurofilament 68 had been used for immunoprecipitation (−ve). The medium used for the negative control was harvested after a 60 min chase period, and the cell lysate negative control was harvested at the start of the chase period. (B) SY5Y cells were labeled with 1 mCi/mL [^{35}S]methionine and [^{35}S]cysteine for 4 h in the presence or absence of 20 μM PAD, and then cell samples were harvested and immunoprecipitated with mAb 6E10. Immunoprecipitates were run on 10/15%. Tris/Tricine gels and transferred to PVDF membranes. The sizes of the molecular weight markers (MW lane) are indicated in kDa, and the negative control lane (−ve) was the result of immunoprecipitation of cell lysate with anti-neurofilament 68 antibody. The blurred area visible at approximately 37 kDa (*) is distortion of the gel that occurs on transfer (at the 10% and 16% acrylamide interface). Quantitation was not carried out as the background levels in the cell samples were too high.

would suggest that the βA4 is being produced intracellularly and is then secreted. The results showed that intracellular βA4 could be detected for up to 90 min chase periods but was not detectable after longer chase periods, while the media levels of βA4 increased over time (Figure 4A). SY5Y cell lysate immunoprecipitations were also carried out using the polyclonal R1280 antibody as well as mAb 6E10, and results were compared. Both antibodies immunoprecipitated 4 kDa bands, whereas appropriate negative control antibodies failed to do so, suggesting that the cellular 4 kDa band is indeed the βA4 peptide (gels not shown). These results are further evidence that the βA4 is produced intracellularly and subsequently secreted into an extracellular compartment.

Phosphoramidon Increases Recovery of Intracellular βA4 Peptide. Of the several possible mechanisms by which PAD might increase βA4 recovery from SY5Y cells, some can be easily eliminated. We established that phosphoramidon had no effect on the immunoprecipitation procedure (results not shown). Moreover, the effect of PAD was unlikely to be due to inhibition of extracellular breakdown of βA4, as we found that secreted radioactively labeled βA4 added to nonlabeled SY5Y cells was not broken down within a 1 h period, which is longer than the 40 min chase period used in our experiments (results not shown).

As there is a possibility that PAD increases the βA4 recovered in the medium by inhibiting intracellular breakdown prior to secretion, experiments were conducted to detect intracellular βA4 using PAD as a putative βA4 protease inhibitor. SY5Y cells were labeled with 1 mCi/mL [^{35}S]methionine and [^{35}S]cysteine for 4 h with and without PAD, and then cells and media were harvested. All cell samples were lysed in buffers containing 20 μM PAD.

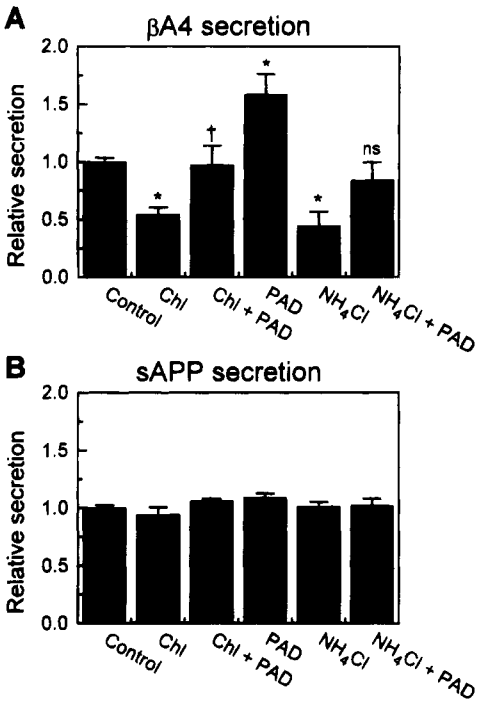

FIGURE 5: The effect of phosphoramidon and lysosomotropic agents on the secretion of βA4 and sAPP from SY5Y cells. SY5Y cells were labeled for 3 h with 0.5 mCi/mL [^{35}S]methionine and [^{35}S]-cysteine and then chased in unlabeled methionine-rich medium for 40 min in the absence (Control) or presence of NH_4Cl, chloroquine (Chl), and/or phosphoramidon (PAD). Media samples were immunoprecipitated with mAb 6E10. Immunoprecipitates were run on 10/15% Tris/Tricine gels and transferred to PVDF membranes. Quantitation was carried out on the phosphorimager, and results for βA4 and sAPP are summarized in (A) and (B), respectively. The mean control values for sAPP and βA4 were assigned the value of 1.00, and other values were normalized accordingly. Each bar represents the average for four samples, with error bars indicating standard error. "*" indicates significance of $p \leq 0.05$ compared to control, "†" indicates $p < 0.05$ compared to Chl samples (Wilcoxon's Rank Sum test), and "ns" indicates not significantly different from NH_4Cl.

Once again, immunoprecipitation of the media samples showed on approximately 2-fold increase in secreted βA4 peptide levels on treatment with PAD with no change in the secretion of sAPP (Figure 3c). Although the background radioactivity levels were high in the immunoprecipitated cell lysate samples, βA4 was detected in the untreated SY5Y cell lysates, and a higher level of βA4 was detectable in the cells that were treated with PAD during the labeling period (Figure 4B).

The Effect of Phosphoramidon Is Not Decreased by Lysosomotropic Agents. It has been previously reported that the lysosomotropic agents chloroquine and NH_4Cl can cause a decrease in secretion of βA4 peptide (Haass et al., 1993; Shoji et al., 1992). These results suggested that an acidic compartment (lysosomal/late endosomal) is involved in the production of the βA4 peptide.

SY5Y cells were labeled and then treated with these agents in the chase periods. Chloroquine (50 μM) and NH_4Cl (10 mM) caused a decrease of up to 60% in βA4 secretion without affecting the secretion of sAPP (Figure 5). On addition of both chloroquine and PAD (20 μM) or both NH_4Cl and PAD, the decrease in βA4 release was not as pronounced as with the lysosomotropic agents alone. Another way to express this result would be that PAD still produced a 2-fold increase in βA4 release despite the presence of the lysosomotropic agents.

DISCUSSION

Protein kinase C activation produced by either phorbol esters or activation of muscarinic acetylcholine receptors has been shown to increase the secretion of both sAPP and the p3 peptide into the medium of cell cultures (Caporaso et al., 1992; Buxbaum et al., 1990), and to concomitantly decrease βA4 secretion (Buxbaum et al., 1993; Jacobsen et al., 1994; Hung et al., 1993). It has therefore been proposed that the APP secretory pathway and the amyloidogenic processing pathway are inversely coupled and that compounds which increase protein kinase C activity may conceivably be useful in efforts to slow the development of Alzheimer's disease.

In our studies we used the untransfected SY5Y human neuroblastoma cell line and found that increased sAPP secretion due to activation of protein kinase C was not accompanied by a decrease in βA4 secretion. Recent work by Dyrks et al. (1994) showed similar results, using SY5Y cells transfected with APP cDNA. Our results were obtained using untransfected cells, suggesting that the results obtained by Dyrks et al. (1994) were not due to a peculiarity of their specific transfected cell line. On treating our cells with the protease inhibitor PAD, there was a significant increase in βA4 secretion with no effect on sAPP secretion. If the two pathways were coupled in the SY5Y cell line and PAD were somehow directing APP toward an amyloidogenic degradative pathway, as indicated by increased βA4 secretion, then a decrease in flow toward the sAPP secretory pathway would have been expected. Alternatively, if PAD were inhibiting the intracellular breakdown of soluble βA4, the accumulation of APP breakdown products might have been expected to cause an increase in the proportion of APP destined for secretion. Yet despite a significant increase in βA4 secretion from SY5Y cells on treatment with PAD, the amount of sAPP secreted did not change. We have therefore demonstrated a double dissociation between βA4 secretion and sAPP secretion in this cell line.

Intracellular βA4 has been detected by only one research group to date, in a human teratocarcinoma cell line terminally differentiated with retinoic acid (Wertkin et al., 1993). The lack of data on intracellular βA4 may be due to rapid secretion of βA4 following its production, resulting in very low cellular levels of the peptide. Intracellular βA4 may also be degraded upon lysis of cells unless appropriate protease inhibitors are included in the cell lysis buffers. We show here that low levels of βA4 can be detected in an untransfected (and undifferentiated) neuronal cell line and that the peptide is produced intracellularly with subsequent secretion by the cells. The hypothesis that βA4 is produced intracellularly in SY5Y cells is reinforced by the fact that βA4 release is inhibited when cells are treated with the lysosomotropic drugs chloroquine and NH_4Cl, indicating that acidic compartments are required to produce and/or secrete the βA4 peptide. Despite inhibition of βA4 release by up to 60%, there was no change in sAPP secretion, indicating that the lower βA4 secretion was not due to nonspecific impairment of cell metabolism.

Metalloproteases have already been linked to APP processing (for a review see Evin et al., 1994). A human brain enzyme identified as metalloprotease 24:15 (EC 3.4.24:15)

was shown to cleave a synthetic peptide spanning the β-secretase site of APP (McDermott et al., 1992), and purified human 24:15 identified by sequencing was shown to cleave recombinant APP (Papastoitsis et al., 1994). There is also evidence that APP α-secretase may be a metalloprotease (Roberts et al., 1994; McDermott et al., 1991). Phosphoramidon (PAD) is known to inhibit a number of thermolysin-like bacterial metalloproteases, but inhibits only a small number of mammalian enzymes, and to date has only been shown to inhibit metalloproteases (Powers & Harper, 1986). PAD does not inhibit 24:15 (Papastoitsis et al., 1994), but is a potent inhibitor of the cell surface metalloprotease, neutral endopeptidase (24:11 (NEP) (Mumford et al., 1981; Orlowski et al., 1983; for a review see wilkins et al., 1993), as well as the metalloprotease endothelin converting enzyme (ECE) (McMahon et al., 1991a,b; Shimada et al., 1994). Thiorphan and CPPL are also potent inhibitors of NEP (Olins et al., 1989; Fournié-Zaluski et al., 1982), but thiorphan is a much less effective inhibitor of ECE (Olins et al., 1989). Thiorphan and CPPL did not increase βA4 recovery in our medium, suggesting that NEP is unlikely to be a βA4 degrading enzyme; but an ECE-like enzyme is a possible candidate. The cell membrane permeabilities of thiorphan and CPPL were not relevant when testing NEP inhibition in cell culture as NEP is a cell surface enzyme. Furthermore, it is unlikely that PAD exerts its effect on a putative extracellular βA4 degrading enzyme such as NEP, as there was no detectable breakdown of βA4 once it had been secreted using our assay system. The increased amount of βA4 recovered intracellularly as well as extracellularly after treatment with PAD suggests that it is not increasing the recovery of βA4 in the medium by nonspecifically activating the secretion from the cells of previously formed βA4. Our results also show that PAD still increases the release of βA4 in the presence of lysosomotropic agents, when compared to the lysosomotropic agents alone. If PAD is inhibiting intracellular βA4 degradation, then the enzyme responsible is unlikely to be in an acidic compartment. Alternatively, PAD may be exerting its effect by channeling a higher proportion of cellular APP toward the degradative compartments of the cell that produce βA4, for example, by increasing cell surface APP endocytosis, as endocytosis of APP has been shown to increase βA4 secretion (Koo et al., 1994). Further work is required to establish the site of PAD inhibition and to characterize the proteases involved in the metabolism of APP.

PAD has been considered and tested as a possible therapeutic drug for the treatment of hypertension due to its inhibition of NEP as well as its inhibition of ECE (Vemulapalli et al., 1993; Wilkins et al., 1993). Neutral endopeptidase degrades the atrial natriuretic peptide (Olins et al., 1989), and thus inhibition of NEP raises the circulating levels of atrial naturiuretic peptide, causing a decrease in blood pressure in hypertensive patients (Le Francois et al., 1990). Treatment with PAD has also been shown to lower the blood pressure of spontaneously hypertensive rats (McMahon et al., 1991a), due to ECE inhibition presumably causing a fall in endothelin-1 levels (Ikegawa et al., 1990). Considering that PAD treatment may also result in long-term increased levels of βA4 and that higher levels of βA4 may accelerate the formation and deposition of amyloid, the use of PAD and similar inhibitors as candidates for antihypertensive therapy should be reevaluated in light of these findings.

ACKNOWLEDGMENT

We wish to thank Dr. D. J. Selkoe for the rabbit polyclonal antibody R1280, and Dr. D. Small for helpful discussions.

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BI9426874