Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 31, Issue of November 5, pp. 22959-22962, 1993

Activation of Protein Kinase C Inhibits Cellular Production of the Amyloid β-Protein*

(Received for publication, July 23, 1993, and in revised form, September 2, 1993)

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The 39–43-amino acid amyloid β-protein (Aβ), which is progressively deposited in cerebral plaques and blood vessels in Alzheimer's disease (AD), is released by cultured human cells during normal metabolism. Here we show that agents which activate protein kinase C or otherwise enhance protein phosphorylation caused a substantial decrease in Aβ production *in vitro*. Protein kinase C activation also markedly decreased Aβ release from cells that express mutant forms of the β-amyloid precursor protein genetically linked to familial AD. Inhibition of Aβ secretion could also be effected by direct stimulation of m1 muscarinic acetylcholine receptors with carbachol. These results demonstrate that activation of the protein kinase C signal transduction pathways down-regulates the generation of the amyloidogenic Aβ peptide. Pharmacologic agents that activate this system, including a variety of first messengers, could potentially slow the development or growth of some Aβ plaques during the early stages of AD.

An invariant pathologic feature of Alzheimer's disease (AD)[1] is the deposition of fibrillar aggregates of the amyloid β-protein (Aβ) in the brain and cerebral blood vessels. This 39–43-amino acid peptide is generated by proteolytic cleavage of the β-amyloid precursor protein (βAPP), a 100–140-kDa integral membrane protein encoded by a gene on human chromosome 21 (Kang *et al.*, 1987). Aβ comprises a region of βAPP beginning 28 residues outside the membrane and including 11–15 amino acids of the transmembrane domain (Fig. 1*A*). Alternative splicing of a single βAPP pre-mRNA generates three major isoforms containing 695, 751, or 770 amino acids (Kang *et al.*, 1987; Ponte *et al.*, 1988; Tanzi *et al.*, 1988; Kitaguchi *et al.*, 1988). Proteolytic processing of βAPP gives rise to a ~90–100-kDa soluble derivative (APP_s) in most cell types examined to date (Weidemann *et al.*, 1989; Schubert *et al.*, 1989). This derivative is released following cleavage between residues 612 and 613 of $βAPP^{695}$ (Esch *et al.*, 1990; Sisodia *et al.*, 1990; Wang *et al.*, 1991). Because this proteolytic event occurs within the Aβ domain (between residues 16 and 17 of Aβ), secretion of APP_s presumably precludes Aβ generation and deposition. In contrast to this secretory pathway, some full-length βAPP molecules are reinternalized from the cell surface into endosomes and lysosomes (Haass *et al.*, 1992a), where they are apparently processed into a number of potentially amyloidogenic carboxyl-terminal derivatives (Estus *et al.*, 1992; Golde *et al.*, 1992; Haass *et al.*, 1992a). The relative utilization of these two pathways appears to differ among various cell types (Haass *et al.*, 1991; Hung *et al.*, 1992). However, the pathway that actually produces Aβ and the cellular mechanisms regulating its formation are unknown.

Aβ was recently found to be continuously produced and released by a variety of cultured human cells under normal metabolic conditions (Haass *et al.*, 1992b; Seubert *et al.*, 1992; Shoji *et al.*, 1992). Consequently, the regulation of Aβ production and secretion can now be studied *in vitro*, and the effects of various physiological and pharmacological modulators can be readily assessed. Because stimulation of protein kinase C (PKC) by phorbol esters has been shown to increase APP_s secretion (Caporaso *et al.*, 1992; Gillespie *et al.*, 1992), we examined whether protein kinase activation decreases the formation of the Aβ fragment, which derives from a proteolytic processing mechanism other than the principal secretory cleavage. We show that stimulation of PKC, either directly by addition of phorbol esters or indirectly by activation of PKC-coupled cell surface receptors, inhibits production of Aβ.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—293 cells stably transfected with $βAPP^{695}$ and primary human skin fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (HyClone). For generation of cell lines expressing mutant forms of βAPP, the K595N/M596L (Citron *et al.*, 1992) and V642I amino acid substitutions were introduced into the $βAPP^{695}$ expression vector pCMV695 (Selkoe *et al.*, 1988) by oligonucleotide-directed mutagenesis and transfected into 293 cells using Lipofectin (Life Technologies, Inc.), as described by the manufacturer. Cells were cotransfected with βAPP plasmids (10 μg) and the neomycin resistance plasmid pRc/CMV (InVitrogen) (0.5 μg), and selected in medium containing 400 μg/ml G418 for 2–3 weeks. Pulse-chase experiments were performed on pools of stably transfected clones. βAPP expression in the various mutants was comparable, as determined by immunoblotting of total cellular extracts (data not shown). To analyze the effect of carbachol on Aβ production, 293 cells stably expressing the m1 subtype of muscarinic acetylcholine receptors (Nitsch *et al.*, 1992) were transiently transfected with pCMV695 using Lipofectin. Cells were then used for pulse-chase experiments 60–72 h after transfection.

Antibodies—The polyclonal antibody R1736 was raised against a synthetic peptide of $βAPP^{595-611}$, containing the last 17 amino acids of APP_s (Haass *et al.*, 1992b). R1280 is a polyclonal antiserum raised against synthetic $Aβ^{1-40}$ (Tamaoka *et al.*, 1992) and precipitates Aβ, p3,

* This work was supported in part by grants from the National Institute on Aging, the National Institute of Mental Health, Athena Neurosciences, Inc., and the Center for Brain Sciences and Metabolism Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from Merck Sharp and Dohme Research Laboratories.

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** Supported by the Max Planck Gesellschaft.

[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, amyloid β-protein; βAPP, β-amyloid precursor protein; APP_s, soluble βAPP; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle medium; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; Tricine, *N*-tris(hydroxymethyl)methylglycine; AChR, acetylcholine receptor.

and small amounts of APP_s. Because R1280 precipitates only a small, variable quantity of all APP_s secreted by cells, R1736-immunoprecipitable material more accurately represents total APP_s release.

Pulse-Chase Experiments—βAPP^{695}-expressing 293 cells grown to near confluence were incubated in methionine-free DMEM for 30 min, labeled with 60–100 μCi/ml [^{35}S]methionine (DuPont NEN) in serum-free, methionine-free DMEM in the absence of drug, and then chased in DMEM containing 10% fetal calf serum without (control) or with drug. PDBu and PMA (Sigma) were prepared as 10 mM stock solutions in dimethyl sulfoxide and diluted in medium prior to addition to cells. For APP_s analysis, cells in 6-cm dishes were labeled for 20 min and chased for 60 min. For Aβ analysis, 10-cm dishes were labeled for 2 h and chased for 5–6 h; the prolonged chase period was used to ensure processing and release of all labeled βAPP species. Phorbol esters and okadaic acid (Life Technologies, Inc.) were included only during the chase period. Staurosporine was added to the medium at a concentration of 1 μM during both labeling and chase periods. To inactivate PKC prior to labeling (Ballester and Rosen, 1985; Young *et al.*, 1987), cells were preincubated for 15 h with 1 μM PDBu. The medium was then discarded, and fresh medium containing 1 μM PDBu was used throughout the labeling and chase periods. Because 293 cells were less adherent in the presence of okadaic acid, conditioned media from all samples were centrifuged prior to immunoprecipitation to remove detached cells and cell debris. For primary fibroblasts, which synthesize only small amounts of βAPP, the conditioned media from three 10-cm dishes were pooled. Immunoprecipitations of conditioned media with R1736 or R1280 were performed as described (Haass *et al.*, 1992b), separated by 10% SDS-polyacrylamide gel electrophoresis (for APP_s) or on 10–20% Tris-Tricine gels (for Aβ; Novex), and subjected to autofluorography. For immunoprecipitations, R1280 was used at a dilution of 1:300. Quantitation of the effect of PDBu on Aβ release was similar at antibody concentrations between 1:150 and 1:1200.

To analyze the effect of carbachol, m1-expressing 293 cells transiently transfected with βAPP^{695} were pulse-labeled as above and chased for 3 h in DMEM containing 10% fetal calf serum in the absence or presence of 1 μM PDBu or 1 mM carbachol (Sigma). When added, 10 μM atropine (Sigma) was included during both the labeling and chase periods. For each sample, the conditioned media from three independently transfected 10-cm dishes were combined to maximize signal intensities and to minimize differences in transfection efficiency among dishes. To confirm the efficacy of the various drug treatments, a small aliquot of the chase medium was immunoprecipitated with antibody R1736 (Fig. 4*A*). The remainder of the chase medium was precipitated with antibody R1280 (Fig. 4*B*) and analyzed as above.

Quantitation of Aβ Release—Aβ release from untreated and drug-treated cells was quantitated using a PhosphorImager 400A and ImageQuant software (Molecular Dynamics). Each individual protein band corresponding to Aβ immunoprecipitated from the conditioned media of treated cells was quantitated three separate times, averaged, and compared to the corresponding untreated control within the same experiment.

RESULTS AND DISCUSSION

To examine the role of protein kinase C in the regulation of Aβ production, we used human embryonic kidney 293 cells stably transfected with a βAPP^{695} cDNA (Selkoe *et al.*, 1988). Like a number of other cell types, including PC12 pheochromocytoma cells (Caporaso *et al.*, 1992) and Hs 683 human glioma cells (Buxbaum *et al.*, 1992), 293 cells demonstrate altered βAPP processing upon PKC activation (Gillespie *et al.*, 1992). βAPP^{695}-transfected 293 cells were pulse-labeled with [^{35}S]methionine and then chased in the absence or presence of various agents affecting protein phosphorylation. In parallel with an increase in APP_s secretion (Fig. 1*B*), addition of 1 μM phorbol 12,13-dibutyrate (PDBu) to βAPP^{695}-transfected cells led to a substantial decrease in the amounts of Aβ released during the chase period (Fig. 1*C*, compare *lanes 1* and *2*). This result was confirmed with a second activator of PKC, phorbol 12-myristate 13-acetate (PMA) (Fig. 1*C*, *lane 3*). Furthermore, these agents increased the secretion of a 3-kDa truncated Aβ species (p3) (Fig. 1*C*), the amount of which has been shown to parallel that of APP_s released from cells (Haass *et al.*, 1992b, 1993). Quantitation of Aβ release by phosphorimaging (Fig. 1*D*) showed that addition of either PDBu or PMA during the chase period

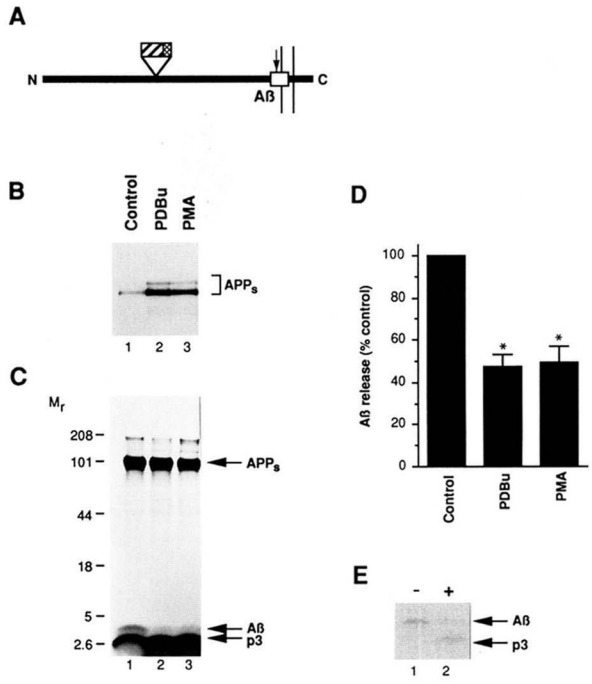

FIG. 1. **Aβ release is inhibited upon addition of phorbol esters.** *A*, schematic diagram of βAPP. The positions of Aβ (*white box*) and the additional 56-amino acid (*hatched box*) and 19-amino acid (*cross-hatched box*) inserts in the alternatively spliced βAPP^{751} and βAPP^{770} are shown. *Vertical lines* indicate transmembrane region. The *arrow* indicates the position of the proteolytic cleavage that generates APP_s. *B*, immunoprecipitates of APP_s from the conditioned media of [^{35}S]methionine-labeled βAPP^{695}-transfected 293 cells with antibody R1736. Note the increase in secretion of both the endogenous APP$^{751/770}$ forms (*upper band*) and the transfected APP^{695} form (*lower band*) of APP_s (indicated by *bracket*) upon PDBu (*lane 2*) or PMA (*lane 3*) treatment. *C*, immunoprecipitates of conditioned media from the βAPP^{695}-transfected 293 cells with antibody R1280. Lanes are as in *B*. *Arrows* indicate APP_s, Aβ, and p3. R1280 precipitates small, variable amounts of APP_s and therefore, in contrast to R1736, does not provide a reliable indicator of APP_s levels. The band migrating at ~200 kDa is nonspecific and is precipitated by preimmune serum (Haass *et al.*, 1992b). No other bands were detected below p3. The gel in *B* was exposed for 24 h, whereas the gel in *C* was exposed for 7 days. *D*, quantitation of Aβ from the gel in *C* and additional gels was carried out by phosphorimaging. Signals from the media of phorbol ester-treated cells were compared to those of untreated cells for each individual experiment. Each column represents the mean ± S.E. of six to seven independent experiments. *Asterisks* indicate significant decrease in Aβ release ($p < 0.0001$) compared to control. *E*, immunoprecipitates of conditioned media from primary human skin fibroblasts with antibody R1280, without (*lane 1*; indicated by –) or with (*lane 2*; indicated by +) treatment with 1 μM PDBu.

resulted in a decrease in secreted Aβ levels to less than one half of control levels. Stimulation of primary human skin fibroblasts with PDBu similarly decreased Aβ release and increased p3, confirming the results obtained from the transfected cells (Fig. 1*E*).

To confirm the role of phosphorylation in Aβ regulation, we treated cells with agents that either enhance or block the effect of phorbol esters. Treatment of the transfected 293 cells with PDBu plus 0.5 μM okadaic acid, an inhibitor of protein phosphatases PP1 and PP2A (Cohen, 1989), augmented the inhibition of Aβ release by PDBu, whereas okadaic acid alone had lesser effects on release of the 4-kDa peptide (Fig. 2*A*, *lanes 3* and *4*). Treatment of the cells with PDBu plus staurosporine, an inhibitor of protein kinases, largely abolished the decrease in Aβ levels observed with PDBu alone (Fig. 2*B*, compare *lanes 2* and *3*). As an additional control, we preincubated the 293 cells with 1 μM PDBu for 15 h prior to labeling. This has been shown to down-regulate endogenous PKC, thus preventing its activation upon subsequent treatment with additional phorbol

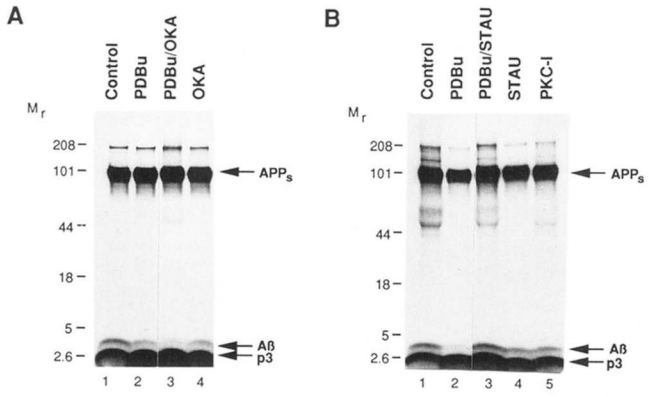

FIG. 2. **Addition of protein phosphatase or kinase inhibitors modulates Aβ release.** *A*, effect of okadaic acid on Aβ production. Immunoprecipitations of Aβ with R1280 from the conditioned media of βAPP^{695}-transfected 293 cells in the absence of drug (*lane 1*) or in the presence of: 1 μM PDBu (*lane 2*), 1 μM PDBu plus 0.5 μM okadaic acid (*lane 3*), or 0.5 μM okadaic acid alone (*lane 4*). *B*, effect of kinase inhibitors on PDBu-mediated inhibition of Aβ release. Conditioned media from untreated cells (*lane 1*) or from cells treated with 1 μM PDBu (*lane 2*), 1 μM PDBu plus 1 μM staurosporine (*lane 3*), or 1 μM staurosporine alone (*lane 4*) were immunoprecipitated with antibody R1280. The cells labeled in *lane 5* were chronically treated for 15 h with 1 μM PDBu prior to labeling to inactivate endogenous protein kinase C (*PKC-I*). The positions of APP$_s$, Aβ, and p3 are indicated with *arrows*. Each panel is representative of three to four independent experiments.

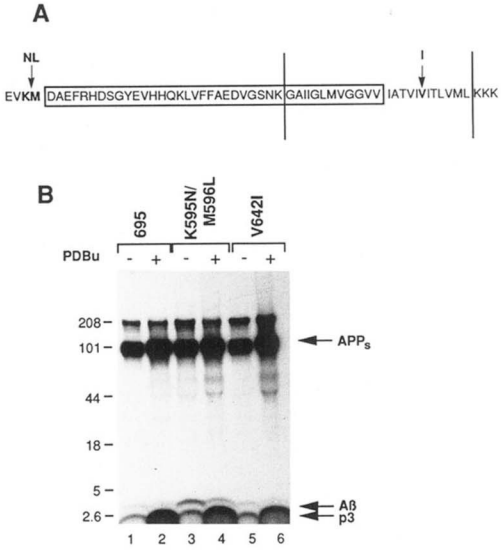

FIG. 3. **Release of Aβ derived from mutant forms of βAPP linked to familial Alzheimer's disease is inhibited by PKC activation.** *A*, schematic representation of the FAD-linked βAPP mutations (*arrows*) examined in this study. *Box* represents Aβ$^{1-40}$. *Vertical lines* indicate transmembrane region. *B*, immunoprecipitates with antibody R1280 from conditioned media of [^{35}S]methionine-labeled 293 cells stably transfected with either wild-type βAPP^{695} (*lanes 1* and *2*), or βAPP mutants K595N/M596L (*lanes 3* and *4*) or V642I (*lanes 5* and *6*), and treated without phorbol ester (denoted by –; *lanes 1*, *3*, and *5*) or with 1 μM PDBu (denoted by +; *lanes 2*, *4*, and *6*). APP$_s$, Aβ, and p3 are indicated. Similar results were obtained with four independent experiments.

ester (Ballester and Rosen, 1985; Young *et al.*, 1987). Under these conditions, little decrease in Aβ or increase in p3 release was observed, despite the inclusion of PDBu during both the labeling and chase periods (Fig. 2*B*, *lane 5*).

In view of the substantial lowering of Aβ production induced by phorbol esters, we asked whether PDBu could have a similar effect on Aβ release from cells expressing mutant forms of βAPP genetically linked to early onset familial AD (Fig. 3*A*). A double missense mutation immediately amino-terminal to the Aβ sequence (Lys → Asn at residue 595 and Met → Leu at

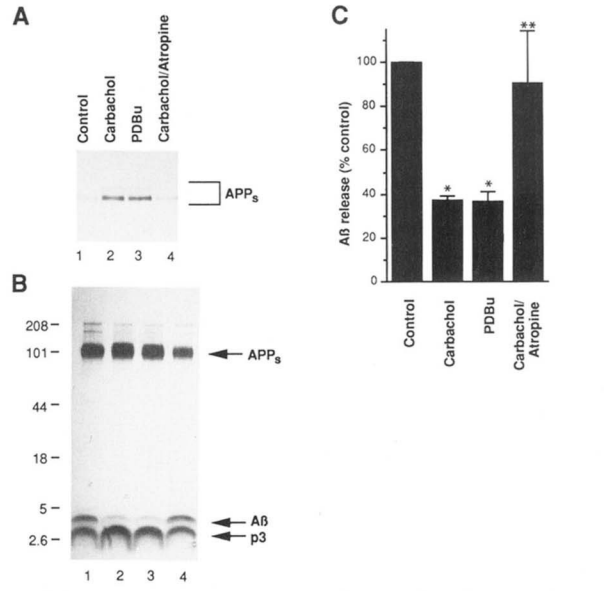

FIG. 4. **Stimulation of muscarinic m1 receptors in transfected 293 cells inhibits Aβ production.** *A*, immunoprecipitation of APP$_s$ (endogenous APP$^{751/770}$ and transfected APP^{695} indicated by *bracket*) with antibody R1736 from the conditioned media of 293 cells stably expressing m1 receptors and transiently transfected with βAPP^{695}. Cells were pulse-labeled with [^{35}S]methionine and chased in the absence (*lane 1*) or presence of 1 mM carbachol (*lane 2*), 1 μM PDBu (*lane 3*), or 1 mM carbachol plus 10 μM atropine (*lane 4*). *B*, immunoprecipitates of conditioned media from βAPP^{695}-transfected m1 cells with antibody R1280. Lanes are as in *A*. APP$_s$, Aβ, and p3 are indicated. *C*, quantitation of the effect of carbachol and atropine treatment on Aβ release from m1-expressing 293 cells. Signals corresponding to Aβ were measured by phosphorimaging and compared to the untreated control (set to 100%) within the same experiment. *Bars* represent the mean ± S.E. of three to four independent experiments. *Asterisks* indicate statistical significance of $p < 0.0001$ compared to control (*) or $p < 0.05$ compared to carbachol alone (**).

residue 596 of βAPP^{695}; K595N/M596L), which was identified in a large Swedish kindred with early onset AD (Mullan *et al.*, 1992), has recently been shown to cause a marked increase in Aβ levels (Citron *et al.*, 1992; Cai *et al.*, 1993). Addition of 1 μM PDBu to 293 cells stably transfected with a βAPP^{695} cDNA bearing the K595N/M596L mutation resulted in a consistent decrease in the elevated secretion of Aβ and a concurrent increase in p3 (Fig. 3*B*, *lanes 3* and *4*). Aβ production from another βAPP mutation linked to familial AD, a valine to isoleucine substitution at position 642 of βAPP^{695} (V642I) (Goate *et al.*, 1991), which does not appear to significantly increase levels of secreted Aβ (Cai *et al.*, 1993), was similarly attenuated by addition of phorbol ester (Fig. 3*B*, *lanes 5* and *6*), indicating that protein kinase C activation can down-regulate Aβ release from cells expressing AD-associated mutant forms of βAPP.

Our findings suggest that a variety of physiologic agonists may normally modulate Aβ release *in vivo* by stimulating cell surface receptors coupled to signal transduction pathways which activate protein kinase C. In particular, stimulation of muscarinic m1 and m3 acetylcholine receptors (AChR) and other first messenger systems linked to phospholipase C/protein kinase C have recently been shown to increase APP$_s$ release (Nitsch *et al.*, 1992; Buxbaum *et al.*, 1992). To examine directly the effect of muscarinic receptor stimulation on Aβ production, we transiently transfected m1 AChR-expressing 293 cells (Nitsch *et al.*, 1992) with a wild-type βAPP^{695} cDNA. The cells were pulse-labeled with [^{35}S]methionine and chased in the presence of the muscarinic agonist carbachol. Treatment with carbachol increased the secretion of APP$_s$ (Fig. 4*A*) and inhibited Aβ release (Fig. 4*B*), similar to PDBu. In addition, treatment of the receptor-expressing cells with the competitive

antagonist atropine blocked the carbachol-mediated stimulation of APP_s (Fig. 4A, *lane 4*) and inhibition of Aβ (Fig. 4B, *lane 4*), indicating that this effect is directly attributable to a ligand-receptor interaction.

Our data demonstrate that activation of protein kinase C alters the proteolytic processing of βAPP to enhance secretory cleavage within the Aβ domain, resulting in increased release of APP_s and the complementary p3 peptide and decreased release of the Aβ peptide. The fact that Aβ production decreases substantially after such activation supports growing evidence that cells process βAPP molecules in a regulated manner via at least two alternative but normal proteolytic cleavage events, at either Met^{596} or Lys^{612}. After either mode of βAPP cleavage, some of the resultant carboxyl-terminal fragments apparently undergo an additional cleavage in the region Val^{636} to Thr^{639}, creating the carboxyl termini of both the Aβ and p3 peptides. This event seems to be associated with rapid release of these fragments from the cell, since they appear to be undetectable within cells (Haass *et al.*, 1992b, 1993). It is unclear whether the generation of Aβ occurs within the same cellular trafficking pathway that generates conventional APP_s (*e.g.* in the late Golgi or in a secretory vesicle destined for the cell surface) or whether βAPP molecules are diverted from this route to another compartment. Our data also indicate that Aβ release may be modulated physiologically and pharmacologically by a number of agonists whose receptors are linked to phospholipase C and protein kinase C activation. In concert with the recent observation that neuronal depolarization increases APP_s secretion (Nitsch *et al.*, 1993), this finding suggests that neuronal activity and neurotransmitter release may directly regulate Aβ production, perhaps variably in different brain regions depending on the local profile of neurotransmitters and their corresponding receptors.

The mechanism by which stimulation of the phospholipase C/protein kinase C signaling pathway causes increased cleavage of βAPP at Lys^{612} and decreased cleavage at Met^{596} is only partially understood. Although *in vitro* activation of exogenous PKC by phorbol esters has been reported to phosphorylate βAPP at Ser^{655} in a semi-intact PC12 system (Suzuki *et al.*, 1992), we have recently found that phorbol ester-induced APP_s secretion and Aβ inhibition is not mediated by a change in the phosphorylation of βAPP itself in intact 293 cells.[2] Indeed, mutation of potential intracellular phosphate acceptor sites or deletion of the cytoplasmic domain failed to abolish the modulation of βAPP processing by PKC. Moreover, we detected no phosphorylation of the cytoplasmic domain either before or after PKC activation. Consequently, other proteins must serve as the substrate of PKC in the experiments described here: *e.g.* proteases that cleave βAPP or proteins that are involved in the anchoring or movement of secretory vesicles containing βAPP. In addition, signaling pathways other than that utilizing PKC may be involved in the regulation of Aβ formation, perhaps in a cell type-dependent manner. Regardless of the mechanism, the pharmacological activation of specific first messenger systems coupled to PKC might prove useful in lowering regional Aβ production *in vivo*. This hypothesis can now be potentially tested directly by administering m1 AChR-specific cholinergic agonists (or other first messengers) to animals and assaying soluble Aβ levels in brain tissue and cerebrospinal fluid (Seubert *et al.*, 1992).

[2] A. Hung and D. Selkoe, submitted for publication.

Acknowledgment—We are grateful to S. Field for help with phosphorimaging analysis.

Note Added in Proof—Findings that are similar in part to ours have been obtained by Buxbaum *et al.* (Buxbaum, J. D., Koo, E. H., and Greengard, P. (1993) *Proc. Natl. Acad. Sci. U. S. A.*, in press).

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