Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia
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Metabotropic Glutamate Receptors Increase Amyloid Precursor Protein Processing in Astrocytes: Inhibition by Cyclic AMP

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Abstract: Neurotransmitter receptors that increase phosphatidylinositol hydrolysis generate second messengers that activate protein kinase C. Here, we used metabotropic glutamate receptor agonists to increase both phosphatidylinositol hydrolysis and secretion of the soluble extracellular fragment of amyloid precursor protein (APPs) from cortical astrocyte cultures. The increase in APPs secretion was mimicked by direct activation of protein kinase C with phorbol ester and was suppressed by the metabotropic glutamate receptor antagonist L-(+)-2-amino-3-phosphonopropionic acid or by the protein kinase C inhibitor GF109203X. Ionotropic glutamate agonists did not increase APPs secretion. Forskolin or dibutyryl cyclic AMP inhibited the increase in APPs secretion caused by metabotropic glutamate receptor agonists or by phorbol ester treatment but did not affect basal APPs levels. Therefore, glutamatergic agonists that increase protein kinase C activation or decrease cyclic AMP formation may enhance the conversion of full-length APP to nonamyloidogenic APPs in Alzheimer's disease. **Key Words:** Neurotransmitters—Dexnorfenfluramine—Adenylate cyclase—Protein kinase C—Phosphatidylinositol. *J. Neurochem.* **68,** 1830–1835 (1997).

Senile plaques found in Alzheimer's disease (AD) are principally composed of abnormal aggregates of amyloid peptides (Aβs), which are released by proteolytic cleavage of a much larger transmembrane protein, the amyloid precursor protein (APP). The first 28 amino acid residues of Aβ, like the large N-terminus of APP, extend into the extracellular space. The remaining 12–15 residues of Aβ are anchored within the cell membrane and are attached to the short, cytoplasmic C-terminus of APP (Selkoe, 1994). Constitutive APP processing creates a cleavage within the Aβ peptide to prevent amyloid formation; the soluble extracellular fragment of APP (APPs) is then secreted into the extracellular medium. APP overexpression or missense mutations may increase Aβ formation by altering constitutive APP processing pathways (Checler, 1995). Because reinternalization of APP retaining the intact Aβ domain may increase Aβ production, strategies directed at increasing APP processing or APPs secretion have been proposed to prevent amyloid plaques in AD.

Neurotransmitter agonists coupled to phosphatidylinositol (PI) hydrolysis can increase APPs secretion and decrease Aβ formation (Buxbaum et al., 1992; Nitsch et al., 1992; Lee et al., 1995*a*; Wolf et al., 1995). This form of nonamyloidogenic processing is mediated in large part by the second messengers derived from PI hydrolysis, diacylglycerol and inositol trisphosphate, which activate protein kinase C (PKC).

Astrocytes express abundant levels of APP but, unlike neurons and most cell lines, undergo low levels of constitutive processing and secrete relatively small quantities of APPs (Haass et al., 1991). Moreover, astrocytes, unlike neurons, reportedly do not increase APPs secretion in response to direct activation of PKC (Gabuzda et al., 1993). Instead, astrocytes secrete abundant levels of Aβs (Busciglio et al., 1993). Thus, astrocytes found in proximity to senile plaques and degenerating neurites may be a significant nonneuronal source of Aβ.

Astrocytes express various neurotransmitter receptors that are coupled to ion channels or second messengers. We explored the possibility that metabotropic glutamate receptor (mGluR) agonists, which activate the PI/PKC cascade, may stimulate APPs secretion in astrocyte cultures. Because mGluR can potentiate cy-

Received November 11, 1996; revised manuscript received December 12, 1996; accepted December 13, 1996.

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Abbreviations used: Aβ, amyloid peptide; ACPD, *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentane dicarboxylic acid; AD, Alzheimer's disease; L-AP3, L-(+)-2-amino-3-phosphonopropionic acid; APP, amyloid precursor protein; APPs, soluble extracellular fragment of amyloid precursor protein; cAMP, cyclic AMP; dBcAMP, dibutyryl cyclic AMP; L-Glu, L-glutamate; MCPG, α-methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; QA, quisqualate.

clic AMP (cAMP) formation (Schoepp and Conn, 1993), we also examined the effect of cAMP signaling on APPs secretion. Parts of this work have been presented in abstract form (Lee et al., 1995*b*).

EXPERIMENTAL PROCEDURES

Cortical astrocyte cultures

Neonatal rat pups (1–3 days old) were overdosed with ketamine hydrochloride (Sigma) before decapitation. Astrocytes were obtained from dissected cortices using protocols described by McCarthy and de Vellis (1980). In brief, cortices were incubated in Hanks' buffered saline solution (GIBCO) containing 0.5% trypsin/0.1% deoxyribonuclease I (both from Sigma) for 30 min at 37°C. The cell pellet collected after centrifugation was resuspended in minimum essential medium (GIBCO) containing 10% horse serum and plated onto poly-L-lysine-coated 35-mm-diameter culture dishes ($\sim 10^5$ cells/cm^2). Cells were kept at 37°C in a humidified 5% CO_2/air incubator. Confluent astrocytes were used for experiments. Immunocytochemical staining with an antibody for glial fibrillary acidic protein (Boehringer Mannheim) labeled >95% of the cultured cells.

Pharmacological treatments

The following drugs were dissolved in water, ethanol, or dimethyl sulfoxide and diluted to working concentrations from frozen stock solutions (10^{-2}–10^{-4} *M*): GF109203X from LC Laboratory; phorbol 12-myristate 13-acetate (PMA), forskolin, dibutyryl cAMP (dBcAMP) from Calbiochem; and L-glutamate (L-Glu), *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentane dicarboxylic acid (ACPD), quisqualate (QA), *S*(−)-5-fluorowillardiine, NMDA, L-(+)-2-amino-3-phosphonopropionic acid (L-AP3), and α-methyl-4-carboxyphenylglycine (MCPG) from Research Biochemicals International. When appropriate, an equivalent volume of vehicle was included in the control group.

APPs level measurements

Astrocytes were exposed for 1 h to 1.5 ml of serum-free media containing the drug(s) to be tested. Secreted APPs was quantified using protocols similar to those described by Lee et al. (1995*a*). In brief, a cocktail of protease inhibitors (Nitsch et al., 1992) was added to the media, which were then desalted in columns containing Sephadex G25 beads (Bio-Rad) and lyophilized in a rotary evaporator. Cells remaining in the dishes were rinsed (3 × 1 ml) and scraped into 1 ml of ice-cold phosphate-buffered saline for measurements of protein concentration using the bicinchoninic acid assay. Lyophilized media were dissolved in 20 μl of extraction buffer (50 m*M* Tris buffer, 5 m*M* EDTA, and 150 m*M* NaCl, pH 7.6) containing 2% Nonidet P-40 and 2% Triton X-100 and diluted with 20 μl of loading buffer (125 m*M* Tris buffer, 4% sodium dodecyl sulfate, 20% glycerol, 5% 2-mercaptoethanol, and 0.01% bromphenol blue, pH 6.8). Samples were boiled (5 min) before electrophoresis. The amount of protein loaded on 12% sodium dodecyl sulfate polyacrylamide gels for each sample was corrected for the total amount of cell protein per dish.

The proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore), which were subsequently incubated overnight in monoclonal antibody 22C11 or Alz-90 (from Boehringer Mannheim) or the polyclonal antiserum R1285 (a gift from Dr. D. J. Selkoe, Harvard Medical School). Antibody 22C11 and Alz-90 and antiserum R1285 are directed against N-terminus residues 60–100 (Weidemann et al., 1989), 511–608 (Boehringer Mannheim), and 527–540 (Haass et al., 1991) of APP, respectively (numbering according to APP695).

Immunolabeling was visualized by a secondary antibody conjugated to horseradish peroxidase (Amersham). Enhanced chemiluminescence (Du Pont NEN) was used to detect antibody immunoreactivity, and the transfer blots were exposed to Kodak X-Omat film. Labeled APP proteins were quantified by laser scanning densitometry (LKB, Sweden). When immunoreactive bands appeared as doublets on western blots, the densitometry signals from both bands were summed.

PI hydrolysis assay

Inositol phospholipids were quantified using protocols similar to those described by Lee et al. (1995*a*). In brief, confluent astrocyte cultures were incubated for 24 h with serum-free minimum essential medium containing 2 μCi of *myo*-[2-^{3}H]inositol (Du Pont NEN; 1 Ci = 37 GBq). The cells were rinsed in serum-free minimum essential medium containing 10 m*M* LiCl before pharmacological treatments, which lasted 1 h. The cells were scraped into 1 ml of chloroform and 0.5 ml of water. After vortex-mixing the solution, the aqueous and organic phases were separated by centrifugation. One milliliter of the aqueous phase was loaded onto columns containing AG1-X8 anion-exchange resin (Bio-Rad) for separation of [^{3}H]inositol-containing compounds. Free [^{3}H]inositol was eluted into scintillation vials with 4 ml of 1 *M* ammonium formate/0.1 *M* formic acid. PI hydrolysis was estimated from the amount of radioactivity measured on a Beckman model LS7500 liquid scintillation counter.

Data analysis

Measurements of APPs levels and PI hydrolysis in different treatment groups were standardized against those of control groups. Differences between groups were evaluated by Student's *t* tests and ANOVA ($p < 0.05$).

RESULTS

Effects of mGluR agonists and antagonists on APPs secretion and PI hydrolysis

Treatment of astrocytes with L-Glu, QA, or ACPD (10 μ*M*–1 m*M*) caused highly significant and concentration-dependent increases in PI hydrolysis ($p < 0.05$). The relative order of potency of these mGluR agonists was QA > L-Glu > ACPD (Fig. 1). At 1 m*M*, the highest concentration tested, QA, L-Glu, and ACPD increased PI hydrolysis by 650, 530, and 480%, respectively.

The amount of secreted APPs, detected as a ~100-kDa protein band in the media using monoclonal antibody 22C11 or Alz-90 or antiserum R1285, was increased by the mGluR agonist ACPD (Fig. 2). L-Glu, ACPD, and QA significantly increased APPs levels by ~1.5-fold relative to baseline levels ($p < 0.05$). Similar increases in APPs levels by ACPD stimulation were detected by antibody 22C11 or Alz-90 or antiserum R1285. The increase in content of APPs stimulated by mGluR agonists was not concentration-dependent in the range examined, i.e., 10 μ*M*–1 m*M*. Also, 1 μ*M*

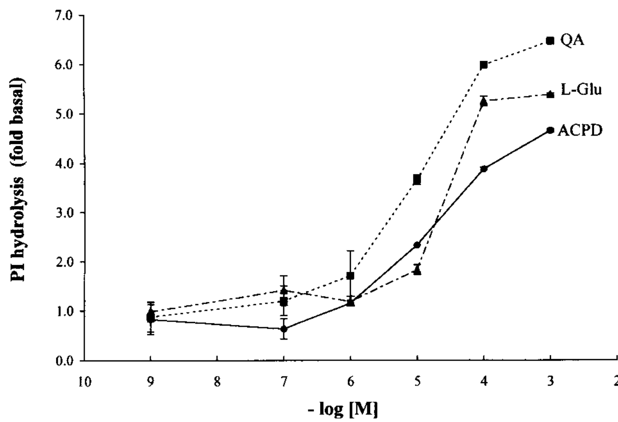

FIG. 1. Effect of mGluR agonists on PI hydrolysis in confluent astrocytes. Triplicate dishes of astrocytes were treated with various concentrations of the mGluR agonist L-Glu, QA, or ACPD for 1 h, in serum-free medium containing 10 m*M* lithium. PI hydrolysis was estimated from the accumulation of radiolabeled inositol phosphates in the aqueous phase of cell extracts. Data are mean ± SEM (bars) values from four independent experiments ($p < 0.05$).

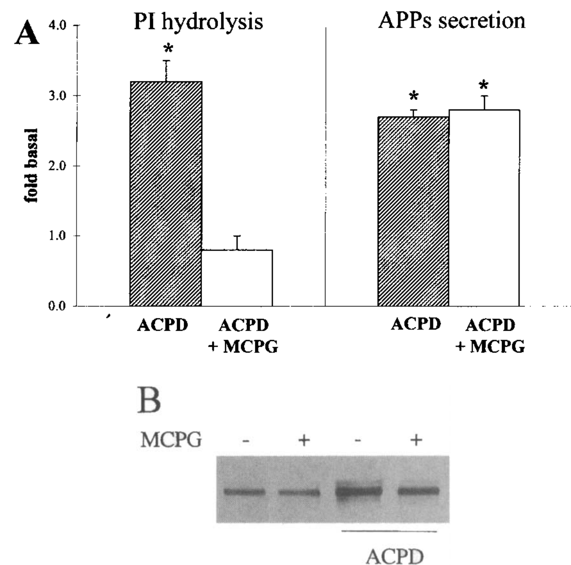

FIG. 3. Effect of MCPG on PI hydrolysis and APPs secretion caused by ACPD. **A:** PI hydrolysis and APPs secretion by astrocyte cultures prepared as in Fig. 1 were measured in dishes containing ACPD (100 μM), with or without MCPG (500 μM), or neither drug. Data are given ("fold basal") as the ratio of PI hydrolysis or APPs secretion in drug-treated dishes to that in dishes containing neither drug. Data are mean ± SEM (bars) values from three independent experiments. MCPG blocked the ACPD-induced increase in PI hydrolysis but not the increase in APPs secretion (*$p < 0.05$). **B:** Representative western blot using antibody 22C11 shows increased APPs secretion caused by ACPD, with or without MCPG.

concentrations of mGluR agonists failed to increase APPs secretion or PI hydrolysis. In confirmation of previous findings on hippocampal neurons (Lee et al., 1995*a*), activation of ionotropic glutamate receptors with *S*(−)-5-fluorowillardiine or NMDA failed to affect PI hydrolysis or APPs secretion.

Exposing astrocytes to the mGluR antagonist MCPG (500 μM) blocked the threefold increase in PI hydrolysis caused by 100 μM ACPD ($p < 0.05$) but not the stimulation of APP processing; APPs levels in the medium were increased in the presence of ACPD, with or without MCPG (Fig. 3). Another mGluR antagonist, L-AP3 (100 μM), had no significant effect on basal or stimulated PI hydrolysis but did completely suppress the stimulation of APPs secretion by 100 μM L-Glu, QA, or ACPD (Fig. 4; $p < 0.05$).

Effect of PKC and cAMP on APPs secretion

PMA (5 μM) mimicked the stimulatory effects of mGluR agonists, increasing APPs secretion by ~1.7-fold relative to that by untreated control cells ($p < 0.05$). The PKC inhibitor GF109203X inhibited the APPs secretion elicited by mGluR agonists or PMA (Fig. 5).

APPs secretion in response to mGluR agonists or to PKC activation by PMA (5 μM) was potently inhibited ($p < 0.05$) by exposing astrocytes to the membrane-permeant dBcAMP or to forskolin (both 100 μM). Although forskolin or dBcAMP occasionally did suppress basal APPs secretion, this inhibitory effect was not consistent in the absence of mGluR agonists or PMA (Fig. 6). The enhancement of PI hydrolysis by ACPD (100 μM) was partially but significantly suppressed by dBcAMP (100 μM) treatment, i.e., by ~20%; however, the production of ^{3}H-inositol phosphates remained significantly above baseline ($p < 0.05$).

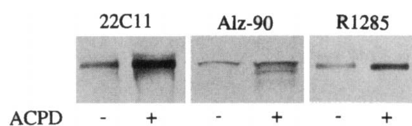

FIG. 2. Effect of ACPD on APPs secretion. APPs was detected on western blots by monoclonal antibody 22C11 or Alz-90 or antiserum R1285. The content of an ~100-kDa band was increased by ACPD stimulation of cortical astrocytes.

DISCUSSION

These data show that exposure of short-term astrocyte cultures to the metabotropic glutamate agonist L-Glu, QA, or ACPD promotes nonamyloidogenic APP processing and increases APPs secretion. L-Glu, QA, and ACPD also stimulated PI hydrolysis, suggesting that neurotransmitter agonists coupled to activation of the PI/PKC cascade promote constitutive APP processing and, possibly, decrease Aβ formation (Nitsch et al., 1992; Lee et al., 1995*a*; Wolf et al., 1995). In contrast, activation of ionotropic glutamate receptors by NMDA or *S*(−)-5-fluorowillardiine did not increase APPs secretion or PI hydrolysis. The regu-

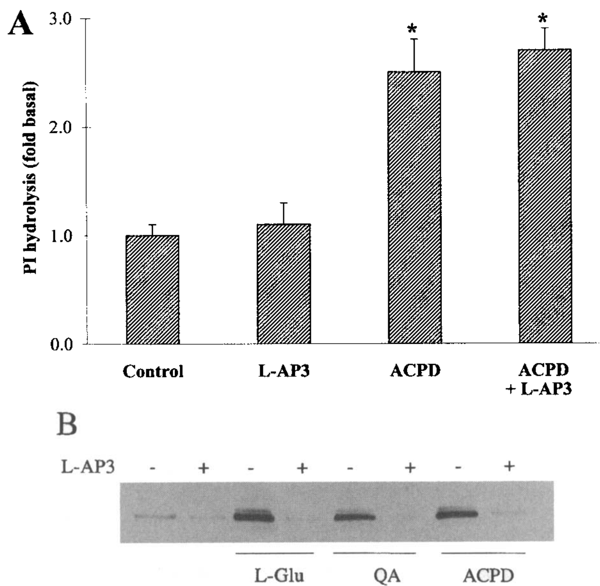

FIG. 4. Effect of L-AP3 on PI hydrolysis and APPs secretion caused by mGluR agonists. **A:** Inositol trisphosphate secretion by astrocyte cultures prepared as in Fig. 1 was measured in culture dishes containing ACPD (100 μM) with or without L-AP3 (100 μM) or neither drug (*$p < 0.05$). Data are mean ± SEM (bars) values. **B:** APPs secretion in culture dishes containing L-Glu, QA, or ACPD (all 100 μM) with or without the putative mGluR antagonist L-AP3 (100 μM). APPs was detected on western blots using antibody 22C11.

lation of APP processing by mGluRs but not ionotropic receptors in astrocytes is consistent with our previous observation that ion channels on neurons do not regulate APP processing (Lee et al., 1995*a*). Hence, increases in APPs secretion produced by the nonspecific glutamate agonists L-Glu and QA are probably not mediated by their actions on ionotropic glutamate receptors.

The nonselective antibody 22C11, which was used to quantify astrocytic APPs, also recognizes a highly homologous polypeptide, APLP2 (Slunt et al., 1994). We confirmed that the peptide secreted during stimulation by mGluR agonists was indeed APPs by using an antibody, Alz-90, and a polyclonal antiserum, R1285, that are directed at epitopes specific to the extracellular domain of APP (Haass et al., 1991; Storey et al., 1996). Immunoblotting with these three antibodies confirmed that mGluR activation increased APPs secretion.

The stimulatory effects of mGluR agonists on astrocytic APPs were suppressed by the PKC inhibitor GF109203X (Toullec et al., 1991) and mimicked by phorbol ester, suggesting that promotion of APP processing by mGluR agonists can involve PI/PKC signaling. Our data contradict the previous report that human astrocytes and glioma cells do not respond to PKC activation by increasing APPs secretion (Gabuzda et al., 1993) and suggest that the regulation of APP processing by phorbol ester may be specific for certain cell types. We previously showed that APPs secretion stimulated by PKC is associated with a decrease in the levels of cell-associated APP holoprotein and is not dependent on APP synthesis (Slack et al., 1993). Moreover, neurotransmitters typically increase APPs secretion from cells within 5–15 min of stimulation (Nitsch et al., 1992), suggesting that de novo APP synthesis is not required for increased APPs secretion. None of our drug treatments increased the levels of cell-associated APP in cultured astrocytes (authors' unpublished data). Because neurotransmitters coupled to cAMP but not to PKC activation stimulated APP synthesis in astrocytes (Lee et al., 1996), we suggest that the increase in APPs secretion following mGluR or phorbol ester treatment observed in our study results from proteolytic processing of preexisting APP and not from increased APP synthesis.

Although neurotransmitter agonists can stimulate the secretion of soluble and truncated forms of APP, e.g., APPs, cholinergic stimulation of primary bovine chromaffin cells resulted in the secretion of APP holoprotein (~130 kDa) (Efthimiopoulos et al., 1996*b*). In our study on astrocytes, the APP whose release is induced by mGluR agonists is probably a truncated APP fragment, i.e., APPs, and not full-length APP. On western blots, antibody 22C11 or Alz-90 and antiserum R1285 detected a protein band of ~100 kDa from the media that had faster mobility than full-length APP (~130 kDa) obtained from rat brain homogenates. Astrocytes express the larger APP transcripts, such as APP751 and APP770, in addition to the smaller APP695 isoform that is expressed by brain neurons (Haass et al., 1991). Therefore, APP holoprotein in the media of astrocytes might be expected to have slower mobility on western blots compared with APP

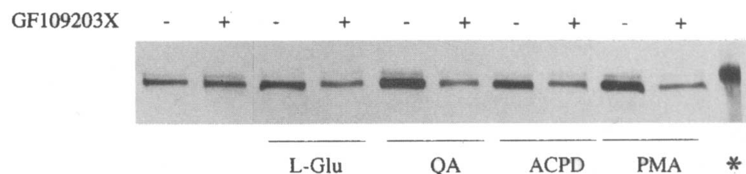

FIG. 5. Effect of PKC inhibition on the increase in APPs secretion caused by mGluR agonists or phorbol ester. APPs secretion from astrocytes prepared as in Fig. 1 was assayed by immunoblotting with antibody 22C11. Culture dishes contained the mGluR agonist L-Glu, QA, or ACPD (all 100 μM) or PMA (5 μM), with or without the PKC inhibitor GF109203X (2.5 μM). PKC inhibition blocked the increase in APPs secretion caused by the mGluR agonists or PMA. Note that truncated APPs had faster mobility, i.e., lower molecular weight, than full-length APP (*) obtained from rat brain.

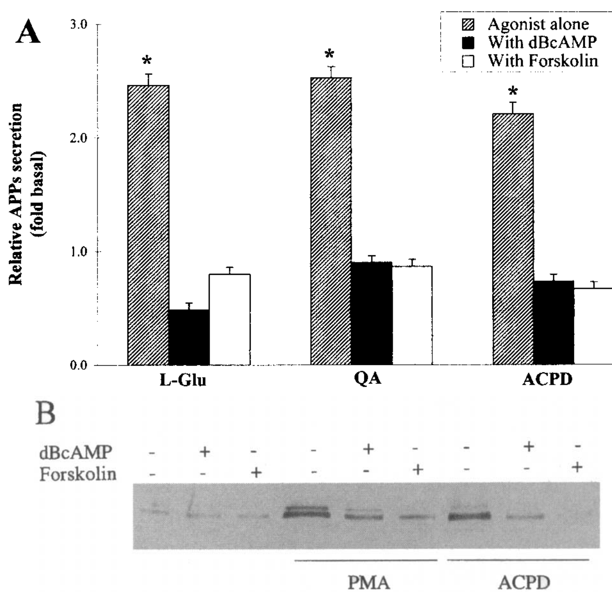

FIG. 6. Effect of dBcAMP or forskolin on APPs secretion caused by mGluR agonists or PMA. **A:** Cultured astrocytes prepared as in Fig. 1 were exposed to the mGluR agonist L-Glu, QA, or ACPD (all 100 μM), with or without dBcAMP and forskolin (both 100 μM). All three mGluR agonists significantly increased APP secretion as measured by western blots using antibody 22C11 (*$p < 0.05$), and this increase was blocked by dBcAMP or forskolin (both 100 μM). Data are mean ± SEM (bars) values accumulated from four independent experiments. **B:** Representative western blot of APP secretion in cells exposed to PMA or ACPD, with or without dBcAMP, or neither drug.

holoprotein from rat brains. However, this was not the case. APP secreted by astrocytes had faster mobility than APP holoprotein from rat brain, suggesting that PMA or mGluR agonists stimulated the release of C-terminal truncated forms of APP, i.e., APPs. Antibodies directed against the C-terminus of APP did occasionally detect low levels of APP in the media, suggesting that both APP holoprotein and APPs are released by cultured astrocytes (authors' unpublished data). However, the levels of APP bearing intact C-terminal fragments did not appear to be significantly altered by drug treatments, suggesting that mGluR or PKC activation did not increase the secretion of APP holoprotein. Thus, the increase in APP levels observed with mGluR or PKC activation most likely results from accelerated constitutive APP processing and increased APPs secretion.

L-Glu, QA, and ACPD act on various mGluR subtypes to generate various second messengers (Nakanishi, 1992). Glutamate-stimulated APP processing in astrocytes must involve other signal transduction systems independent of the PI/PKC signaling cascade, inasmuch as MCPG inhibited the increase in PI content caused by mGluR activation without suppressing the increase in APPs secretion. Also, the putative mGluR antagonist L-AP3 effectively inhibited the stimulation by mGluR agonists of APPs secretion but not of PI hydrolysis, indicating that PI hydrolysis and APPs secretion are not necessarily coupled in astrocytes. The dissociations between APPs secretion and PI hydrolysis suggest that the mGluR antagonists we have used may not be specific for the particular mGluRs that are coupled to PI hydrolysis (Manev et al., 1993). Alternatively, L-Glu, QA, and ACPD may be generating intracellular signals, e.g., phospholipase A_2, independent of the PI/PKC cascade, to increase APPs secretion (Emmerling et al., 1993).

An increase in number of β_2-adrenergic receptors coupled to cAMP formation has been detected in the postmortem AD brain (Kalaria et al., 1989). The effects of cAMP signaling on APP processing or synthesis are not known. Recently, Efthimiopoulos et al. (1996*a*) reported that elevations in intracellular cAMP level inhibit constitutive and phorbol ester-stimulated APPs secretion from C6 cells transfected with APP751. In contrast, Xu et al. (1996) showed that forskolin or activation of protein kinase A mimicked the action of phorbol esters, increasing APPs secretion from PC12 cells. The discrepant effect of cAMP signaling on constitutive APPs secretion has not been resolved.

In our study, dBcAMP or forskolin inhibited the increase in APPs secretion caused by mGluR agonists or phorbol ester. In both C6 cells (Efthimiopoulos et al., 1996*a*) and cortical astrocytes (Lee et al., 1996), the stimulation of cAMP production by the β-adrenergic agonist isoproterenol also opposed the stimulatory effect of phorbol ester, indicating that activation of receptors that elevate the intracellular cAMP level inhibits APPs secretion. The inhibitory effects of forskolin or dBcAMP on APPs secretion is not related to nonspecific toxicity, as neither drug had a significant effect on basal APPs levels. Furthermore, prolonged treatment (~24 h) of cortical astrocytes with dBcAMP or forskolin did not cause cell death but actually promoted the synthesis of APP mRNA and protein (Lee et al., 1996). Because PMA stimulation of APPs secretion was suppressed by dBcAMP or forskolin, cAMP signaling may act downstream of the PI/PKC cascade to inhibit APP processing. However, elevations in intracellular cAMP concentrations may also interfere with the formation of such second messengers as diacylglycerol and inositol trisphosphate, because dBcAMP partially suppressed the PI hydrolysis caused by ACPD treatment.

Astrocytes are a significant source of amyloid and can contribute to the neuropathology by up-regulating APP expression during neuronal injury (Siman et al., 1989). In addition to mGluR agonists, which increase APPs secretion, serotoninergic agonists, e.g., dexnorfenfluramine, a metabolite of the antiobesity drug dexfenfluramine, can also stimulate PI hydrolysis and APPs secretion in astrocyte cultures (authors' unpublished data) as has also been shown in 3T3 fibroblasts expressing 5-HT2a or 5HT2c receptors (Nitsch et al., 1996). Thus, activation of various cell surface recep-

tors on astrocytes can be used to promote nonamyloidogenic processing of APP.

In summary, the present study shows that enhancing glutamatergic transmission in astrocytes can accelerate APP processing to form APPs. Inasmuch as the neurodegeneration and synapse loss of AD may be associated with an up-regulation of astrocytic APP (Siman et al., 1989) and with decreased glutamate levels (Francis et al., 1993), glutamatergic agonists that increase PKC activation or inhibit cAMP formation may have therapeutic usefulness in AD by promoting nonamyloidogenic APP processing and improving synaptic transmission.

Acknowledgment: We are grateful to Alice Cox and Benson Yang for assistance and Dr. Wataru Araki for comments on the manuscript. This work was funded by grant MH-28783 from the National Institutes of Health and by The Center for Brain Sciences and Metabolism Charitable Trust.

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