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Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors

(phosphatidylinositol phosphate/protein kinase C/diacylglycerol/arachidonic acid/calcium)

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ABSTRACT **Stimulation of muscarinic m1 or m3 receptors can, by generating diacylglycerol and activating protein kinase C, accelerate the breakdown of the amyloid precursor protein (APP) to form soluble, nonamyloidogenic derivatives (APP_s), as previously shown. This relationship has been demonstrated in human glioma and neuroblastoma cells, as well as in transfected human embryonic kidney 293 cells and PC-12 cells. We now provide evidence that stimulation of metabotropic glutamate receptors (mGluRs), which also are coupled to phosphatidylinositol 4,5-bisphosphate hydrolysis, similarly accelerates processing of APP into nonamyloidogenic APP_s. This process is demonstrated both in hippocampal neurons derived from fetal rats and in human embryonic kidney 293 cells transfected with cDNA expression constructs encoding the mGluR 1α subtype. In hippocampal neurons, both an mGluR antagonist, L-(+)-2-amino-3-phosphonopropionic acid, and an inhibitor of protein kinase C, GF 109203X, blocked the APP_s release evoked by glutamate receptor stimulation. Ionotropic glutamate agonists, *N*-methyl-D-aspartate or *S*(−)-5-fluorowillardiine, failed to affect APP_s release. These data show that selective mGluR agonists that initiate signal-transduction events can regulate APP processing in bona fide primary neurons and transfected cells. As glutamatergic neurons in the cortex and hippocampus are damaged in Alzheimer disease, amyloid production in these regions may be enhanced by deficits in glutamatergic neurotransmission.**

The senile plaques found in Alzheimer disease (AD) are primarily composed of abnormal neurites and dystrophic terminals that surround an amyloid core. The major component of these plaques is an ≈4-kDa amyloid β-protein (Aβ), which resides within a much larger amyloid precursor protein (APP), encoded by a gene that maps to human chromosome 21 (1). APP is a transmembrane glycoprotein constitutively expressed in many types of mammalian cells. The long N terminus of APP extends extracellularly, whereas its short C-terminal region lies in the cytoplasm. Within APP, a single membrane-spanning region of 39–42 aa represents the amyloidogenic Aβ peptide (2).

Conventional proteolysis of APP involves proteolytic cleavage within Aβ at the cell surface and in the trans-Golgi network to preclude the formation of amyloidogenic Aβ peptides. Soluble APP (APP_s) fragments of ≈110–140 kDa are released into the extracellular medium, and the membrane-associated derivative is retained for subsequent cleavage and endocytotic processing (3–5). Overexpression of APP, as occurs in trisomy 21/Down syndrome, as well as inherited mutations of the APP gene can accelerate Aβ secretion and plaque formation (6). The *in vitro* expression of a double "Swedish" mutation is associated with an increase in Aβ secretion (7, 8). In high concentrations or in the presence of amyloidotrophic factors, these Aβ peptides can form insoluble aggregates that may be toxic to neurons (9–11).

Neurotransmitters can regulate APP processing to favor the secretion of APP_s (12). In human embryonic kidney (HEK) 293 cells stably expressing the human muscarinic receptor subtypes m1 or m3, stimulation with the muscarinic agonist carbachol increased APP_s secretion. The muscarinic receptors mediating this effect (m1 and m3 but not m2 or m4) are coupled to intracellular signaling pathways via second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$), which are generated by phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) hydrolysis. In other established cell lines, accelerated APP metabolism produced by direct stimulation of protein kinase C (PKC) or inhibition of phosphatase activity also increased APP_s secretion (13–16). In HEK 293 cells overexpressing m1 receptors, there was a reciprocal relationship between APP_s and Aβ secretion after receptor activation with carbachol (17); thus, receptor activation can enhance APP_s secretion and also suppress Aβ formation.

Abundant levels of both APP message and protein are present in neurons. The detection of APP_s in plasma and cerebrospinal fluid (18, 19) suggests that proteolysis of APP into soluble derivatives occurs in the central nervous system. Because receptor activation can regulate APP processing, impaired neurotransmission could conceivably exacerbate amyloid formation in AD, particularly in cortex and hippocampus. In addition to the loss of cholinergic basal forebrain neurons (20), glutamatergic corticocortical connections and major projections of the hippocampus are especially vulnerable to damage in AD (21). These pathways atrophy during the early stages of the disease, and in postmortem brain glutamate concentrations are decreased by as much as 80% (22). Because glutamate activates PKC in nervous tissue (23, 24), we tested the hypothesis that glutamate might divert APP processing from amyloidogenic pathways and, instead, favor increased APP_s secretion.

Metabotropic glutamate receptors (mGluR) are coupled to the formation of multiple second messengers via activation of phospholipase enzymes (23–25). Nonselective glutamate agonists [e.g., L-glutamate, quisqualate (QA)] can interact with both mGluR and ionotropic glutamate receptors (iGluR), but the selective mGluR agonist, *trans*-(*1S,3R*)-1-amino-1,3-cyclopentane dicarboxylic acid (ACPD) initiates signal transduction without affecting the iGluR. mGluR exist as seven subtypes and are categorized into three major groups on the

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Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; APP_s, soluble APP; DAG, diacylglycerol; $InsP_3$, inositol 1,4,5-trisphosphate; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; HEK, human embryonic kidney; mGluR, metabotropic glutamate receptor(s); iGluR, ionotropic glutamate receptor(s); $InsP_x$, inositol phosphates; mAb, monoclonal antibody; Aβ, amyloid β-protein; QA, quisqualate; ACPD, *trans*-(*1S,3R*)-1-amino-1,3-cyclopentane dicarboxylic acid; L-AP3, L-2-amino-3-phosphonopropionate; DEDA, 7,7-dimethyleicosadienoic acid; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate; *InsP*, inositol phosphate; NMDA, *N*-methyl-D-aspartate.

basis of sequence similarities, agonist selectivities, and signal-transduction characteristics. In cells expressing mGluR1α and mGluR5 (25, 26) and in mammalian brains (23, 24), L-glutamate, QA, and ACPD stimulated PtdInsP_2 hydrolysis and Ca^{2+} mobilization. ACPD treatment also evoked arachidonic acid release in brain slices (23, 24) and in cells expressing mGluR1α (25). The other five mGluR subtypes are coupled to cAMP and can be distinguished by agonist selectivities: mGluR2 and mGluR3 react with ACPD, whereas mGluR4, mGluR6, and mGluR7 are most responsive to 2-amino-4-phosphonobutyrate (27–29).

Muscarinic regulation of APP processing has been demonstrated in HEK 293 cells (12), human glioma and neuroblastoma cells, rat pheochromocytoma (PC-12) cells (13), and, more recently, NT2N neurons derived from a human teracarcinoma cell line (30). Although various mGluR subtypes, including those coupled to phospholipase activation, are expressed in the mammalian brain (31–33), it is not known whether mGluR can also regulate APP processing in normal neuronal tissue. In transfected cells, brain slices, and cultured cells, ACPD stimulates PtdInsP_2 hydrolysis to generate DAG and InsP_3 (23, 24); because both these second messengers can activate PKC, activation of PtdInsP_2-coupled mGluR would be predicted to promote conventional APP processing in normal brain tissue, as do m1 and m3 muscarinic receptors (12, 13).

To study the role of mGluR in APP processing, we measured the effects of glutamate agonists on APP_s levels in culture medium of fetal rat hippocampal neurons and of HEK 293 cells expressing the cDNA encoding mGluR1α (31). Both transfected and endogenous mGluR rapidly stimulated secretory APP processing and increased APP_s secretion into the medium.

MATERIALS AND METHODS

Subcloning and Expression of mGluR1α in HEK 293 Cells. To express the cDNA for mGluR1α in HEK 293 clones, cDNA coding for mGluR1α (from Shigetada Nakanishi, Kyoto University School of Medicine), was digested with *Not* I and *Sal* I (New England Biolabs), purified by agarose gel electrophoresis and silica gel extraction (Qiagen, Chatsworth, CA), and inserted into the linearized (*Not* I, *Sal* I) pBK-CMV phagemid vector (Stratagene) using T4 DNA ligase (GIBCO/BRL). The *Escherichia coli* strain XL1-Blue MRF′ (Stratagene) was transformed with DNA generated from the ligation reaction and grown overnight on agar plates containing kanamycin, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), and isopropyl β-D-thiogalactoside. White colonies were grown over night in liquid medium, and plasmid DNA was prepared by using the Wizard DNA purification resin (Promega). Identity of the purified expression plasmids was analyzed by restriction mapping. Plasmids containing both the 4.35-kb mGluR1α insert and the 4.5-kb pBK-CMV vector were used for subsequent transfections. HEK 293 cells were plated on poly(D-lysine)-coated culture dishes and grown to ≈70% confluence in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (GIBCO) supplemented with 10% fetal bovine serum. Cells were washed with serum-free medium and transfected with plasmid DNA at 5 μg per dish, using calcium precipitation of DNA followed by 15% (vol/vol) glycerol shock (34). Cells were grown for 48 hr in DMEM/F-12 supplemented with 10% fetal calf serum. Twelve hours before the experiments, growth medium was exchanged for glutamate- and serum-free DMEM. Experiments were done with serum-free medium. Undigested pBK-CMV without the mGluR1α insert was treated identically and used in parallel for control transfections.

Hippocampal Neuron Cultures. Dissociated hippocampal neurons from fetal rats were cultured as described (35) with minor modifications. Briefly, embryonic day-18 to -19 fetal pups were removed after pregnant rats were overdosed with ketamine. Dissected hippocampi were incubated with 0.25% trypsin in minimum essential medium (MEM; GIBCO) followed by light trituration with a Pasteur pipette to dissociate cells. After centrifugation, supernatant fluids were aspirated to prevent further digestion. The cell pellet was resuspended using a flame-narrowed Pasteur pipette, and fresh MEM/10% horse serum was added. Cells were plated on poly(L-lysine)-(Sigma) treated culture dishes (10,000 cells per cm^2). When the cells were well attached, the medium was replaced by MEM/5 mM glutamine/B27 components (GIBCO). Fetal bovine serum (5%) was added to promote neuronal growth, and cytosine arabinoside (5 μM) was used to arrest proliferation of nonneuronal cells. Cells were maintained for as long as 4–5 weeks in a humidified incubator (5% CO_2/95% air; 37°C). Immunocytochemical methods with cell-specific antibodies (neural cell typing set; Boehringer Mannheim) were used to determine neuronal-culture purity.

Pharmacological Manipulations. The following drugs were dissolved in serum-free medium: L-glutamate, QA, and ACPD; a glutamate antagonist [L-2-amino-3-phosphonopropionate (L-AP3)]; the ionotropic agonists, *S*(−)-5-fluorowillardiine or *N*-methyl-D-aspartate (NMDA).

EGTA was used to chelate extracellular Ca^{2+}. Phospholipase A_2 (PLA_2) inhibitors tested were quinacrine, manoalide, and 7,7-dimethyleicosadienoic acid (DEDA). Activation and inhibition of PKC were produced using phorbol 12-myristate 13-acetate (PMA) and GF 109203X (40), respectively. Stock solutions of manoalide, DEDA, PMA, and GF 109203X were dissolved in dimethyl sulfoxide. In experiments involving these drugs, an equivalent volume of vehicle was added to medium of control and agonist-treated groups. In experiments involving antagonists (L-AP3, GF 109203X, manoalide, DEDA, and quinacrine), cells were pretreated with the drug for 15 min. Medium containing the antagonist was aspirated and replaced with one containing both the antagonist and/or agonist. Cells were incubated with 1.5 ml of the test medium for 1 hr at 37°C in a 5% CO_2/air incubator. Experiments were replicated at least three times unless otherwise stated. All drugs were purchased from either Research Biochemicals (Natick, MA), Sigma, or LC Laboratories (Woburn, MA), and used in the micromolar range.

Measurement of APP_s Release. Phenylmethylsulfonyl fluoride was added to the collected medium, which was then applied to Sephadex G-25 desalting columns at 4°C and eluted with 4 ml of chilled water. Column eluates were frozen, dried by vacuum centrifugation, and resuspended in an extraction buffer (15). The amount of protein per culture dish was determined by the bicinchoninic acid protein assay. The amount of medium loaded for SDS/2% PAGE (Bio-Rad) was corrected for amount of protein per dish. To detect APP_s, proteins from gels were transferred to poly(vinylidene difluoride) membranes (Millipore), which were then immersed in Tris-buffered saline/0.05% Tween 20 (TBST) containing monoclonal antibody (mAb) 22C11 (Boehringer Mannheim) as the primary antibody. After overnight incubation, membranes were rinsed in TBST before being treated for 1 hr with a peroxidase-linked secondary antibody. An enhanced chemiluminescence method (Amersham) was used to visualize protein bands. Optical densities of the bands were quantitated by laser scanning densitometry (LKB). The amounts of APP_s released by different treatments were standardized by comparing them with release from a control group loaded onto the same blot.

mAb 22C11 (Boehringer Mannheim) recognizes an epitope on both the extracellular domain of full-length APP (2) and amyloid precursor-like protein 2 (36). To confirm that the peptide released into the medium is principally APP_s and not amyloid precursor-like protein 2, we also used mAb Alz-90 (Boehringer Mannheim). Immunoblotting with mAb 22C11

Table 1. Stimulation of APP_s release and PtdInsP_2 turnover by HEK 293 cells transfected with mGluR1α using the pBK-CMV phagemid vector

	Control		mGluR1α	
	Unstimulated	Glutamate	Unstimulated	Glutamate
[^{3}H]InsP_x, cpm	2220 ± 530 (4)	3100 ± 1100 (4)	6830 ± 2380† (6)	12,530 ± 4200*† (6)
APP_s, fold basal	1.00 ± 0.21 (3)	1.10 ± 0.28 (3)	1.76 ± 0.71 (5)	2.95 ± 1.96*‡ (5)

Control transfection was with vector alone. Glutamate was used at 500 μM. Data are means ± SD; *n*, number of independent experiments, is in parentheses.
*$P < 0.02$ vs. unstimulated cells transfected with mGluR1α.
†$P < 0.01$ vs. unstimulated control cells.
‡$P < 0.05$ vs. unstimulated control cells.

and mAb Alz-90 produced similar results (R.K.K.L., A.J.C., and R.J.W., unpublished data).

Measurement of PtdInsP_2 Hydrolysis. Inositol phospholipids were labeled with *myo*[2-^{3}H]inositol (DuPont/NEN) by incubating each dish of cells for 16–24 hr with 2 μCi (1 Ci = 37 GBq) in 2 ml of MEM. Before stimulation, cells were rinsed and incubated with serum-free MEM/10 mM LiCl for 30 min. After 1 hr, the stimulating medium was aspirated, and 1 ml of ice-cold methanol was squirted onto the cells. Cells were scraped and collected in 1 ml of chloroform and 0.5 ml of water. After being mixed, the suspension was centrifuged at 6000 × *g* for 10 min to increase phase separation. PtdInsP_2 hydrolysis was estimated from the total amount of [^{3}H]inositol phosphates ([^{3}H]InsP_x) in the aqueous phase of the cell extracts (37). One milliliter of sample was loaded onto columns containing AG1-X8 anion-exchange resin (Bio-Rad). Free [^{3}H]inositol was removed by washing the columns with water (1 ml six times). [^{3}H]InsP_x were then eluted with 4 ml of 1 M ammonium formate in 0.1 M formic acid. The radioactivity in samples was measured by using a scintillation counter.

Data Analysis. Measurements of PtdInsP_2 hydrolysis and APP_s release in different treatment groups were normalized against the control group. ANOVA and student's *t* tests were used to evaluate differences between groups (significance level, $P = 0.05$).

RESULTS

Glutamate Stimulation of PtdInsP_2 Turnover in and APP_s Release from HEK 293 Cells Transiently Expressing mGluR1α. After transient transfection of HEK 293 cells with cDNA expression constructs encoding mGluR1α, glutamate approximately doubled PtdInsP_2 turnover, as compared with that in unstimulated transfected cells, and caused an ≈4-fold greater stimulation in transfected than in control cells (Table 1; only a high, 500 μM concentration was tested). The latter difference partly reflected an ≈3-fold increase in InsP_x formation in unstimulated transfected cells, suggesting that expression of mGluR1α alone enhanced PtdInsP_2 turnover in the absence of exogenous glutamate (Table 1). This stimulation might be caused by release of endogenous glutamate (e.g., from glutamine or histidine), which were present in DMEM at 584 μg/ml and 42 μg/ml, respectively. Stimulation of the cells transiently expressing mGluR1α with 500 μM glutamate doubled the amount of APP_s recovered from the medium after 1 hr compared with that released from unstimulated cells (Table 1). Neither cells transfected with the control expression constructs nor the untransfected parent cell line responded to glutamate by increasing APP_s secretion.

Immunocytochemical Characterization of Primary Hippocampal Neurons. Immunocytochemical assay revealed that the primary hippocampal cultures contained >95% neurons. Of the neurons positive for the antineurofilament antibody, 90–95% resembled mature pyramidal neurons; the remainder were probably γ-aminobutyric acid-releasing hippocampal interneurons (35). Antibodies directed against glial fibrillary acidic protein and galactocerebroside, respectively, showed that the major contaminants in hippocampal cultures were astrocytes and oligodendrocytes (3–5%). Antifibronectin, directed against fibroblasts, identified <1% of the cells.

Glutamate Stimulation of Hippocampal Neurons. The effects of L-glutamate, QA, and ACPD, and various antagonists on APP_s secretion in hippocampal neurons is shown in Fig. 1. All three glutamate agonists stimulated APP_s release by ≈2.7-fold compared with baseline release. This release was blocked by L-AP3 or GF 109203X. PMA was as effective as L-glutamate, QA, or ACPD in stimulating APP_s secretion (Fig. 2), suggesting that the stimulatory effects of these agents were mediated, at least in part, by the phospholipase C (PLC)/PKC cascade. Because the nonselective mGluR agonists L-glutamate and QA also stimulated APP_s secretion, it seemed that ligand-gated channels might mediate L-glutamate effects on

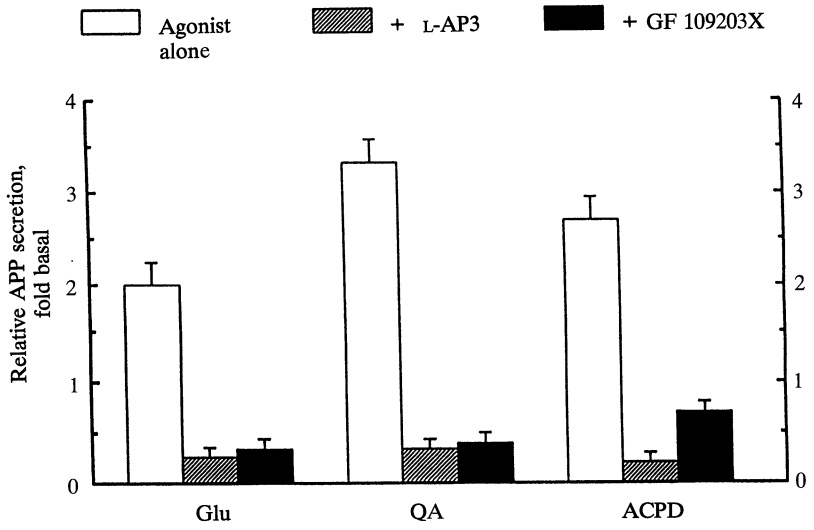

FIG. 1. Effects of mGluR agonists and antagonists on APP_s secretion. L-Glutamate (Glu), QA, and ACPD (all 10 μM) all significantly increased APP_s release (as detected by mAb 22C11) above control levels in hippocampal neurons ($P < 0.05$). L-AP3 (100 μM), a mGluR antagonist, and GF 109203X (2.5 μM), a PKC inhibitor, blocked these stimulatory effects ($P < 0.05$). APP_s levels in all groups were normalized to basal release by the control group (equal to a value of 1.0) within the same blot. Values are means and SEMs of three separate experiments done in triplicate.

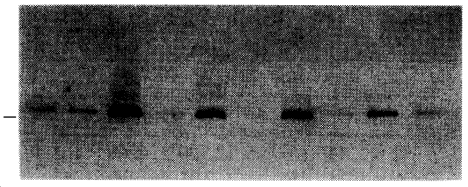

FIG. 2. PMA (1 μM) mimics L-glutamate (Glu), QA, and ACPD (all 10 μM) in stimulating APP_s secretion above control (Con) levels. Neurons treated with GF 109203X (2.5 μM) for 15 min before stimulation suppressed APP_s into the medium in response to PMA or agonists.

APP proteolysis. However, because neither NMDA nor *S*(−)-5-fluorowillardiine increased APP_s secretion across a range of concentrations (1 μM to 1 mM), this hypothesis seems unlikely.

Activation of glutamate receptors by L-glutamate, QA, or ACPD significantly increased PtdInsP_2 hydrolysis, such that [^{3}H]InsP_x levels were ≈1.8-fold those of controls (Table 2). L-AP3, which inhibited APP_s release, attenuated the increases in PtdInsP_2 hydrolysis caused by L-glutamate, QA, or ACPD, but PtdInsP_2 hydrolysis remained significantly above baseline levels. The PLA_2 inhibitors manoalide and DEDA consistently suppressed APP_s secretion; they also inhibited PtdInsP_2 hydrolysis, suggesting that they inhibit PLC activation, as well as that of PLA_2. Quinacrine did not block agonist-stimulated PtdInsP_2 hydrolysis or APP_s secretion (Table 2). Glutamate agonists continued to stimulate PtdInsP_2 hydrolysis in the presence of EGTA (5 μM); however, the effect was reduced compared with that accumulated when extracellular Ca^{2+} was present. EGTA also failed to block the effects of L-glutamate, QA, or ACPD on APP_s secretion.

DISCUSSION

These data show that mGluR activation can accelerate nonamyloidogenic APP processing in both hippocampal neurons derived from fetal rats and HEK 293 cells expressing transfected mGluR1α. It is possible that L-glutamate and QA, which are nonspecific glutamate receptor agonists, could enhance APP_s secretion by stimulating Ca^{2+} influx via iGluR. However, this seems unlikely inasmuch as selective stimulation of ionotropic NMDA and amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate receptors with NMDA and *S*(−)-5-fluorowillardiine, respectively, did not increase APP_s secretion, whereas ACPD, a selective mGluR agonist, had this effect (Fig. 1). The glutamate agonists that stimulated APP_s release also accelerated PtdInsP_2 turnover in both cell types. Furthermore, in cultured hippocampal neurons, all pharmacological agents that inhibited PtdInsP_2 hydrolysis also inhibited APP_s secretion. These observations extend earlier findings from established cell lines (12–17) that cell-surface receptors that regulate APP processing are coupled to PtdInsP_2 hydrolysis via GTP-binding proteins as part of their signal-transduction cascade. It is important to note that mAb/22C11, used to detect secreted APP_s, also shows low affinity for amyloid precursor-like protein 2 (36). However, because similar increases in APP_s release after mGluR activation were obtained with mAb Alz-90, an antibody specific for the APP molecule, the principal polypeptide detected in our experiments was probably APP_s.

Direct activation of PKC by PMA in hippocampal neurons mimicked the stimulation of APP_s release by L-glutamate, QA, or ACPD. This finding agrees with previous observations that second messengers generated by PtdInsP_2 hydrolysis, such as DAG and InsP_3/Ca^{2+} mobilization, increased APP_s secretion and decreased Aβ formation in various cell lines (12–17). The incomplete suppression of APP_s release by muscarinic agonists in transfected HEK 293 or CHO cells after PKC inhibition or down-regulation was interpreted as suggesting that PKC-independent pathways also regulate APP processing (38, 39). GF 109203X has been used to inhibit PKC in human platelets (40), Swiss 3T3 cells (39), and hippocampal cells (41). In our study, inhibition of PKC by GF 109203X blocked the ability of PMA and ACPD to stimulate APP_s release in hippocampal neurons, suggesting that PKC plays a major role in mGluR induction of APP_s secretion.

Regulation of APP_s secretion by mGluR in hippocampal neurons was sensitive to inhibition by the mGluR antagonist L-AP3. However, although L-AP3 usually blocks PtdInsP_2 hydrolysis in cortical and hippocampal slices (24), this drug did not suppress the PtdInsP_2 hydrolysis mediated by L-glutamate, QA, and ACPD in primary hippocampal cultures. The inability of L-AP3 to fully antagonize the metabotropic actions of glutamate has also been shown in other types of brain tissue and established cell lines (24–26). Recently, patch-clamp recordings of cultured hippocampal pyramidal neurons revealed a class of mGluRs that are insensitive to L-AP3 but are nevertheless coupled to PtdInsP_2 hydrolysis (42). This result suggests that in the mammalian brain, L-AP3 may act through an independent receptor to inhibit the actions of mGluR agonists. The ability of L-AP3 to suppress APP_s secretion without affecting PtdInsP_2 hydrolysis in our study might result from an action on an independent receptor or perhaps on events downstream to PtdInsP_2 hydrolysis. In support of this possibility, we observe that forskolin and dibutyryl cAMP can block APP_s release in glutamate-treated neurons without suppressing PtdInsP_2 hydrolysis (43). In neurons treated with either GF 109203X or L-AP3, APP_s release was reduced below baseline levels, suggesting that, in the control groups, low concentrations of endogenous glutamate may constitutively stimulate APP_s release.

Although both mGluR1α and mGluR5 are linked to PtdInsP_2 hydrolysis and expressed in rat hippocampus, mGluR1α immu-

Table 2. APP_s release and PtdInsP_2 hydrolysis in hippocampal neurons stimulated by L-glutamate, QA, and ACPD (all 10 μM)

	Pretreatment					
	None*	L-AP3	Quinacrine	Manoalide	DEDA	EGTA
APP release	2.7 ± 0.21†	0.5 ± 0.1‡	2.5 ± 0.1†	0.2 ± 0.02‡	0.3 ± 0.03‡	2.3 ± 0.2†
PtdInsP_2 hydrolysis	1.8 ± 0.12†	1.4 ± 0.1†‡	1.5 ± 0.2†	0.6 ± 0.1†‡	0.48 ± 0.1†‡	1.3 ± 0.1‡

Values are relative to control levels (equal to 1.0). ANOVA revealed that the three glutamate agonists did not differ with respect to stimulating APP_s secretion or PtdInsP_2 hydrolysis; hence, data from L-glutamate, QA, and ACPD studies are combined. The pooled means and SEMs are presented. The ability of the agonists to enhance APP_s release was blocked by L-AP3 (100 μM), manoalide (5 μM), and DEDA (100 μM). Manoalide, DEDA, and EGTA (5 μM) also blocked the agonist effects on PtdInsP_2 hydrolysis, whereas L-AP3, quinacrine (10 μM), and EGTA reduced these effects.

*Neurons were incubated with L-glutamate, QA, or ACPD.

†Value is significantly different from control ($P < 0.01$).

‡Value is significantly different from agonist alone ($P < 0.01$).

noreactivity is detectable in <1% of cultured hippocampal neurons and in only a subpopulation of γ-aminobutyric acid-releasing interneurons (44). mGluR5, on the other hand, is highly expressed in hippocampal pyramidal cells and is coupled to PtdInsP_2 hydrolysis via Ca^{2+}-insensitive phospholipase C (26). mGluR5 may thus be responsible for mediating PtdInsP_2 hydrolysis and APP_s secretion in hippocampal neurons.

Activation of mGluR may lead to the activation of other phospholipases besides phospholipase C–e.g., PLA_2, which could hydrolyze membrane phospholipids to produce arachidonic acid. In CHO cells transfected with m1 muscarinic receptor subtypes, activation of PLA_2 by melittin increased APP_s secretion (45). In transfected cells and brain slices, mGluR are coupled to arachidonic acid production via PtdInsP_2-independent pathways as well as by InsP_3-mediated elevations in intracellular Ca^{2+} (25, 46). Because arachidonic acid can act by itself or synergistically with DAG to stimulate PKC (47), PLA_2 activation may also affect APP proteolysis. Exposure of our hippocampal neurons to the PLA_2 inhibitors manoalide and DEDA suppressed APP_s formation in agonist-treated cells. However, the inhibitory effect of manoalide and DEDA may be due to their inhibitory action on PtdInsP_2 hydrolysis. Quinacrine appeared to be a more specific PLA_2 inhibitor in hippocampal neurons, as it did not interfere with PtdInsP_2 hydrolysis. The stimulatory effects of glutamate agonists on APP_s release were not blocked by quinacrine, suggesting that PLA_2 is not significantly involved in regulating APP processing in hippocampal neurons. In confirmation, addition of EGTA, which chelates extracellular Ca^{2+}, also failed to block glutamate-induced increases in APP_s secretion. Because extracellular Ca^{2+} is required for PLA_2 activation (48, 49), these data support the view that phospholipase C products, and not arachidonic acid, are primarily responsible for the mGluR effects.

This study demonstrates that neuronal glutamate receptors are coupled to APP proteolysis and APP_s secretion, and that activation of neurotransmitter receptors can enhance APP_s release in mammalian neurons, as well as in transfected cell lines. Experiments in which both PtdInsP_2 hydrolysis and APP_s secretion were measured showed that glutamate agonists stimulate PtdInsP_2 hydrolysis at concentrations that also increase APP_s release. Secretory processing of APP by mGluR may be mimicked by phorbol esters and blocked by PKC inhibitors, suggesting that second messengers generated by PtdInsP_2 hydrolysis mediate APP_s release via PKC activation.

In AD brains, glutamatergic transmission is severely altered by early degeneration of corticocortical connections and hippocampal projections (21). Because both of these regions accumulate amyloid and are components of neural systems involved in learning and memory, the decrease in glutamatergic signaling may contribute to the accumulation of amyloid plaques and, secondarily, to memory dysfunction and progressive dementia.

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