MITOGEN-ACTIVATED PROTEIN KINASE IS INVOLVED IN N-METHYL-D-ASPARTATE RECEPTOR REGULATION OF AMYLOID PRECURSOR PROTEIN CLEAVAGE

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Abstract—Glutamate is the principal excitatory neurotransmitter in the mammalian brain. Several lines of evidence suggest that glutamatergic hypoactivity exists in the Alzheimer’s disease brain, where it may contribute to both brain amyloid burden and cognitive dysfunction. Although metabotropic glutamate receptors have been shown to alter cleavage of the amyloid precursor protein, little attention has been paid to the role of N-methyl-d-aspartate receptors in this process. We now report that activation of N-methyl-d-aspartate receptors in transiently transfected human embryonic kidney 293 cells increases production of the soluble amyloid precursor protein derivative. Moreover, using both pharmacological and gene transfer techniques, we show that this effect is largely due to activation of the mitogen-activated protein kinase cascade, specifically the pathway leading to activation of extracellular signal-regulated protein kinase but not other mitogen-activated protein kinases.

These observations further our understanding of the pathways that regulate amyloid precursor protein cleavage, and buttress the notion that regulation of amyloid precursor protein cleavage is critically dependent upon the mitogen-activated protein kinase cascade.

Key words: Alzheimer’s disease, APP, sAPP, MAPK, NMDA.

Several lines of evidence suggest that glutamate plays a role in the pathophysiology of Alzheimer’s disease. Corticocortical connections and the major projection pathways of the hippocampus utilize glutamate as a neurotransmitter and degenerate early in the disease process,15 and post mortem analysis reveals that glutamate concentrations are significantly decreased in the brains of Alzheimer’s disease patients.24 Because these brain regions accumulate amyloid deposits and are intimately involved in learning and memory,45 it has been suggested that glutamatergic hypoactivity may contribute both to increased brain amyloid burden and memory dysfunction.33 Finally, glutamate receptors are heavily implicated in the molecular mechanisms underlying cognitive function, in particular long-term potentiation, the premier cellular model of learning.52 For these reasons, investigating the role of glutamate receptors in the regulation of amyloid precursor protein (APP) processing43 is of significant theoretical and clinical importance.

Glutamate is the principal excitatory neurotransmitter in the mammalian brain. Glutamate acts upon both ionotropic receptors, which mediate transmembrane ion fluxes, and G-protein-coupled receptors, which initiate cascades leading to activation of intracellular effectors.46,56 Glutamatergic G-protein-coupled receptors linked to the phospholipase C/protein kinase C (PKC) signaling system regulate release of the soluble N-terminal ectodomain of APP (sAPP).27,32,33,49,61 These findings are in keeping with previous data demonstrating that activation of serotonergic or cholinergic G-protein-coupled receptors increases sAPP production and concomitantly decreases β-amyloid production.4,23,33,48,49,60,66

Less attention has been paid to the role of ionotropic glutamate receptors in the regulation of APP cleavage. Among the ionotropic glutamate receptors, the N-methyl-d-aspartate (NMDA) receptor is unique in that it is highly permeable to Ca2+.40 Because Ca2+-dependent regulation of APP cleavage has been demonstrated in a variety of cell lines,4,50–52 we reasoned that activation of NMDA receptors might result in altered cleavage of APP. Indeed, stimulation of Ca2+-permeable nicotinic receptors increases sAPP release from PC12 cells.26

Ca2+ regulation of sAPP release has been shown to occur in a PKC-independent manner,4 possibly via activation of tyrosine kinases,50 but the signaling pathway mediating this effect has not been well characterized. The mitogen-activated protein kinase (MAPK) pathway, involving sequential activation of p21Ras, Raf, mitogen-activated protein kinase kinase (MEK) and an MAPK family member known as extracellular signal-regulated protein kinase (ERK), is a likely candidate as it is activated by Ca2+.54,55 regulates APP cleavage in both a PKC-dependent and -independent manner,11,22,42 and requires activation of tyrosine kinases. For these reasons, we hypothesized that (i) stimulation of NMDA receptors regulates APP processing, and (ii) ERK is required for NMDA receptor-mediated regulation of APP cleavage. In order to test these hypotheses, we transiently transfected human embryonic kidney 293 (HEK 293) cells with NMDA receptors and inhibited the MAPK pathway using either PD 98059, a selective inhibitor of MEK1,1,12,31 or transient expression of a kinase-dead MEK1 mutant.57

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Abbreviations: APP, amyloid precursor protein; APV, N-methyl-2-amino-5-phosphonovalerate; ERK, extracellular signal-regulated protein kinase; HEK, human embryonic kidney; JNK/SAPK, c-Jun N-terminal kinase stress-activated protein kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; NMDA, N-methyl-d-aspartate; PKC, protein kinase C; sAPP, soluble ectodomain of APP.
EXPERIMENTAL PROCEDURES

Cell culture and transfections

HEK 293 cells were cultured in minimal essential medium supplemented with 10% fetal calf serum, as described previously. 42 Fully functional NMDA receptors having a high Ca<sup>2+</sup> conductance are found by the expression of both NMDA receptor subunits NR1 and NR2A. 53 Therefore, HEK 293 cells were transiently transfected with pCMV695 (an expression vector for APP<sub>695</sub>) 58, pCDNANR1 and pCDNANR2A (expression vectors for the NMDA receptor subunits), and either pCDNAN97A (an expression vector for kinase-inactive MEK) or vector expressing bacterial β-galactosidase (Clontech Laboratories) using a high-efficiency calcium phosphate transfection protocol. Average transfection efficiency was assessed by staining for β-galactosidase according to the method of Raymond <i>et al.</i> 53 At the end of the 12-h incubation with the DNA-containing solution, cells were washed and 2 × 10<sup>6</sup> cells were replated on to poly-d-lysine precoated 60-mm dishes in complete media. 1,2-Amino-5-phosphonovalerate (APV; 1 mM) was added to the media to prevent excitotoxicity due to chronic activation of NMDA receptor channels by low levels of aspartate, glutamate and glycine in the serum-containing media. 53 Approximately 16 h before stimulation, cells were exposed to culture media containing 1 mM APV and 10% charcoal-inactivated calf serum. At the end of this period, HEK 293 cells were exposed to drugs in Ringer’s solution containing (in mM): 140 NaCl, 5.4 KCl, 1.4 CaCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 21 glucose and 26 NaHCO<sub>3</sub>. Media for control cultures contained 1 mM APV and 1 mM Mg<sup>2+</sup>. 52 to ensure that NMDA receptors were completely inactive following overexpression, 53 while stimulated cultures contained the NMDA receptor co-agonists NMDA (100 μM) and glycine (50 μM). PD 98059 was diluted from a 10 mM stock made up in dimethyl sulfoxide.

Quantification of the secreted form of amyloid precursor protein in culture media

Following drug exposure, media were centrifuged for 10 min at 16 000 × g to remove cellular debris. Media were subsequently desalted and concentrated by centrifugation in the presence of protease inhibitors (17 μg/ml phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin, 10 μg/ml aprotinin and 2 μg/ml pepstatin) according to the method of Mills and Reiner. 42 APV was detected by western blot analysis using an anti-APP N-terminal antibody (anti-PreA4 monoclonal antibody; Boehringer Mannheim) or WO-2, a monoclonal antibody generated against the first 16 amino acids of the N-terminal region of β-amyloid, 42 as described previously. 42 The anti-PreA4 monoclonal antibody (22C11) detects secreted APP and amyloid precursor-like protein, while WO-2 is selective for sAPP. Densitometric measurements for each trial were taken from the same blot: each trial represents one transfection and cell plating. Following densitometric analysis, a one-way ANOVA followed by Fisher’s post hoc analysis was used to determine the significance of observed differences. All of the data were scaled to the average of the control and have been expressed as mean ± S.E.M.

Western blots of mitogen-activated protein kinases, N-methyl-d-aspartate and cellular amyloid precursor protein

Cells were lysed in an extraction buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 4 mM P-nitrophenylphosphate and 1 mM sodium vanadate, and the lysate was briefly sonicated on ice. Cellular protein (25 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12.5% low bis (acrylamide : bis ratio 118.5:1) mini gels for western blots of ERK, MEK, c-Jun N-terminal kinase stress-activated protein kinases (JNK/SAPKs), p38/HOG1, APP or NR1. Following gel electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane and probed using an antibody specific for phosphorylated ERK (1:1000; New England Biolabs), phosphorylated p38/HOG1 (1:500; phospho-p38, New England Biolabs), phosphorylated JNK/SAPK (1:1000; phospho-JNK/SAPK, New England Biolabs), APP (1:200; anti-PreA4 monoclonal antibody, Boehringer Mannheim) or NR1 (1:500; anti-rat NR1, CT, Upstate Biotechnology). Western blots of cell lysates, taken from the same trial in which sAPP was measured, were run as markers, were run as markers, were run as markers, were run as markers, were run as markers, or were run as markers and are representative of three to five separate trials. Representative western blots of secreted samples and cellular samples do not always correspond to the exact same trial.

Kinase-inactive mitogen-activated protein kinase kinase antagonizes N-methyl-d-aspartate receptor stimulation of soluble amyloid precursor protein release

Overexpression of the K97A MEK mutant has proven to be a powerful tool for studying the role of the MAPK signaling pathway in various cellular processes. This kinase-inactive mutant, when overexpressed, acts in a “dominant negative” fashion, inhibiting stimulation of endogenous MEK and its downstream substrate ERK. 57 Stimulation of sAPP was measured in HEK 293 cells transiently expressing the NMDA receptor and APP<sub>695</sub> together with either the dominant negative MEK mutant or vector expressing β-galactosidase. Two sAPPs were recognized by either 22C11 or WO-2 (Fig. 2A), indicating that they result from α-secretase cleavage and are most likely due to variable post-translational modification. 59 Densitometric analysis revealed that levels of sAPP in the culture media during a 15-min exposure period increased significantly in the presence of 100 μM NMDA as detected by either 22C11 or WO-2 (1.00 ± 0.10 and 1.98 ± 0.20 or 1.00 ± 0.18 and 1.81 ± 0.34, respectively; n = 5, P < 0.05; Fig. 1A). This increase was significantly inhibited in the presence of the kinase-dead MEK1 mutant K97A as compared with vector alone (22C11: 1.98 ± 0.20 and 1.15 ± 0.21; WO-2: 1.81 ± 0.34 and 1.09 ± 0.15; n = 5, P < 0.05; Fig. 1A). Constitutive release of sAPP from HEK 293 cells transfected with cDNA expression constructs encoding β-galactosidase or K97A were not significantly different from one another. Transfection efficiency, as measured by β-galactosidase staining, was 66 ± 4 (n = 4).

N-Methyl-D-aspartate receptor stimulation increases extra-cellular signal-regulated protein kinase phosphorylation but not other family members: antagonism by a kinase-inactive mutant

NMDA receptor stimulation of ERK activity has been shown previously to be inconsistent. 2,14,62,67 To ensure that NMDA receptor stimulation increased ERK activation, 5 we utilized a phospho-specific ERK antibody to probe cell lysates from each trial via western blot analysis. Immunoreactivity of phospho-ERK1 and phospho-ERK2 were both increased by NMDA receptor stimulation, and this increase was inhibited by K97A overexpression (Fig. 1B). Basal levels of phospho-ERK activity also appeared to be increased by expression of the NMDA receptor (this change in ERK activity is unlikely to be caused by activation of NMDA receptors by release of endogenous glutamate, as the control experiments were carried out in the presence of the NMDA antagonists APV and Mg<sup>2+</sup>). Overexpression of the K97A mutant was confirmed using a rabbit polyclonal antibody raised against the N-terminal of MEK1 (Upstate Biotechnology; Fig. 1B). Cellular expression levels of either APP<sub>695</sub> or the NMDA receptor were not affected by overexpression of the dominant negative MEK1 mutant (Fig. 1B).

The mammalian MAPK family members have been expanded to include JNK/SAPK 0.30 and p38/HOG1. 21 Moreover, activation of JNK/SAPK or p38/HOG1 has been shown to occur following receptor-mediated rises in intracellular Ca<sup>2+</sup> in several cell types. 25,29,44,55,64 Notably, activation of JNK/SAPK or p38/HOG1 did not appear to be increased by
NMDA receptor stimulation, even though the same lysates contained elevated ERK activity (Fig. 1B).

Pharmacological inhibition of mitogen-activated protein kinase kinase antagonizes N-methyl-D-aspartate receptor stimulation of amyloid precursor protein release

Inhibition of NMDA receptor stimulation of ERK using the
MEK1 antagonist PD 98059 complemented the results obtained using the kinase-dead MEK mutant. Specifically, NMDA receptor stimulation of sAPP release was antagonized in the presence of 50 μM PD 98059 (3.49 ± 0.48 and 1.62 ± 0.42; n = 3, P < 0.05; Fig. 2A). Constitutive sAPP release in the presence of this same concentration of PD 98059 did not differ from vehicle alone. Western blots of the cell lysate from each trial, probed using a phospho-specific MAPK antibody, indicate that ERK1 and ERK2 were both increased by NMDA receptor stimulation, and this increase was antagonized in the presence of 50 μM PD 98059 (Fig. 2B). These changes were not due to altered cellular expression levels of either APP965 or the NMDA receptor, as seen in western blots run in parallel (Fig. 2B).

**DISCUSSION**

We provide evidence that NMDA receptor stimulation promotes α-secretase processing of APP and that the MAPK pathway is involved in this regulation. Specifically, pharmacological and molecular inhibition of MEK1 antagonized NMDA receptor stimulation of both sAPP release and ERK activity. These results extend an earlier study from our laboratory showing that MAPK is necessary for PKC and growth factor regulation of APP catabolism. Our findings indicate that the MAPK pathway also underlies NMDA receptor regulation of APP processing and complements a recent finding indicating that MAPK is involved in muscarinic receptor-mediated release of sAPP.

Regulation of APP processing by the neurotransmitter glutamate has been observed in cell lines, primary neuronal cultures and rat brain slices. These studies have emphasized the role of the metabotropic glutamate receptor in regulating sAPP production, and when examined, have found that the NMDA receptor was not involved. However, these experiments were carried out using concentrations of Mg2+ that would be expected to result in voltage-dependent blockade of NMDA receptors. Moreover, in the presence of 0.2 mM Mg2+, NMDA receptor stimulation of primary rat cortical cultures does not increase MAPK activity, consistent with our observation that the effects of NMDA receptor stimulation upon sAPP production are MAPK dependent.

Given that the NMDA receptor is a ligand-regulated Ca2+ channel, it is likely that NMDA receptor-dependent changes in sAPP production are due to changes in intracellular Ca2+. Indeed, Ca2+-dependent regulation of APP processing has been amply demonstrated, although the results are somewhat contradictory. For example, blocking Ca2+ re-uptake with thapsigargin has varied effects on sAPP release, and increasing intracellular Ca2+ with inositol 1,4,5-trisphosphate, A23187 or caffeine produces differing effects upon β-amyloid release. However, the present data are congruent with an earlier study indicating that stimulation of nicotinic receptors, which also function as ligand-gated Ca2+ channels, regulates APP processing.

MAPKs are central transducers of a wide variety of cellular signals, including those mediated by neurotransmitters and Ca2+. Several different MAPK pathways are now known to exist, including ERKs, JNK/SAPK and p38/HOG1. Traditionally, these kinases have been categorized as those activated during differentiation (ERKs) or those activated by stress (JNK/SAPK and p38/HOG1). While selective activation of MAPKs by extracellular signals does not always occur, our data clearly demonstrate that NMDA receptor activation selectively activates ERK, arguing for a discrete mechanism of NMDA receptor regulation of ERK in these cells. The mechanism may be mediated by a rise in intracellular Ca2+, which has been shown to activate the small guanine nucleotide-binding protein p21Ras, in turn leading to activation of the MAPK cascade. However, because we have not formally demonstrated that Ca2+ is required for the effects we have observed, it remains possible that NMDA receptors may directly couple to signaling proteins upstream of ERK activation, thereby altering APP cleavage. Distinguishing between these possibilities remains a challenge for future studies.

**CONCLUSIONS**

The biological activities of sAPP have been shown to include promotion of neuronal cell survival, adhesive interactions, neurite outgrowth, synaptogenesis and synaptic plasticity. sAPP has been shown to regulate intraneuronal Ca2+, an effect which may underlie some of its purported physiological roles. In particular, sAPP decreases influx of Ca2+ via NMDA receptor-gated channels and voltage-operated calcium channels. Thus, NMDA receptor regulation of sAPP release may provide a negative feedback mechanism for decreasing NMDA receptor-mediated Ca2+ influx. Given the potential role played by the NMDA receptor and APP in synaptic plasticity and cognitive function, such a hypothesis is of particular relevance to the pathophysiology of Alzheimer’s disease.

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