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GLUTAMATE STIMULATES GLUTAMATE RECEPTOR INTERACTING PROTEIN 1 DEGRADATION BY UBIQUITIN-PROTEASOME SYSTEM TO REGULATE SURFACE EXPRESSION OF GluR2

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Abstract—The glutamate receptor interacting protein 1 (GRIP1) is a scaffolding protein in postsynaptic density (PSD), tethering AMPA receptors to other signaling proteins. Here we report that glutamate stimulation caused a rapid reduction in protein levels of GRIP1, but not that of glutamate receptor (GluR) 1, GluR2 and protein interacting with C kinase 1 (PICK1) in rat primary cortical neuron cultures. Down-regulation of GRIP1 by glutamate was blocked by carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), a proteasome inhibitor and by expression of K48R-ubiquitin, a dominant negative form of ubiquitin. The GRIP1 reduction was inhibited by MK-801, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, but not by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor antagonist. EGTA and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra acetic acid tetrakis (BAPTA), two Ca^{2+} chelators, but not nifedipine, an L-type Ca^{2+} channel blocker, prevented GRIP1 degradation. Furthermore, MG132 prevented glutamate-stimulated reduction in surface amount of GluR2, and knockdown of GRIP1 by RNAi against GRIP1 reduced surface GluR2 in neurons. Our results suggest that glutamate induces GRIP1 degradation by proteasome through an NMDA receptor–Ca^{2+} pathway and that GRIP1 degradation may play an important role in regulating GluR2 surface expression.

Key words: GRIP1, degradation, glutamate, AMPA receptor, UPS, GluR2.

The remodeling of synapses is a fundamental mechanism for information storage and processing in the brain (Luscher et al., 2000; Muller et al., 2002). Much of this remodeling occurs at the postsynaptic density (PSD), a specialized biochemical apparatus containing glutamate receptors and associated scaffolding proteins that organize signal molecules at the postsynaptic membrane (Tomita et al., 2001; Sheng and Kim, 2002).

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Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra acetic acid tetrakis; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; GluR, glutamate receptor; GRIP1, glutamate receptor interacting protein 1; HRP, horseradish peroxidase; LTD, long term depression; MDC, monodansylcadaverine; MG132, carbobenzoxyl-leucinyl-leucinyl-leucinal; NMDA, *N*-methyl-D-aspartate; PDZ domain, PSD95/DglA/ZO-1-like domains; PI, propidium iodide; PICK1, protein interacting with C kinase 1; PSD, postsynaptic density; RT, reverse transcription; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin; UPS, ubiquitin–proteasome system.

The glutamate receptor interacting protein 1 (GRIP1), a scaffolding protein in PSD with seven PSD95/DglA/ZO-1 (PDZ) domains, was initially identified by its interaction with the C-terminus of glutamate receptor (GluR) 2 and GluR3 subunits of AMPA receptors (Dong et al., 1997). Knockdown of GRIP1 in hippocampal neurons suppresses dendritic formation (Hoogenraad et al., 2005). At glutamatergic synapses in brain, synapse modifications can be expressed as a change in the number of postsynaptic AMPA receptors (Barry and Ziff, 2002; Contractor and Heinemann, 2002; Malinow and Malenka, 2002; Narisawa-Saito et al., 2002; Bredt and Nicoll, 2003), but the mechanism by which glutamate controls surface amount of AMPA receptors is incompletely understood. It has been shown that GRIP1 binding to GluR2 is important for surface accumulation of AMPA receptors, suggesting that GRIP1 is critical for maintaining synaptic accumulation of GluR2, possibly by limiting its endocytosis (Osten et al., 2000; Braithwaite et al., 2002; Steiner et al., 2005). However, the mechanism by which GRIP1 affects surface amount of GluR2 in response to glutamate remains unknown.

The ubiquitin–proteasome system (UPS), a complex known to mediate protein degradation, stimulated by synaptic activity affects composition of postsynaptic proteins (Ehlers, 2003). It has been shown that proteasome inhibitors prevent agonist-induced AMPA receptor internalization and synaptically induced-long term depression (LTD) (Colledge et al., 2003; Patrick et al., 2003; Bingol and Schuman, 2004), indicating that UPS-mediated protein degradation plays a role in regulating the number of synaptic AMPA receptor. Further, subcellular distribution of AMPA receptor is rapidly regulated by glutamate in cultured neurons (Nusser, 2000; Ye et al., 2000). We thus asked whether glutamate induces GRIP1 degradation by UPS to regulate redistribution of AMPA receptor. In this report, we provided evidence to support a notion that *N*-methyl-D-aspartate (NMDA) receptor-mediated and Ca^{2+}-dependent GRIP1 degradation by UPS may be a new mechanism by which glutamate regulates surface amount of AMPA receptor.

EXPERIMENTAL PROCEDURES

Materials and reagents

Rabbit polyclonal antibodies against GRIP1 (1:1000, cat. #06-986); GluR2 (1:1000, cat. #06-307); protein interacting with C kinase 1 (PICK1) (1:500, cat. #07-293) and NR2B(1:1000, cat. #06-600) were obtained from Upstate Biotechnology (Lake Placid,

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NY, USA); mouse monoclonal antibody against ubiquitin (1:500, cat. #sc-8017), rabbit polyclonal antibodies against actin (1:1000, cat. #sc-10731) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal antibody against GRIP1 (1:500, cat. #611318) from BD Biosciences Pharmingen (San Diego, CA, USA); rabbit polyclonal antibody against synapsin1 (1:1500, cat. #AB1543) and mouse monoclonal antibody against PSD-95 (1:500, cat. #MAB1596) from Chemicon (Temecula, CA, USA). Secondary antibodies conjugated with Texas Red; Alexa Fluor 488 and Alexa Fluor 546 against mouse or rabbit IgG were from Molecular Probes (Eugene, OR, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibodies were from Amersham (Piscataway, NJ, USA). Glutathione-agarose immobilized GST-S5a (UW 8635) was from Biomol (Plymouth Meeting, PA, USA). Purified ubiquitin (U6235) and all other reagents were from Sigma (St. Louis, MO, USA).

Primary neuron cultures and treatment

The primary cortical neurons were obtained by trypsinization of dissected cortex from 18-day-old fetal S.D. rats (Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China) under ethyl anesthesia following the animal protocol approved by the animal committee. All animal experiments were conducted in accordance with the Chinese Academy of Science guidelines for the care and use of laboratory animals and approved by the institutional animal care and use committee at the Institute of Neuroscience. Every effort was made to minimize the number of animals used for the experiments and to reduce their suffering. In brief, the cells were plated at 1.0×10^6 cells/ml on poly-D-lysine-coated dishes in minimal essential medium (MEM) with high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% horse serum, 50 U/ml penicillin and 50 μg/ml streptomycin. After 3 days, 15 mg/ml 5-fluoro-2-deoxyuridine and 35 mg/ml uridine were added to inhibit non-neuronal cell growth. The neurons were used for experiments at 10–13 days *in vitro* (DIV10-13). For neuron stimulation, cultures were bath-incubated with glutamate (50 μM), AMPA (100 μM), NMDA (30 μM) or ionomycin (10 μM) solutions. Carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) (20–50 μM), epoxomicin (10 μM), E64d (10 μM), MK-801 (20 μM), APV (20 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (100 μM), tetrodotoxin (TTX) (1 μM), EGTA (2 mM), 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra acetic acid tetrakis (BAPTA) (200 μM), nifedipine (5 μM), monodansylcadaverine (MDC) (100 μM), anisomycin (10 μg/ml) and DMSO were pre-incubated with neurons 20–60 min before stimulation.

Reverse transcription (RT)-PCR

Total RNA was isolated from the cultured neurons using Trizol-reagent (Invitrogen). The cDNAs were synthesized using Revert Aid M-MuLV reverse transcriptase (MBI) in a total 20 μl reaction containing 5 μg of total RNA and 0.5 μg oligoDT primer. The PCR was performed in a 25 μl reaction using *Taq* (Promega, Madison, WI, USA). The primers were as following: 5′-ACCATGTGAAAATTCAGAGG-3′ and 5′-ATTCCAAAGCCAGTGACAGG-3′ 30 cycles for GRIP1; 5′-GGATTGGTCTGTTGTGACTTGC-3′ and 5′-TGGCTTGTACAGACACCAGACA-3′ 20 cycles for β-actin. The PCR products were electrophoresed in 2% agarose gel with 0.5 μg/ml ethidium bromide and imaged with a CCD camera.

Transient transfection and propidium iodide (PI) staining

Primary neurons in 35 mm dish were transfected with 2–3 μg constructs by the NucleofectorIIusing Rat Neuron Nucleofector Kit (Amaxa Biosystems, Köln, Germany) before plating. Neuron viability was determined by PI-staining. The PI dissolved in PBS was directly added to the culture medium to give a final concentration of 10 μg/ml, after incubation for 10 min, images were taken by the inverted microscope (10× objective, Olympus IX51; Olympus, Tokyo, Japan).

Western blot analysis and immunoprecipitation

Neurons were extracted with RIPA buffer (1 mM sodium phosphate, pH 7.2, 1% Nonidet P-40 (NP-40), 1% deoxylate acid, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 2 mM EDTA, and protease inhibitors). The extracts were centrifuged at 13,000 r.p.m. for 10 min at 4 °C and the supernatants were obtained for further analysis after determination of protein concentration using the Bradford assay (Bio-Rad, Richmond, CA, USA). The proteins were separated by SDS-6-10% PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and probed with primary antibodies followed with HRP-conjugated secondary antibodies. The bands were then visualized by an Amersham ECL system. The density of the bands was analyzed by the Software ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA). Signal intensities were normalized against the internal control (β-actin). Representative examples of the Western blots were performed at least three times. For immunoprecipitation, total cell lysates were incubated with the primary antibody overnight at 4 °C. For ubiquitin blocking experiments, the anti-ubiquitin antibody was incubated with the purified ubiquitin and the protein-G sepharose was then added and incubated for 4 h at 4 °C. The immunocomplexes were collected, washed and Western blotted with the indicated antibodies.

GST-S5a pull-down assay

Total cell lysates were incubated with the glutathione–agarose immobilized GST-S5a beads in TBS buffer (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% Nonidet P-40, and protease inhibitors) overnight at 4 °C. Precipitated complexes obtained by a sequential wash of the beads with TBS buffer followed by centrifugations were solubilized in a 2% SDS solution, heated at 100 °C for 10 min and analyzed by Western blotting using the indicated antibodies.

Immunocytochemistry analysis

The neurons grown on coverslips were washed and fixed with 4% paraformaldehyde (PFA) containing 4% sucrose for 10 min. The fixed neurons were washed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 5 min and then blocked with 10% new born bovine serum. After probed with the primary antibodies, the neurons were rinsed with 0.05% Tween-20 and incubated with the fluorescent secondary antibodies. The coverslips were mounted with the mounting medium (Sigma, cat. #G0918). The images were taken with a Zeiss LSM510 confocal microscope (Plan-Neofluar 40×/1.30 Oil, Inverted Axiovert 200M; Carl Zeiss, Oberkochen, Germany) and analyzed with the Image Pro-Plus software. The GRIP1 fluorescence intensity was obtained by subtraction of the mean background fluorescence from mean fluorescence intensity of the cell area.

Biotinylation measurement of surface receptors

Briefly, cultured cortical neurons with a high-density were treated with glutamate for 10 min and incubated for 30 min at 37 °C to allow endocytosis to occur. The cultures were washed three times with ice cold PBS (containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$) on ice and incubated with 2 ml of freshly prepared EZ-LINK-Sulfo-NHS-LC-Biotin (0.25 mg/ml, Pierce, Rockford, IL, USA) for 45 min at 4 °C, followed by addition of glycine (10 mM). After incubation for 20 min at 4 °C, the cultures were washed three times and lysed in 350 μl the RIPA buffer. The extracts were centrifuged and 50 μl supernatant was removed for determination of protein concentra-