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ATPA induced GluR5-containing kainite receptor S-nitrosylation via activation of GluR5–Gq–PLC–IP_3R pathway and signalling module GluR5·PSD-95·nNOS

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ABSTRACT

GluR5-containing kainite receptor (GluR5–KAR) plays an important role in the pathophysiology of nervous system diseases, while S-nitrosylation exerts a variety of effects on biological systems. However, the mechanism of GluR5–KAR S-nitrosylation is still unclear up to now. Here our researches found that GluR5–KAR selective agonist ATPA stimulation activated the nonclassical GluR5–KAR–Gq–PLC–IP_3R pathway and the signalling module GluR5·PSD-95·nNOS (the former is more important), led to Ca^{2+} release from intracellular calcium stores endoplasmic reticulum (ER) to cytoplasm and extracellular calcium indrawal, respectively, which further resulted in nNOS activation and GluR5–KAR S-nitrosylation, and then inhibited GluR5-mediated whole-cell current attenuation and induced apoptosis in primary cultured hippocampal neurons. Clarification of the primary mechanisms of GluR5–KAR S-nitrosylation induced by ATPA and identification of critical cysteine for GluR5-2a S-nitrosylation (Cys231 and Cys804) open up a brand-new field for revealing downstream signalling pathway of GluR5–KAR and its molecular characteristics, exploring the pathogenesis of neurological diseases and searching for promising therapies.

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1. Introduction

Recent discoveries related to the function of kainite receptors and their involvement in synaptic physiology brought about profound changes in this field: kainite receptors are now regarded as key players in synaptic integration, synaptic plasticity, regulation of neurotransmitter release and control of neuronal excitability as well as in synaptogenesis and synaptic maturation (Lauri and Taira, 2011; Melyan and Wheal, 2011; Rozas, 2011; Sihra and Rodriguez-Moreno, 2011). Certainly, the proper subcellular localization of kainite receptors in specific functional domains of neurons, as well as complicated molecular and cellular mechanisms involved, is essential for all these functions (Pinheiro and Mulle, 2006). Once the indispensable role of kainite receptors in the pathology and physiology is fully appreciated, some promising therapeutic targets for the development of antiepileptic and analgesic drugs can be obtained (Matute, 2011). Five types of kainate receptor subunits, GluR5, GluR6, GluR7, KA1 and KA2, are found in KA receptor (Hollmann and Heinemann, 1994). These subunits are arranged in different ways to form a tetramer. GluR5–7 can form homomers (receptor composed entirely of GluR5) and heteromers (receptor composed of both GluR5 and GluR6). However, KA1 and KA2 can only form functional receptors by combining with one of the GluR5–7 subunits (Bloss and Hunter, 2010).

S-nitrosylation (coupled with denitrosylation), a redox-based modification of cysteine thiol side chains by nitric oxide (NO), constitutes a ubiquitous mechanism in signal transduction (Hess and Stamler, 2012). Accumulating evidence indicates that S-nitrosylation exerts profound cardiovascular, pulmonary, musculoskeletal and neurological effects, as well as causes cancer by hypo- or hyper- S-nitrosylation or denitrosylation of specific protein targets (Nakamura and Lipton, 2011). Since exogenous mediators of protein S-nitrosylation or denitrosylation can extensively affect the emergence and development of diseases, potential therapeutic agents which modulate S-nitrosylation might bring broad clinical utility (Nakamura and Lipton, 2011; Park et al., 2004).

GluR5-containing kainate receptor (GluR5–KAR) has only been regarded as a receptor operated channel (ROC) transmitting neurotransmitter and Ca^{2+} for quite a long time (Jane et al., 2009). Yet, recent research has shown that the GluR5-mediated Ca^{2+} increase was mediated largely via a noncanonical mechanism through activation of G protein, phospholipase C, and release of

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Ca^{2+} from intracellular stores by activation of inositol triphosphate (IP_3) receptors, which was defined as Gq–PLC–IP_3 pathway (Gq, guanine nucleotide binding regulatory protein Gq) and changed completely the traditional understanding of GluR5–KAR (Ouardouz et al., 2009a,b). However, little research has been done on GluR5–KAR S-nitrosylation both in the physiology and in the pathology.

This paper aims to enunciate the primary mechanisms of GluR5–KAR S-nitrosylation, explore the effect of GluR5–KAR S-nitrosylation on ion channel function and neuronal apoptosis and identify critical cysteine for GluR5-2a S-nitrosylation. In addition, we will compare calcium permeability of N-methyl-D-aspartate receptor (NMDAR) and GluR5–KAR, which directly leads to the activation of neuronal nitric oxide synthase (nNOS) and then GluR5–KAR S-nitrosylation. All the researches were performed in vitro, and this will help to reveal the downstream signalling pathway of GluR5 and search for new drug targets for neurodegenerative diseases.

2. Materials and methods

2.1. HEK293 cell culture and transfection

HEK293 cells or HEK293 cells stably expressing PSD-95 were cultured under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and cultured in 5 cm × 5 cm or 5 cm × 7 cm culture flasks in a humidified incubator. HEK293 cells were transfected with wild GluR5-2a or its mutant plasmids, and HEK293 cells stably expressing PSD-95 were transfected with the mixture of GluR5-2a and nNOS plasmids at the time of 90–95% confluent by Lipofectamine™ 2000. After cultured for 48 h, the cells were taken for experiment.

2.2. Hippocampal cell culture

Neurons from hippocampi of fetal Sprague-Dawley rat (19 days gestation) were prepared as described previously with a little modification. Briefly, hippocampi were meticulously isolated in ice-cold high-glucose DMEM. Hippocampal cells were dissociated by trypsinization [0.25% (w/v) trypsin and 0.05% EDTA in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution] at 37 °C for 15 min, followed by gentle triturating in plating medium (Neurobasal medium supplemented with 2% B-27, 25 μM glutamate, 25 μM β-mercaptoethanol and 0.5 mM glutamine). Cells were seeded onto poly-L-lysine-coated wells or coverslips at a density of 0.8×10^5 cells/cm^2 and incubated at 37 °C in 5% CO_2 atmosphere. After 18–24 h, cells were incubated in growing medium (Neurobasal supplemented with 2% B-27, 25 μM β-mercaptoethanol and 0.5 mM glutamine), and then half-replaced twice every week. Cultures were used for series of pharmacological experiments after 20 days in vitro. Cultures were taken for patch clamp recording after culturing for 14 days in vitro (Xu et al., 2008).

2.3. Sample preparation

The hippocampi or HEK293 cells sample for the detection of S-nitrosylated proteins were homogenized with ultrasonic wave in approximately 20 volumes of HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, protease inhibitor mixture), otherwise in approximately 20 volumes of normal homogenate containing 50 mM 3-(N-morpholino) pro-panesulphonic acid (MOPS) (pH 7.4), 100 mM KCl, 320 mM sucrose, 50 mM NaF, 0.5 mM $MgCl_2$, 0.2 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM sodium pyrophosphate, 20 mM b-phosphoglycerol, 1 mM p-nitrophenyl phosphate (PNPP), 1 mM benzamidine, 1 mM phenylmethylsulphonylfluoride (PMSF) and 5 mg/ml each of leupeptin, aprotinin and pepstatin A. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C. Supernatants were collected, and protein concentrations were determined using the BCA method. Samples were stored at −80 °C until use.

2.4. Biotin switch assay

The biotin switch assay was performed as described previously by Jaffrey et al. (2001), using low-light conditions and opaque tubes. Briefly, the sample (800 μg) was adjusted to 0.4% chaps with 10% chaps stock. Free thiols were blocked by methyl methanethiosulphonate (MMTS). Unreacted MMTS was removed by protein precipitation in 10 volumes of acetone (−20 °C). S-nitrosylated cysteine residues were converted to free thiols with sodium ascorbate (1 mM final concentration), which does not alter the methylated thiols. The free thiols were then biotinylated with biotin-hexyl pyridyldithiopropionamide (HPDP) at 25 °C for 1 h. Thus, the S-nitrosylated cysteines were switched for biotin. Proteins were precipitated by chilled acetone, and the pellet was resuspended in HENS buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% sodium dodecyl sulphate (SDS)). Biotinylated proteins were precipitated with streptavidin-agarose (Sigma), eluted from the beads with a solution containing 20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM 2-mercaptoethanol and subjected to Western blot analysis (Hu et al., 2012; Jaffrey et al., 2001; Zhang et al., 2011).

2.5. Immunoblot

Equal amounts of protein were separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking for 3 h in Tris–buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 3% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibodies in TBST for 2 h and developed using NBT/BCIP colour substrate. The densities of the bands on the membrane were scanned and analysed with an image analyser.

2.6. Immunoprecipitation

Cell homogenates (400 mg of protein) were diluted 4-fold with 50 mM HEPES buffer (pH 7.4), containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40 and 1 mM each of EDTA, EGTA, PMSF and Na_3VO_4. Samples were preincubated for 1 h with 20 ml protein A Sepharose CL-4B at 4 °C, and then centrifuged to remove proteins adhered non-specifically to protein A. The supernatants were incubated with 1–2 mg primary antibodies for 4 h or overnight at 4 °C. Protein A was added to the tube for another 2 h incubation. Samples were centrifuged at 10,000 × *g* for 2 min at 4 °C and the pellets were washed with immunoprecipitation buffer three times. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS-PAGE loading buffer and then isolated by centrifuge. The supernatants were used for immunoblot analysis.

2.7. Site-directed mutagenesis

Eukaryotic expression vector pAdTrack-cmv was obtained from Addgene. GluR-2a cDNA is cloned in our laboratory and nNOS cDNA is a gift form Professor Y. Watanabe. Site-directed mutagenesis of GluR5 was performed with a QuikChange kit (Stratagene) and the following mutagenic primers: C231G up, 5′CTATGTGATATTTGATgGTTCGCACGAAACAG3′; C231G down,

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