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Signaling through EAAT-1/GLAST in cultured Bergmann glia cells

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ABSTRACT

Glutamate, the major excitatory amino acid, activates a wide variety of signal transduction cascades. Synaptic plasticity relies on activity-dependent differential protein expression. Ionotropic and metabotropic glutamate receptors have been critically involved in long-term synaptic changes, although recent findings suggest that the electrogenic Na^+-dependent glutamate transporters, responsible of its removal from the synaptic cleft, participate in glutamate-induced signaling. Transporter proteins are expressed in neurons and glia cells albeit most of the glutamate uptake occurs in the glial compartment. Within the cerebellum, Bergmann glial cells are close to glutamatergic synapses and participate actively in the recycling of glutamate through the glutamate/glutamine shuttle. In this context, we decided to investigate a plausible role of Bergmann glia glutamate transporters as signaling entities. To this end, primary cultures of chick cerebellar Bergmann glial cells were exposed to D-aspartate (D-Asp) and other transporter ligands and the serine 2448 phosphorylation pattern of the master regulator of protein synthesis, namely the mammalian target of rapamycin (mTOR), determined. An increase in mTOR phosphorylation and activity was detected. The signaling cascade included Ca^{2+} influx, activation of the phosphatidylinositol 3-kinase and protein kinase B. Furthermore, transporter signaling resulted also in an increase in activator protein-1 (AP-1) binding to DNA and the up-regulation of the transcription of an AP-1 driven gene construct. These results add a novel mediator of the glutamate effects at the translational and transcriptional levels and further strengthen the notion of the critical involvement of glia cells in synaptic function.

1. Introduction

Excitatory neurotransmission in the vertebrate central nervous system (CNS) is mediated largely by glutamate (Glu). Two main subtypes of Glu receptors have been defined: ionotropic (iGluRs) and metabotropic receptors (mGluRs). Three iGluRs exist: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate (AMPA) and kainate (KA) receptors (Hollmann and Heinemann, 1994). Metabotropic receptors are divided in terms of sequence similarity, signal transduction mechanisms and pharmacology in three groups. Group I receptors are coupled to the stimulation of phospholipase C with the consequent release of intracellular Ca^{2+}, while Groups II and III are coupled to the inhibition of adenylate cyclase. These three groups are activated preferentially by (RS)-3,5-dihydroxyphenylglycine (DHPG) for Group I, (S)-4-carboxy-3-hydroxyphenylglycine (S)-4C3HPG activates Group II while L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) acts upon Group III (Coutinho and Knopfel, 2002).

Bergmann glia cells (BGCs) are the most abundant glia cells in the cerebellum, comprising more than 90% of the cerebellar glia. These cells span the entire cerebellar molecular layer and encapsulate neuronal somata, dendrites and axons. BGC are involved in neurotransmitter uptake, K^+ homeostasis and pH regulation due to the expression of a battery of receptors and transporters (Lopez-Bayghen et al., 2007). In terms of glutamatergic transmission, BGC are in a very short proximity to the parallel fiber-Purkinje cell synapses, and are involved in the Glu/glutamine shuttle that assures the Glu supply to the presynaptic terminals. In this sense, BGC respond to glutamatergic stimulation, as we have been able to characterize over the years (Barrera et al., 2010).

Activity-dependent gene expression regulation stabilizes the synaptic changes that underlie the late phase of long-term potentiation (Pittenger and Kandel, 1998). Transcription and translation are essential for long-term memory (Hu et al., 2006). While most studies have focused in gene expression regulation at the transcriptional level, regulation of protein synthesis has a crucial role in synaptic plasticity (Cammalleri et al., 2003). Translational

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control offers the possibility of a rapid response to external stimulus without mRNA synthesis and transport. Therefore, immediacy is the most conspicuous advantage of translational over transcriptional control.

The mammalian target of rapamycin (mTOR) is a master regulator of protein synthesis (Proud, 2007). It is a multi-domain serine/threonine kinase that phosphorylates a wide array of proteins like phosphatase 2A and Huntingtin. It forms the catalytic core of two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). During acute exposure, rapamycin inhibits mTORC1 but not mTORC2. In a rather simplified scenario, mTORC1 mediates the mTOR effects that are rapamycin-sensitive. The canonical pathway that leads to mTOR serine 2448 phosphorylation and thus activation, includes phosphatidylinositol 3-kinase (PI3-K), which produces phosphatidylinositol 3,4,5-triphosphate (PIP3), that anchors phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB) to the cell membrane (Sabatini, 2006). PKB is activated through a sequential phosphorylation cascade by PDK1 and a PDK2 activity that now it has been shown to correspond to mTORC2 (Bayascas and Alessi, 2005). Phosphorylated PKB activates mTORC1 that acts upon several translation components like eukaryotic initiation factor 4E binding protein-1 (4EBP1) and the 70 kDa S6 ribosomal kinase ($p70^{S6K}$) increasing protein synthesis (Bayascas and Alessi, 2005; Foster and Fingar, 2010).

Glu is removed from the synaptic cleft by a family of electrogenic sodium-dependent transporters expressed in neurons and glia cells (Danbolt, 2001). Five subtypes of transporters named excitatory amino acids transporters 1–5 (EAAT-1–5) have been characterized. The glial transporters EAAT-1 (GLAST) and EAAT-2 (GLT-1) account for more than 80% of the Glu uptake activity in the brain (Eulenburg and Gomeza, 2010; Swanson, 2005). Within BGC, EAAT-1/GLAST is the predominant transporter (Maragakis et al., 2004). Evidences suggest that Glu transporters might also participate in the signaling transactions triggered by this amino acid. In fact, Glu regulates the uptake process in a receptor-independent manner (Gonzalez and Ortega, 2000). More recently, it has also been reported that EAAT-1 is coupled to the Na^+/K^+ ATPase (Gegelashvili et al., 2007; Rose et al., 2009). To provide further evidence for a role of EAAT-1/GLAST in Glu signaling, in the present contribution we challenged the plausible participation of Glu transporters in gene expression regulation. We show here that Glu uptake is linked to an increase in the translation process and that it is also coupled to the transcriptional activation of an AP-1 driven construct. These results are discussed in terms of the physiological significance of an alternative signaling entity to Glu receptors and the identity of the genes regulated. A preliminary description of a D-Asp-dependent mTOR phosphorylation was reported earlier (Zepeda et al., 2009).

2. Materials and methods

2.1. Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). A23187 (5-(methylamino)-2-({(2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5] undec-2-yl}methyl)-1,3-benzoxazole-4-carboxylic acid), Wortmannin, Amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazine-2-carboxamide), KB-R7943 (2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothioureamesylate), DL-TBOA (DL-threo-β-Benzyloxyaspartic acid), THA (threo β–hydroxyaspartate), DNQX (6,7-Dinitroquinoxaline-2,3-dione), LAP5 (L-(+)-2-Amino-5-phosphonopentanoic acid); CPCCOEt (7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester), CPPG ((RS)-α-cyclopropyl-4-phosphonophenylglycine), PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine, genistein and D-aspartate (D-Asp) and Glu were all obtained from Tocris-Cookson (St. Louis, MO, USA). PDC (L-trans-Pyrrolidine-2,4-dicarboxylic acid) was purchased to Sigma–Aldrich (St. Louis, MO, USA); ^{45}Ca was from Perkin Elmer (Boston, MA, USA). Polyclonal anti-phospho-mTOR (Ser 2448) and anti mTOR antibodies (05–235) were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal phospho-4EBP1 (Thr 70) was purchased from Santa Cruz Biotech, (Santa Cruz, CA, USA, sc-18092-R). Monoclonal anti-actin antibodies were kindly donated by Prof. Manuel Hernández (Cinvestav-IPN). Horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies, and the enhanced chemiluminescence reagent (ECL), were obtained from Amersham Biosciences (Buckinghamshire, UK). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and stimulation protocol

Primary cultures of cerebellar BGC were prepared from 14-day-old chick embryos as previously described (Ortega et al., 1991). Cells were plated in 6 or 24-well plastic culture dishes in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and gentamicin (50 μg/ml) and used on the 4th to 7th day after culture. Before any treatment, confluent monolayers were switched to non-serum DMEM media containing 0.5% bovine serum albumin (BSA) for 30 min and then treated as indicated. Inhibitors were added 30 min before agonists. The cells were treated with Glu analogues added to culture medium for the indicated time periods; after that, in the case of transfected cells, the medium was replaced with DMEM/0.5% albumin.

2.3. SDS–PAGE and Western blots

Cells from confluent monolayers were harvested with phosphate-buffer saline (PBS) (10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4) containing phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4). The cells were lysed with RIPA buffer (50 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsufonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1% NP-40, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4 pH 7.4). Cell lysates were denaturized in Laemmli's sample buffer, and equal amount of proteins (100 μg as determined by the Bradford method) were resolved through a 6% SDS–PAGE and then electroblotted to nitrocellulose membranes. Blots were stained with Ponceau S stain to confirm that protein content was equal in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated in TBS containing 5% dried skimmed milk and 0.1% Tween 20 for 60 min to block the excess of non-specific protein binding sites. Membranes were then incubated overnight at 4 °C with the particular primary antibodies indicated in each figure, followed by secondary antibodies. Immunoreactive polypeptides were detected by chemiluminescence and exposed to X-ray films. Densitometry analyses were performed and data analyzed with Prism GraphPad Software (San Diego, CA, USA).

2.4. $^{45}Ca^{2+}$ Influx

Confluent BGC monolayers seeded in 24-well plates were washed three times to remove all non-adhering cells with 0.5 ml aliquots of solution A containing 25 mM HEPES-Tris, 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$. 0.8 mM $MgCl_2$, 33.3 mM glucose and 1 mM NaH_2PO_4 at pH 7.4. The Glu or D-Asp-induced influx of $^{45}Ca^{2+}$ ions was initiated at $t = 0$ by the addition of 0.5 ml solution A containing 1.5 μCi/ml solution A, Glu or Asp at the specified con-