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Glutamate regulates eEF1A phosphorylation and ribosomal transit time in Bergmann glial cells

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ABSTRACT

Glutamate, the major excitatory transmitter in the vertebrate brain, is involved in neuronal development and synaptic plasticity. Glutamatergic stimulation leads to differential gene expression patterns in neuronal and glial cells. A glutamate-dependent transcriptional control has been established for several genes. However, much less is known about the molecular events that modify the translational machinery upon exposure to this neurotransmitter. In a glial model of cerebellar cultured Bergmann cells, glutamate induces a biphasic effect on [^{35}S]-methionine incorporation into proteins that suggests that the elongation phase of protein biosynthesis is the target for regulation. Indeed, after a 15 min exposure to glutamate a transient increase in elongation factor 2 phosphorylation has been reported, an effect mediated through the activation of the elongation factor 2 kinase.

In this contribution, we sought to characterize the phosphorylation status of the eukaryotic elongation factor 1A (eEF1A) and the ribosomal transit time under glutamate exposure. A dose-dependent increase in eEF1A phosphorylation was found after a 60 min glutamate treatment; this phenomenon is Ca^{2+}/CaM dependent, blocked with Src and phosphatidyl-inositol 3-kinase inhibitors and with rapamicyn. Concomitantly, the ribosomal transit time was increased with a 15 min glutamate exposure. After 60 more minutes, the average time used by the ribosomes to complete a polypeptide chain had almost returned to its initial level. These results strongly suggest that glutamate exerts an exquisite time-dependent translational control in glial cells, a process that might be critical for glia–neuron interactions.

1. Introduction

Glutamate (Glu) is the major excitatory amino acid neurotransmitter in the vertebrate brain. Ionotropic as well as metabotropic Glu receptors, both expressed in neurons and glial cells mediate most, albeit not all, of Glu effects. Ionotropic receptors of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate (KA) subtypes are ligand-gated composed of different subunits (Sager et al., 2009). Metabotropic Glu receptors have been subdivided in terms of sequence similarity and signaling mechanisms in three groups. Those that are activated by specific agonists such as (±)-1-aminocyclopentane-trans-1,3-dicarboxilic acid (*trans*-ACPD) and (RS)-3,5-dihydroxyphenilglicine (DHPG) correspond to Group I; (1R,2R)-3-[(1S)-1-amino-2-hydroxy-2-oxoethyl]cyclopropoane-1,2-dicarboxylic acid (DCG-IV) activates those in Group II, while L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) acts upon Group III. Signaling through these receptors is carried out by the phospholipase C and Ca^{2+} for Group I and inhibition of adenylate cyclase for Groups II and III (Wieronska and Pilc, 2009).

Bergmann glial cells (BGC) extend their processes through the molecular layer of the cerebellar cortex enwrapping excitatory and inhibitory synapses (Somogyi et al., 1990). Recent evidence suggests that these cells are involved in neuronal communication and might constitute a neuronal reservoir (Anthony et al., 2004; Hansson and Ronnback, 1995; Malatesta et al., 2003). When cultured, BGC become an excellent model in which the molecular and cellular basis of glial–neuronal signaling can be analyzed (Lopez-Bayghen et al., 2007). In such preparations, Glu acting through its ionotropic and metabotropic receptors, changes gene expression patterns at the transcriptional and translational levels (Gonzalez-Mejia et al., 2006; Rosas et al., 2007). Additionally, it has

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become evident that plasma membrane Glu transporters are also involved in the signaling transactions triggered by this aminoacid transmitter (Gegelashvili et al., 2007; Zepeda et al., 2009). Furthermore, it has been demonstrated *in vivo* that BGC morphology and that of the Purkinje cells that they surround, is modified according to the expression of Ca^{2+}-permeable iGluRs, suggesting a strong and continuous communication between these two cell types (Watanabe, 2002).

Under the influence of Glu, BGC signaling leads to a biphasic effect in overall protein synthesis (Gonzalez-Mejia et al., 2006). Glu treatment modifies [^{35}S]-methionine incorporation into newly synthesized polypeptides in a time-dependent fashion. Moreover, Glu-receptors activity can be associated with a time- and dose-dependent increase in eukaryotic elongation factor-2 phosphorylation. Pharmacological tools established that AMPA and KA, but not NMDA receptors, trigger this phosphorylation. Accordingly, the removal of extracellular Ca^{2+} or pre-treatment with a calmodulin antagonist prevents the Glu effect. As expected, Glu receptors also regulate eukaryotic elongation factor-2 kinase phosphorylation through the involvement of Ca^{2+}/calmodulin/extracellular-regulated protein kinases 1/2 (Barrera et al., 2008). These results suggest that Glu receptors regulate the elongation of peptide chains in glial cells and support the idea that glia cells participate in the activity-dependent changes in the brain protein repertoire.

Translation eukaryotic elongation factor 1A (eEF1A) is another core member of the polypeptide elongation machinery. It provides efficiency and processivity to protein synthesis (Negrutskii and El'skaya, 1998). In fact, eEF1A forms a ternary complex with aminoacyl-tRNA and GTP and delivers the correct aminoacyl-tRNA to the A site of mRNA programmed ribosome in a GTP hydrolysis-dependent mode. The GDP-bound form of eEF1A interacts with deacylated tRNA (Petrushenko et al., 1997) and can transport it to the aminoacyl-tRNA synthetase as the tRNA recycling arm of a tRNA channeling cycle (Petrushenko et al., 2002). eEF1A undergoes threonine phosphorylation (T431) by the Ca^{2+}/diacylglycerol dependent family of protein kinases (PKC), although it must be stated that no conclusive evidence for a role of this modification in eEF1A activity is currently available (Browne and Proud, 2002). In any event, after any pause in elongation, an increase in eEF1A activity might be needed to re-initiate polypeptide elongation (Monnier et al., 2001).

To further characterize Glu-dependent translational control in BGC, we decided to explore the phosphorylation pattern of eEF1A in BGC after the application of the glutamatergic stimuli. Moreover, we evaluated the effect of Glu treatment on the elongation rate. Our results clearly suggest that Glu exerts a tight control on protein synthesis through the regulation of the elongation phase of translation.

2. Materials and methods

2.1. Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). DHPG ((RS)-3,5-Dihydroxyphenylglycine), DNQX (6,7-Dinitroquinoxaline-2,3-dione), AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate), NMDA (N-methyl-D-aspartate), PP2 ((4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)), LY294002 (2-morpholin-4-yl-8-phenylchromen-4-one), Rapamycin, KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine), W7 (N-(6-aminohexyl)-5-chloro-l-naphthalenesulfonamide), BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N^1,N^1-tetraaceticacid), DL-TBOA (DL-threo-b-Benzyloxyaspartic acid), THA (threo-β-hydroxyaspartate) and Glu were all obtained from Tocris-Cookson (St. Louis, MO, USA). Kainate (KA) was obtained from Ocean Produce International (Shelburne, Nova Scotia, Canada). Anti eEF1A monoclonal antibodies (05-235) were purchased from Upstate (Lake Placid, NY, USA); monoclonal anti phospho-threonine agarose conjugated antibodies were from Santa Cruz Biotech., (Santa Cruz, CA, USA; H-2 AC sc-5267), anti Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) antibody (3362) was from Cell Signaling (Boston, MA, USA). Horseradish peroxidase-linked anti-mouse or anti-rabbit 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 60 mm diameter plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, and gentamicin (50 μg/ml) and used on the fourth or fifth day after culture (all obtained from GE Healthcare). 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. Antagonists or inhibitors were added 30 min before agonists. The cells were treated with the Glu analogues added to culture medium for the indicated times; after that, medium was replaced with DMEM/0.5% albumin.

2.3. Cell transfection

Cultured BGC were transiently transfected with the expression vectors for the full length and a truncated (constitutively active) form of the rat CaMKII (CaMKII wt and CaMKII/290, respectively), which expression was driven by the metallothionein promoter (Matthews et al., 1994). Transient transfection assays were performed in 80% confluent BGC cultures using a calcium phosphate protocol with the indicated amount of purified plasmids. Under such conditions, the transfection efficacy was close to 50% determined by a transfection control (β-gal). Treatment with Glu 1 mM for 60 min was performed 16 h post-transfection and then the cell cultures were harvested for immunoprecipitation assays and for Western blot analysis to detect total CaMKII and eEF1A levels.

2.4. Immunoprecipitation 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 deoxycolate, 10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4 pH 7.4). Cell lysates were pre-absorbed with 15 μl of protein A coupled to Sepharose 4B for 25 min at 4 °C. The cleared lysates (1 mg of protein) were incubated with agarose-coupled anti-phosphothreonine or anti-eEF1A antibodies for 10 h, 4 °C and then immunoblotted.

For Western blots, immunoprecipitates were denaturized in Laemmli's sample buffer, resolved through a 10% 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 the adequate secondary antibodies. Immunoreactive polypeptides were detected by chemiluminescence and exposed to X-ray films. Densitometric analyses were performed and data analyzed with the Prism, GraphPad Software (San Diego, CA, USA).

2.5. Ribosomal transit time

Confluent BGC monolayers were serum-starved for 12 h, incubated with 3 μCi of [^{35}S]-methionine for 3, 5, 9, 13, and 15 min in the presence or absence (control) of 1 mM Glu. Alternatively, the cells were incubated with 3 μCi of [^{35}S]-methionine for 5, 15, 30, 45, and 60 min in the presence or absence (control) of 1 mM Glu. In both sets of experiments, the cells were washed with PBS and lysed with 1 ml of extraction buffer (20 mM HEPES, pH 7.2, 100 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml cycloheximide, 0.5% sodium deoxycholate, 0.5% Triton X-100 and *Complete Protease Inhibitor Cocktail* (Roche Diagnostics Corporation Indianapolis, IN, USA). After pelleting the nuclei (16,060 × *g* for 10 min), the supernatant was separated in aliquots to measure the complete peptide chains and the newly synthesized (completed plus ribosome-bound peptide chains) total proteins. Approximately, 0.5 ml of lysate was layered on top of 1 ml of extraction buffer without detergents and containing 0.8 M sucrose. The polysomal fraction was pelleted by centrifugation at 120,000 × *g* for 3 h. Samples of the post-ribosomal supernatant (1.2 ml) were collected to measure [^{35}S]-methionine incorporation into completed proteins. Newly synthesized total proteins containing completed and ribosome-bound elongation peptide chains were measured by recording the incorporation of [^{35}S]-methionine into the post-nuclear supernatant. The transit time was determined from the difference in the positions of the intercepts on the time axis of the plotted data from the total and completed polypeptide chains. RTT is the difference in the interception points is equivalent to one half of the ribosomal transit time (Inamura et al., 2005; Redpath et al., 1996).

2.6. Statistical analysis

Data are expressed as the mean values (average) ± the standard error (S.E.). A nonparametric one-way ANOVA (Kruskal–Wallis test) was performed to determine significant differences between conditions. When these analyses indicated significance (at the 0.05 level), a Dunn's post hoc test was used to determine which conditions were