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GLUTAMATE-DEPENDENT TRANSLATIONAL REGULATION IN CULTURED BERGMANN GLIA CELLS: INVOLVEMENT OF p70^{S6K}

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Abstract—Glutamate, the main excitatory amino acid transmitter in the vertebrate brain is involved in the dynamic changes in protein repertoire that underlie synaptic plasticity. Activity-dependent differential expression patterns occur not only in neurons but also in glial cells. In fact, a membrane to nuclei signaling has been described after ionotropic glutamate receptor stimulation in cultured chick cerebellar Bergmann glia cells. In order to characterize other levels of protein expression regulation, we explored the effect of glutamate treatment in [^{35}S]-methionine incorporation into newly synthesized polypeptides. A time-dependent modification in protein synthesis was found. An important component of translational control is the ribosomal S6 protein kinase. Threonine phosphorylation renders the kinase active increasing translation initiation. Glutamate exposure results in ribosomal S6 protein kinase Thr^{389} phosphorylation in a dose and time-dependent manner that matches perfectly with the overall protein synthesis profile detected upon the excitatory amino acid. Pharmacological characterization of the receptors involved suggests the participation of both ionotropic as well as metabotropic glutamate receptors. The non-receptor tyrosine kinase Src, phosphatidylinositol 3-kinase, protein kinase B and the mammalian target of rapamycin are mediators of the glutamate effect. These results not only demonstrate that glutamate receptors activation is critically involved in translational control in glial cells adjacent to synaptic processes like cerebellar Bergmann glia cells, but also further strengthen the notion of an active participation of glial cells in synaptic transmission. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: p70^{S6K}, glutamate receptors, Bergmann glia, mTOR, translational control.

Excitatory neurotransmission in the mammalian nervous system is mediated to a large extent by glutamate (Glu). Glu receptors are functionally divided into ionotropic (iGluRs) and metabotropic receptors (mGluRs). In terms of Glu analogs, iGluRs have been classified into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate (AMPA) and kainate (KA) receptors (Hollmann and Heinemann, 1994). Based on sequence similarity, signal transduction mechanisms, and pharmacology, mGluRs have been subdivided into three groups. Group I are coupled to the stimulation of phospholipase C and the generation of an intracellular calcium signal, while Groups II and III regulate cyclic AMP levels through the inhibition of adenylate cyclase. These three groups are pharmacological activated by specific agonists such as (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (*trans*-ACPD) and (RS)-3,5-dihydroxyphenylglycine (DHPG) for Group I, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) activates Group II while quisqualic acid (QA) acts upon Group III (Coutinho and Knopfel, 2002). Both iGluRs as well as mGluRs are expressed in Bergmann glial cells (BGC) (Lopez et al., 1998; Gallo and Ghiani, 2000). Stimulation of these glial receptors are likely to have a role in long-term synaptic plasticity in the cerebellum (Iino et al., 2001).

Deficits in glutamatergic neurotransmission have been related to neuronal damage and development of neurodegenerative diseases (Mattson and Chan, 2003; Teismann and Schulz, 2004). Moreover, activation of GluRs results in membrane to nuclei signaling involved in transcriptional and translational regulation in neurons and glial cells (Aguirre et al., 2002; Fields and Stevens-Graham, 2002). While most studies have focused into the molecular mechanisms of gene expression regulation at the transcriptional level, covalent modifications of preexisting proteins and regulation of protein synthesis is fundamental in our understanding of gene expression regulation. In fact, dendritic protein synthesis is believed to play a crucial role in long-term synaptic plasticity (Cammalleri et al., 2003). The fine regulation of the proper amount of a certain protein, might facilitate the long-lasting structural modifications that encode memory (Lamprecht and LeDoux, 2004; Lang et al., 2004). Previous studies in different cell types have suggested that translation regulation can be mediated through

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Abbreviations: Akt I, 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; AMPA, amino α-3-hydroxy-5-methyl-4-isoaxazolepropionate; ANOVA, analysis of variance; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetracetoxy methyl ester; BCG, Bergmann glial cells; Chx, cycloheximide; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline-2,3-dione; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; CPPG, (RS)-α-cyclopropyl-4-phosphonophenylglycine; DHPG, (RS)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; Glu, glutamate; iGluR, ionotropic glutamate receptor; KA, kainic acid/kainate; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; mGluRs, metabotropic glutamate receptors; MK-801, (1)-5-methyl-10,11-dihydro-5Hdibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate; mTOR, mammalian target of rapamycin; NMDA, *N*-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; PI3-K, phosphatidyl inositol 3 kinase; PKC, Ca^{2+}/diacylglicerol protein kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*] pyrimidine; p70^{S6K}, ribosomal protein S6 kinase; PKB, protein kinase B; SDS, sodium dodecyl sulfate.

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phosphorylation of several components of the translational machinery (Wang et al., 2001).

The serine/threonine protein kinase ribosomal protein S6 kinase ($p70^{S6K}$, also named S6K1) is an insulin/mitogen-activated protein kinase member of the AGC kinase family, whose major substrate is the 40S ribosomal subunit protein S6 (Banerjee et al., 1990; Kozma et al., 1990; Avruch, 1998). It is involved in the translational control of the so-called 5′ TOP mRNAs, a subset of mRNAs that contain an oligopirimidine tract in their 5′ untranslated region (Proud and Denton, 1997). TOP mRNAs encode components of the translational machinery such as elongation factors, ribosomal proteins and poly (A)-binding proteins and by these means play a key role in modulating translational efficiency.

Diverse kinases activate $p70^{S6K}$ through a multisite phosphorylation process directed to separated protein domains or modules. Roughly, $p70^{S6K}$ has been divided into four domains. Module I extends from the N-terminus to the beginning of the catalytic domain and confers rapamycin sensitivity to the kinase, module II contains the catalytic domain that includes a mitogen-induced phosphorylation site, module III links the catalytic domain to the carboxy terminus and contains two phosphorylation sites (Pearson et al., 1995; Weng et al., 1995; Pullen and Thomas, 1997). The fourth module comprises a pseudosubstrate domain with four phosphorylation sites (Ferrari et al., 1992). Interactions of these modules lead to interdependent phosphorylation events. Although seven phosphorylation sites have been identified, Thr^{389} phosphorylation is the main event through which full $p70^{S6K}$ activity is accomplished, since the substitution of this residue with Ala fully inactivates the enzyme (Pearson et al., 1995). At least two distinct signaling pathways have been identified in the activation of $p70^{S6K}$. One input is provided by phosphatidylinositol 3-kinase (PI3-K), which is recruited to the activated receptor tyrosine kinases or their substrates in response to insulin or mitogens (Alessi and Downes, 1998; Wymann and Pirola, 1998). Treatment of cells with PI3-K inhibitors such as wortmannin inhibits the activation of the enzyme (Weng et al., 1995). The other pathway contributing to activation of $p70^{S6K}$ described up to date, is the mammalian target of rapamycin (mTOR, also named FRAP, RAFT-1, or RAPT-1) (Schmelzle and Hall, 2000). The immunosuppressant rapamycin is known to cause dephosphorylation and inactivation of $p70^{S6K}$ (Jefferies et al., 1994, 1997; Pearson et al., 1995). Rapamycin, in complex with the cytosolic FK506-binding protein (FKBP12), binds to mTOR and inhibits its function *in vivo*, and previous studies have established that mTOR is the rapamycin-sensitive upstream regulator of $p70^{S6K}$ (Harada et al., 2001; Hay and Sonenberg, 2004).

In chick cerebellum Bergmann glia primary cultures as well as in cerebellar slices, Glu exposure leads to the association of PI3-K and scaffold proteins to tyrosine-phosphorylated AMPA receptors (Millan et al., 2001, 2004), opening the possibility that Glu receptors could be involved in translational control, as has been demonstrated in rat hippocampal slices and synaptoneurosomes (Banko et al., 2004).

In order to gain insight into this problem, the aim of this study was to establish a correlation between a Glu-dependent regulation of protein synthesis and Thr^{389} phosphorylation of $p70^{S6K}$. Our results strongly suggest that $p70^{S6K}$ is involved in the Glu signaling transactions that result in translational control in a PI3-K/PKB/mTOR dependent manner and demonstrate, for the first time that Glu is involved in translational control in glial cells.

EXPERIMENTAL PROCEDURES

Animals

Chick embryos (10 days old) were obtained from Avi-Mex and maintained at 37 °C until used. All experiments conformed to international guidelines on the ethical use of animals and had the specific approval of the Animal Ethics Committee of Cinvestav-Mexico. All efforts were made to reduce the number of embryos used and their suffering.

Chemicals

Tissue culture reagents were from Gibco Invitrogen (Gaithersburg, MD, USA). Wortmannin was obtained from Calbiochem (La Jolla, CA, USA). Polyclonal anti-p-p70 S6 kinase (Thr 389), anti-p70 S6 kinase and poly (ADP-ribose) polymerase (PARP) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase were purchased from Zymed Laboratories (San Francisco, CA, USA). The Bradford and the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) reagents were obtained from Bio-Rad (Hercules, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

Cell culture and stimulation protocol

Chick cerebellar BGC were prepared as detailed elsewhere (Ortega et al., 1991). Briefly, 14-day-old chick embryos were used, and the cerebellum was dissected and homogenized mechanically. Cells were plated at a density of 8×10^5/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in 5% CO_2 and used after 5–6 days in culture. Confluent monolayers were exposed to the indicated concentrations of agonists for varying periods of time; antagonists were added 30 min before the agonists. Incubation was stopped by removing the medium, and samples were processed as detailed below.

Metabolic labeling of proteins

Confluent BGC monolayers were labeled overnight with 1 μCi of L-[^{35}S]-methionine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in methionine-free DMEM. After extensive washing, the cells were treated with Glu for the indicated time periods. Cells were washed twice with ice-cold PBS (10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4) and lysed with ice-cold RIPA buffer (20 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 400 mM NaCl, 2 mM dithiothreitol, 1 mM aprotinin, 1 mM leupeptin, and 100 μM phenylmethylsulfonyl fluoride, 20% glycerol, 1% Triton X-100). After a 10 min centrifugation at top speed in a microfuge, the supernatant fraction (approximately 15 μg of protein) was resolved through SDS-PAGE and visualized by silver staining. The gel was dried and exposed to X-ray films (Kodak X-Omat; Eastman Kodak, Rochester, NY, USA) at −70 °C with intensifying screens. Densitometric analysis was done with software Kodak 1D 3.5.4.