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Glutamate transporter GLAST/EAAT1 directs cell surface expression of FXYD2/γ subunit of Na, K-ATPase in human fetal astrocytes

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Abstract

Na^+-dependent uptake of excitatory neurotransmitter glutamate in astrocytes increases cell energy demands primarily due to the elevated ATP consumption by glutamine synthetase and Na^+, K^+-ATPase. The major pool of GLAST/EAAT1, the only glutamate transporter subtype expressed by human fetal astrocytes in undifferentiated cultures, was restricted to the cytoplasmic compartment. Elevated glutamate concentrations (up to 50 μM) stimulated both glutamate uptake and Na^+, K^+-ATPase activity and concomitantly increased cell surface expression of GLAST and FXYD2/γ subunit of Na^+, K^+-ATPase. Intracellular accumulation of glutamate or its metabolites per se was not responsible for these changes since metabolically inert transport substrate, D-aspartate, exerted the same effect. Nanomolar concentrations of TFB-TBOA, a novel nontransportable inhibitor of glutamate carriers, almost completely reversed the action of glutamate or D-aspartate. In the same conditions (i.e. block of glutamate transport) monensin, a potent Na^+ ionophore, had no significant effect neither on the activation of Na^+, K^+-ATPase nor on the cell surface expression of γ subunit or GLAST. In order to elucidate the roles of γ subunit in the glutamate uptake-dependent trafficking events or the activation of the astroglial sodium pump, in some cultures γ subunit/FXYD2 was effectively knocked down using siRNA silencing. Unlike the blocking effect of TFB-TBOA, the down-regulation of γ subunit had no effect neither on the trafficking nor activity of GLAST. However, the loss of γ subunit effectively abolished the glutamate uptake-dependent activation of Na^+, K^+-ATPase. Following withdrawal of siRNA from cultures, the expression levels of γ subunit and the sensitivity of Na^+, K^+-ATPase to glutamate/aspartate uptake have been concurrently restored. Thus, the activity of GLAST directs FXYD2 protein/γ subunit to the cell surface, that, in turn, leads to the activation of the astroglial sodium pump, presumably due to the modulatory effect of γ subunit on the kinetic parameters of catalytic α subunit(s) of Na^+, K^+-ATPase.

Keywords: Glutamate transporter; Excitotoxicity; siRNA; Synaptic transmission; Na^+, K^+-ATPase

1. Introduction

Glutamic acid, a major excitatory amino acid in the brain, at higher concentrations can trigger aberrant metabolic pathways leading to neurodegeneration. The excess of extracellular glutamate is normally neutralized by high-affinity uptake mechanism executed by a family of glutamate transporter proteins: GLAST/EAAT1, GLT1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5. The energy source for these secondary carriers is the electrochemical Na^+ gradient maintained by Na^+, K^+-ATPase (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Fairman et al., 1995; Arriza et al., 1997; for more references, see Gegelashvili and Schousboe, 1997, 1998; Gegelashvili et al., 2001; Danbolt, 2001).

In some pathological conditions (e.g. amyotrophic lateral sclerosis, ischemia, neurotrauma), this system fails to catch up with gradually increasing load of glutamate either because of the reduced expression of glutamate transporter proteins or due to the sustained alterations in ionic environment affecting kinetic properties of glutamate carriers. Moreover, as a result of severe cell energy deficit, glutamate transporters instead of

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protecting neurons, can aggravate excitotoxic insult by releasing intracellular glutamate through the reversed mode of operation (Choi, 1994; Rothstein et al., 1995; Rossi et al., 2000; Bonde et al., 2003a, for more references, see Yi and Hazell, 2006; Danbolt, 2001; Gegelashvili et al., 2001).

The major site for glutamate uptake in the brain is astroglia (Schousboe, 1981). Translocation of glutamate across the astroglial plasma membrane is executed by two distinct glutamate transporter subtypes, GLAST/EAAT1 and GLT1/EAAT2. Due to the emergence of the astroglial transport system as an obvious therapeutic target, molecular and cellular regulation of GLAST and GLT1 have been extensively studied in recent years (Gegelashvili et al., 1996, 1997, 2000; Swanson et al., 1996; Trotti et al., 1999; Zelenaia et al., 2000; Figiel et al., 2003; Rodriguez-Kern et al., 2003; Bonde et al., 2003b; see more references in Gegelashvili et al., 2001). Binding and subsequent translocation of glutamate into the cytosolic compartment by these two astroglial transporters serve both removal of the excess of glutamate from the perisynaptic space and nutritional supply of astrocytes. At the same time, elevated glutamate uptake dramatically increases energy demands of astrocytes, primarily due to ATP-consuming conversion of glutamate into glutamine as well as increased activity of Na^+, K^+-ATPase (Pellerin and Magistretti, 1994, 1997). The mechanism of glutamate uptake-dependent stimulation of the astroglial sodium pump has been explained by increased net influx of Na^+ through glutamate transporter(s) (Magistretti and Chatton, 2005). Although it has been proposed that functional up-regulation of the catalytic α2 subunit of Na, K-ATPase is responsible for this effect (Pellerin and Magistretti, 1997), no experimental data on the roles of distinct molecular components of the sodium pump in this process have been provided so far. In the present study, we demonstrate that the activity of glutamate transporter GLAST/EAAT1 can effectively regulate cell surface expression of the γ subunit (FXYD2) of Na^+, K^+ ATPase in human fetal astrocytes.

2. Experimental procedures

2.1. Materials

TFB-TBOA was synthesized at Suntory Institute for Bioorganic Research. Biotinylation kit, including sulfo-NHS-biotin and immunopure immobilized monomeric avidin was purchased from Pierce (Rockford, IL, USA). Metabotropic glutamate receptor antagonists: MPEP hydrochloride (for mGluR5), YM298198 (for mGluR1), LY341495 (for group II mGluRs), CPPG (for group III mGluRs), AMPA/kainate selective glutamate receptor anatgonist: CNQX disodium salt, NMDA receptor antagonist: norketamine hydrochloride were purchased from Tocris Cookson, UK. ^{86}RbCl (5 mCi/mg) was purchased from Amersham Biosciences, NJ. Other reagents were obtained from Sigma–Aldrich, St. Louis, MO, USA, except where indicated (for the origin of antibodies, see Section 2.5).

2.2. Astroglial cell cultures

Primary human astroglial cultures were prepared from second-trimester human fetuses obtained from abortions (in compliance with national guidelines). The obtained brain tissue was composed of telencephalon with cortical and ventricular surfaces. After primary dissection, the tissue was transferred to DMEM/F12, containing 20% FCS and 75 μg/ml gentamycin, and mechanically dissociated by carefully passing through a Nitex mesh. Resuspended cells were subsequently filtered through a 230-μm sieve. The suspension was washed twice by centrifugation and thereafter plated into 24- or 12-well culture dishes at cell density of ca. 10^5 cells/ml. Culturing media was changed every 3rd day. One week following the plating weakly adherent cells, including microglia and oligodendrocytes, were removed by circular shaking. After this procedure, cultures were additionally treated for 3 days with acetylated-LDL-saporin and ME20.4-saporin (1 μg/ml and 1 nM, respectively, Advanced Targeting Systems, San Diego, CA) to eliminate remaining microglia and p75NTR-expressing neuronal cells. Astroglial cultures normally reached the stage of confluency by 14 DIV and maintained flat polygonal phenotype for another 4 weeks. siRNA or scrambled oligonucleotides mixed with GeneSilencer transfection reagent (Gene Therapy Systems, San Diego, CA) were continuously administered during the last 2 weeks of culturing. All experiments were performed using 8–16 independent cultures for each treatment paradigm.

2.3. Transport assays for D-aspartate and ^{86}Rb

Excitatory amino acid uptake by astroglial high-affinity transport system was assayed using its radiolabeled substrate D-[2,3-^{3}H]aspartate, essentially as described in Gegelashvili et al. (1996).

Activity of the sodium pump represented by Na^+/K^+-ATPase was assessed by ouabain-sensitive uptake of $^{86}Rb^+$. This radioisotope is commonly used as a tracer for K^+. Briefly, at the defined time points of treatment with pharmacological agents, medium was aspirated, cells rapidly rinsed and fresh serum-free medium with radioactive ^{86}RbCl (3 μM) was added. Some cultures additionally received inhibitor of Na^+/K^+-ATPase, ouabain (1 mM). After 15 min, medium was rapidly aspirated, cultures placed on ice and rinsed three times with ice-cold isoosmolar HEPES buffer, pH 7.4, containing 152 mM choline chloride, 1 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Cells were dissolved in 0.1 M KOH + 0.1% SDS and 250 μl aliquots for the measurement of incorporated radioactivity and protein content were collected. Na^+, K^+-ATPase mediated uptake of ^{86}Rb was determined by subtracting from scintillation counts for ouabain-treated cells from total counts.

2.4. Cell surface biotinylation assay

Cultures were double rinsed with 0.5 ml PBS, pH 7.2 and thereafter incubated with 0.5 ml/well sulfo-NHS-biotin solution (1 mg/ml in PBS supplemented with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) for 25 min at ambient temperature. In order to stop the labeling and quench the excess of the biotinylation reagent, cells were rinsed twice with 0.25× PBS containing 100 mM glycine and additionally incubated with this quenching solution for 30 min at 4 °C. Finally, cells were lysed in 150 μl buffer containing 20 mM NaCl, 125 mM Tris–HCl, pH 6.8, 1% Triton X-100, 0.1% SDS and a protease inhibitor cocktail "Complete" (Boehringer) for 1 h at 4 °C. The lysate was thoroughly triturated in order to reduce viscosity and cleared by centrifugation at 20,000 × *g* for 20 min. Collected supernatant (100 μl) was mixed and incubated with 100 μl suspension of monomeric avidin-conjugated beads (Pierce, Rockford, IL, USA) for 1 h at 4 °C with gentle shaking. The suspension was then centrifuged at 15,000 × *g* for 10 min and the resulting supernatant (depleted of biotinylated proteins) was saved as a cytoplasmic fraction for subsequent Western blot analysis (in figures abbreviated as "C"). The pelleted beads were washed five times in the lysis buffer, finally resuspended in a modified Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 10 mM NaCl, 2% SDS, 0.5 mM EDTA, 5% 2-mercaptoethanol) to a total suspension volume of 200 μl, and incubated for 1 h with vigorous shaking at room temperature. In such conditions, biotinylated proteins normally dissociate from avidin-beads. Finally, the suspension was centrifuged at 15,000 × *g* for 30 min and the resulting supernatant was collected for use in Western blot analysis as a cell surface protein (biotinylated) fraction (in figures designated "M" for the plasma membrane).

2.5. Western blotting and antibodies

Solubilization of astroglial cultures, as well as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of these samples, electrophoretic transfer of separated proteins onto Immobilon membranes (Millipore,