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Riluzole and gabapentinoids activate glutamate transporters to facilitate glutamate-induced glutamate release from cultured astrocytes

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ABSTRACT

We have recently demonstrated that the glutamate transporter activator riluzole paradoxically enhanced glutamate-induced glutamate release from cultured astrocytes. We further showed that both riluzole and the $\alpha_2\delta$ subunit ligand gabapentin activated descending inhibition in rats by increasing glutamate receptor signaling in the locus coeruleus and hypothesized that these drugs share common mechanisms to enhance glutamate release from astrocytes. In the present study, we examined the effects of riluzole and gabapentin on glutamate uptake and release and glutamate-induced Ca^{2+} responses in primary cultures of astrocytes. Riluzole and gabapentin facilitated glutamate-induced glutamate release from astrocytes and significantly increased glutamate uptake, the latter being completely blocked by the non-selective glutamate transporter blocker DL-threo-β-benzyloxyaspartic acid (DL-TBOA). Riluzole and gabapentin also enhanced the glutamate-induced increase in intracellular Ca^{2+} concentrations. Some $\alpha_2\delta$ subunit ligands, pregabalin and L-isoleucine, enhanced the glutamate-induced Ca^{2+} response, whereas another, 3-exo-aminobicyclo[2.2.1] heptane-2-exo-carboxylic acid (ABHCA), did not. The enhancement of glutamate-induced intracellular Ca^{2+} response by riluzole and gabapentin was blocked by the DL-TBOA and an inhibitor of Na^+/Ca^{2+} exchange, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiurea (KB-R7943). Gabapentin's enhancement of Ca^{2+} increase was specific to glutamate stimulation, as it was not mimicked with stimulation by ATP. These results suggest that riluzole and gabapentin enhance Na^+-glutamate co-transport through glutamate transporters, induce subsequent Ca^{2+} influx via the reverse mode of Na^+/Ca^{2+} exchange, and thereby facilitate Ca^{2+}-dependent glutamate release by glutamate in astrocytes. The present study also demonstrates a novel target of gabapentinoid action in astrocytes other than $\alpha_2\delta$ subunits in neurons.

1. Introduction

In the central nervous system, astrocytes regulate extracellular glutamate concentration via two types of glutamate transporters, glutamate transporter-1 (GLT-1) and glutamate-aspartate transporter (GLAST) (Anderson and Swanson, 2000). Under physiological conditions, glutamate transporters take up glutamate from the extracellular space. However, in pathological conditions such as ischemia, astrocytes release large amounts of glutamate via reverse transport, and this increased extracellular glutamate participates in neurotoxicity (Malarkey and Parpura, 2008).

Riluzole, a neuroprotective drug approved for amyotrophic lateral sclerosis (Brooks, 2009), activates GLT-1 and GLAST to enhance glutamate uptake (Frizzo et al., 2004; Fumagalli et al., 2008), and reduces extracellular glutamate concentrations in the spinal cord (Coderre et al., 2007). In contrast to this glutamate lowering effect in the spinal cord, we recently demonstrated that a local injection of riluzole into the locus coeruleus resulted in activation of noradrenergic neurons to induce descending inhibition in rats, possibly via facilitation of glutamate-induced glutamate release from astrocytes (Hayashida et al., 2010). However, the mechanisms by which riluzole might activate glutamate-induced glutamate release from astrocytes is unknown.

Gabapentin inhibits pain transmission via an interaction with $\alpha_2\delta$ subunits of voltage-gated Ca^{2+} channels (Gee et al., 1996). This inhibitory mechanism contrasts with our recent demonstration that gabapentin, like riluzole, activated locus coeruleus neurons via glutamatergic signaling and induced subsequent spinal noradrenaline release in rats and humans (Hayashida et al., 2007; Hayashida et al., 2008). We therefore hypothesized that riluzole and gabapentin may share common mechanisms for glutamate regulation in astrocytes.

Glutamate increases intracellular Ca^{2+} in astrocytes via activation of Ca^{2+} permeable ionotropic glutamate receptors, which respond to AMPA, and metabotropic glutamate receptors, which release Ca^{2+} from internal stores through 1,4,5-inositol-trisphosphate signaling (Hansson et al., 2000; Verkhratsky and Kirchhoff, 2007). In some astrocytes, co-transport of sodium ions and glutamate by glutamate transporters results in Ca^{2+} influx via the reverse mode of Na^+/Ca^{2+} exchange (Kirischuk et al., 1997; Rojas et al., 2007), which in turn

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leads to glutamate release from astrocytes via Ca^{2+}-dependent mechanisms (Malarkey and Parpura, 2008). By this mechanism, glutamate uptake by astrocytes can paradoxically result in glutamate release. We therefore hypothesized that riluzole and gabapentin enhance Ca^{2+}-dependent glutamate release from astrocytes by activation of glutamate transporters and subsequent Ca^{2+} influx via the reverse mode of Na^+/Ca^{2+} exchange. The present study examined whether riluzole, gabapentin, and other $\alpha_2\delta$ subunit ligands increase glutamate uptake through glutamate transporters, enhance glutamate-induced intracellular Ca^{2+} response by activation of glutamate transporters and subsequent Ca^{2+} influx via the reverse mode of Na^+/Ca^{2+} exchange, and thereby facilitate glutamate-induced glutamate release in primary cultured astrocytes.

2. Materials and methods

2.1. Astrocyte cultures

Primary astrocyte cultures were prepared from the cerebral cortices of neonatal rats between postnatal days 1 and 2. Cerebral cortices were mechanically dissociated in ice-cold Hank's buffered salt solution (HBSS, pH = 7.2) by fire-polished glass pipettes and centrifuged at 300×*g* for 5 min. Tissues were re-dissociated in ice-cold HBSS and the procedure was repeated two times using smaller pipette tip diameters. Cells were first seeded onto T-50 flasks and incubated in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine, and 10 ng/ml epidermal growth factor (EGF, BD Biosciences, Bedford, MA, USA) to maintain GLT-1 and GLAST protein expression in astrocytes (Zelenaia et al., 2000), at 37 °C and 5% CO_2. After one week, astrocytes were reseeded onto poly-d-lysine-coated glass coverslips (35 mm in diameter) at a density of 4×10^4 cells for Ca^{2+} imaging experiments and in 24-well plates at a density of 10^4 cells/well for glutamate uptake/release experiments, then cultured for 6 days. In the present study, cells consisted of >98% of flat polygonal astrocytes with positive immunostaining for glial fibrillary acidic protein.

2.2. Ca^{2+} imaging

Ca^{2+} imaging was performed according to our previous study in neurons (Hayashida et al., 2006) with modifications for cultured astrocytes. Astrocytes were incubated with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA) and 0.01% Pluronic F-127 (Molecular Probes) for 30 min at 37 °C, washed with HEPES buffer containing (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 10 glucose, and 10 HEPES, pH = 7.4, and then left at room temperature in a dark environment for 20 min. Coverslips were mounted on a chamber equipped with a pressure valve perfusion system (ALA-VM8, ALA Scientific Instruments, New York, NY, USA) and viewed through an inverted microscope. Fura-2 fluorescence was recorded at 510 nm during alternating excitation at 340 and 380 nm at 1 Hz using a monochromater (PTI Deltascan, Photon Technology International, South Brunswick, NJ, USA). Only cells responding to 300 μM ATP at the end of experiment were included in analysis.

2.3. Glutamate uptake and release

For glutamate uptake experiments, astrocytes were incubated with HEPES buffer for 40 min with a change to fresh buffer at 20 min and treated with test drugs for 5 min at 37 °C. Astrocytes were incubated with 1 μM glutamate (combination of both tritiated and unlabeled glutamate) containing test drugs for 1 min at 37 °C, quickly washed twice, and then lysed with 0.4% Triton X-100 for 10 min. Amount of radioactivity in lysates was measured by scintillation spectrometry (LS6500, Beckman Coulter Inc., Fullerton, CA, USA).

For glutamate release experiments, astrocytes were incubated with Dulbecco's modified Eagle's medium containing 1 μM glutamate (combination of both tritiated and unlabeled glutamate) for 1 h at 37 °C, and washed twice with HEPES buffer. Astrocytes were then incubated with test drugs alone for 5 min, followed by unlabeled 10 μM glutamate in the presence of test drug for 5 min at 37 °C. Supernatants were collected and astrocytes were lysed. The amount of radioactivity in supernatants and lysates was measured as described above.

2.4. Drugs

DL-threo-β-benzyloxyaspartic acid (DL-TBOA, Tocris Bioscience, Ellisville, MO, USA) and 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiurea (KB-R7943, Tocris Bioscience) were dissolved in DMSO and diluted with HEPES buffer. Riluzole (Sigma Chemical Co., St. Louis, MO, USA), gabapentin (Tronto Research Chemicals Inc., North York, ON, USA), pregabalin (gifted from Pfizer Inc., New York, NY, USA), L-isoleucine (Sigma Chemical Co.), and 3-exo-aminobicyclo[2.2.1]heptane-2-exo-carboxylic acid (ABHCA, Acros organics, New Jersey, USA) were dissolved in distilled water, and then diluted with HEPES buffer.

2.5. Data analysis

Data from glutamate uptake and release experiments are normally distributed and presented as mean ± S.E.M. Data from Ca^{2+} imaging experiments were not normally distributed and presented as 25th, 50th, and 75th percentiles of Fura-2 fluorescence ratio response. Differences among groups were determined using two-way analysis of variance (ANOVA) or ANOVA on ranks as appropriate. $P<0.05$ was considered significant.

3. Results

3.1. Glutamate uptake and release

For glutamate uptake, we selected relatively low concentration of glutamate (1 μM) to minimize glutamate-induced glutamate release. As previously shown (Frizzo et al., 2004), riluzole (1 μM) significantly enhanced [^{3}H]-glutamate uptake in astrocytes (Fig. 1, $P<0.01$). The concentration of riluzole in the uptake experiment was determined from the previous study (Frizzo et al., 2004). Gabapentin pretreatment also increased [^{3}H]-glutamate uptake in a concentration-dependent manner. However, when acutely administered, gabapentin (100 μM) did

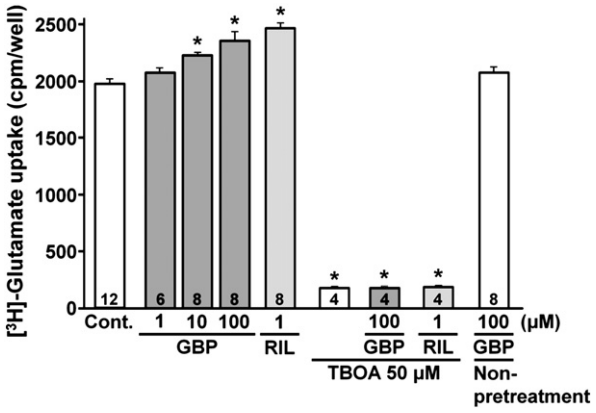

Fig. 1. [^{3}H]-glutamate uptake in astrocytes is presented as a total radioactivity (cpm) per well. Cells were pretreated with buffer (control), gabapentin (GBP, 1–100 μM) or riluzole (RIL, 1 μM) in the presence or absence of TBOA (50 μM) for 5 min and glutamate uptake was performed for 1 min in the presence of test drugs. In non-pretreatment group, GBP (100 μM) was co-applied with glutamate without the pretreatment. Number in each column represents sample size. *$P<0.01$ *vs.* control.