European Journal of Pharmacology 643 (2010) 48–57

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

Bromocriptine, an ergot alkaloid, inhibits excitatory amino acid release mediated by glutamate transporter reversal

Yasufumi Shirasaki [a,*], Masunobu Sugimura [b], Toshiyuki Sato [c]

[a] Biological Research Laboratories, Daiichi-Sankyo Co., Ltd., Tokyo, Japan
[b] Translational Medicine & Clinical Pharmacology Department, Daiichi-Sankyo Co., Ltd., Tokyo, Japan
[c] Frontier Research Laboratories, Daiichi-Sankyo Co., Ltd., Tokyo, Japan

ARTICLE INFO

Article history:
Received 1 February 2010
Received in revised form 11 May 2010
Accepted 4 June 2010
Available online 21 June 2010

Keywords:
Bromocriptine
Glutamate transporter
GLT-1
EAAT2
Ischemia

ABSTRACT

Bromocriptine, a dopamine D_2 receptor agonist, has widely been used for patients with Parkinson's disease. The aim of the present study was to investigate the effect of bromocriptine on glutamate transporter. Since the astroglial glutamate transporter GLT-1 (EAAT2) is the predominant isoform in the forebrain, we generated EAAT2-expressing human embryonic kidney cells and immortalized mouse astrocytes. In the present studies, we observed a GLT-1-immunoreactive band and significant Na^+-dependent D-[^{3}H] aspartate uptake. Furthermore, the glutamate transporter inhibitors, DL-*threo*-β-benzyloxyaspartic acid (TBOA) and dihydrokainate (DHK), displayed a dose-dependent reduction of D-[^{3}H] aspartate uptake in both types of cells. In contrast, cells exposed to either chemical anoxia or high KCl elicited a marked release of D-[^{3}H] aspartate, and the release was inhibited by TBOA and DHK, implying the contribution of glutamate transporter reversal. Interestingly, we found that bromocriptine dose-dependently inhibits D-[^{3}H] aspartate release elicited by chemical anoxia or high KCl, while no changes occurred in the uptake. The inhibitory action of bromocriptine was not affected by sulpiride, a dopamine D_2 receptor antagonist. On the other hand, bromocriptine had no effect on swelling-induced D-[^{3}H] aspartate release, which is mediated by volume-regulated anion channels. In vivo studies revealed that bromocriptine suppresses the excessive elevation of glutamate levels in gerbils subjected to transient forebrain ischemia in a manner similar to DHK. Taken together, these results provide evidence that bromocriptine inhibits excitatory amino acid release via reversed operation of GLT-1 without altering forward transport.

1. Introduction

The excitatory amino acids, glutamate and aspartate, are considered to be the major mediators of excitatory signals in the mammalian central nervous system. The extracellular concentrations of the excitatory amino acids are maintained at low levels to prevent neuronal damage from excessive activation of glutamate receptors. Since glutamate and aspartate are not metabolized in the extracellular space, these amino acids need to be cleared from there. The removal of extracellular glutamate is performed by high-affinity, Na^+-dependent glutamate transporters on the plasma membrane of neurons and surrounding glial cells (Danbolt, 2001; Shigeri et al., 2004). Previous reports have suggested that glia, rather than neurons, mainly contribute to the clearance of extracellular glutamate in the brain (Rothstein et al., 1996; Danbolt, 2001).

In the brain, five subtypes of Na^+-dependent glutamate transporters have been identified so far: EAAT1 (Storck et al., 1992) and EAAT2 (Pines et al., 1992), also known as the human homology of GLAST and GLT-1, respectively; EAAT3 (Kanai and Hediger, 1992), also known as EAAC1; EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). It has been reported that GLT-1 and GLAST are localized in glia (Rothstein et al., 1994; Lehre et al., 1995), whereas EAAC1, EAAT4 and EAAT5 are mainly neuronal (Rothstein et al., 1994; Furuta et al., 1997; Arriza et al., 1997). GLT-1 plays an essential role in the removal of extracellular glutamate in the forebrain regions (Robinson, 1998; Danbolt, 2001), including the hippocampus, which is most vulnerable to neuronal degeneration caused by ischemia.

In contrast to glutamate uptake by a glutamate transporter, its reversal can induce the release of glutamate when the electrochemical gradients for Na^+ and K^+ are disrupted (Szatkowski et al., 1990; Longuemare and Swanson, 1995; Seki et al., 1999). This effect can contribute to the increased extracellular glutamate levels seen in pathological states, such as stroke, cardiac arrest, epilepsy, and spinal cord injury (Hazell, 2007; Sheldon and Robinson, 2007). Several studies have examined the role of GLT-1 in ischemia using pharmacological or genetic approaches. Pharmacological studies have shown that GLT-1

* Corresponding author. Biological Research Laboratories, Daiichi-Sankyo Co., Ltd., 1-16-13 Kita-Kasai, Edogawa-ku, Tokyo 134-8680, Japan. Tel.: +81 3 5696 8604; fax: +81 3 5696 8781.
E-mail address: shirasaki.yasufumi.n7@daiichisankyo.co.jp (Y. Shirasaki).

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.06.007

blocker reduces ischemia-induced glutamate release (Seki et al., 1999), suggesting the contribution of the reversed operation of GLT-1. Furthermore, mice lacking GLT-1 have a lower level of glutamate than wild-type mice during cerebral ischemia (Mitani and Tanaka, 2003).

Bromocriptine is a semisynthetic ergot alkaloid that has been used clinically for parkinsonism therapy (Kartzinel et al., 1976). The effects of bromocriptine are ascribed to the potent agonistic action of dopamine D_2 receptor (Missale et al., 1998). It has been proposed that bromocriptine augments glutamate uptake in HeLaS3 cells expressing high levels of EAAT1 (Yamashita et al., 1998). However, it remains unknown whether bromocriptine affects the function of EAAT2, which is most abundantly expressed in astrocytes of the forebrain.

In the present study, we evaluated the effect of bromocriptine on GLT-1 (EAAT2) in EAAT2-expressing human embryonic kidney cells (HEK293/EcR) and immortalized mouse astrocytes. Here, we report that bromocriptine inhibits the release of glutamate via reversed operation of GLT-1 without altering the uptake of glutamate.

2. Materials and methods

2.1. Materials

Cell culture reagents, Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (PBS), fetal bovine serum, geneticin and zeocin were purchased from Invitrogen Corporation (Carlsbad, CA, USA). D-Aspartate, bromocriptine methansulfonate salt, dihydrokainate (DHK), ponasterone A, tetrodotoxin (TTX) and (−)sulpiride were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). DL-*Threo*-β-benzyloxyaspartate (TBOA) and 4-[(Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid (DCPIB) were purchased from Tocris Bioscience (Ellisville, MO, USA). Antibodies were obtained from the following sources: anti-GLT-1 antibody from Alpha Diagnostic Intl. Inc. (San Antonio, TX, USA), α-tubulin antibody from Sigma-Aldrich and horseradish peroxidase-conjugated secondary antibody from Amersham Biosciences (Piscataway, NJ, USA). D-[2,3-^{3}H]-aspartate (18.0 Ci/mmol) was purchased from NEN Life Science Products Inc. (Boston, MA, USA). L-Glutamate oxidase was obtained from Seikagaku Corporation (Tokyo, Japan).

2.2. Animals

Transgenic C57BL/6 mice harboring a large T-antigen gene of temperature-sensitive mutant simian virus 40 (C57BL/6-SV40T) were bred in our laboratories. Male Mongolian gerbils weighing 60 to 80 g were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). The animals were housed in cages in a regulated environment (23 ± 2 °C, 55 ± 15% relative humidity) under a 12-h light/dark cycle (on 8:00 to 20:00), and given food (F-2; Funabashi Farm, Chiba, Japan) and tap water *ad libitum*. All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi-Sankyo Co., Ltd.

2.3. Establishment of EAAT2-transfected HEK293/EcR cells

Full-length human EAAT2 (574 amino acids) cDNA was synthesized from human brain total RNA (Sawady Technology Co., Ltd., Tokyo, Japan) using an RT-PCR system (RNA LA PCR Kit, Takara Bio Inc., Ohtsu, Shiga, Japan) and cloned into pCR2.1 vectors (Invitrogen). EAAT2 cDNA was subcloned from the pCR2.1 into the pIND (SP1) mammalian expression vector, and the construct was transfected into HEK293/EcR cells (Invitrogen) using Fugene6 (Roche Diagnostics Corporation, Basel, Switzerland). Resistance to geneticin and zeocin was used to select potential positive clones. The EAAT2-transfected cells were maintained in DMEM supplemented with 10% fetal bovine serum, geneticin (0.4 mg/ml) and zeocin (0.4 mg/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. Induction of EAAT2 expression was performed by treatment of the cells with 10 μM ponasterone A for 24 h.

2.4. Establishment of immortalized astrocytes

Immortalized murine astrocytes were established by a modification of the procedure reported previously (Tetsuka et al., 2001). Primary astrocyte cultures were prepared from the cerebral cortex of neonatal C57BL/6-SV40T mice (postnatal day 1) and grown in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent primary astrocytes were subcultured and then conditionally immortalized by a temperature-sensitive SV40 large T antigen at 33 °C. The confluent immortalized cells were seeded onto 48-well plates (10–20 cells/well) to establish cell lines that possess the functional characteristics of astrocytes, which are determined by the Na^+-dependent glutamate uptake. For the selected clones, unless otherwise indicated, the cells were cultured at 33 °C and then allowed to express glutamate transporters under incubation at 37 °C for 48 h.

2.5. Western blot

The cells were solubilized with SDS-PAGE sample buffer (Invitrogen) in the presence of a protease inhibitor mixture (Sigma-Aldrich). The lysates were boiled for 5 min and centrifuged at $5000 \times g$ for 15 min, and the supernatants were subjected to SDS-PAGE. The protein concentration in each sample was determined by a BCA protein assay reagent (Perce, Rockford, IL, USA) with bovine serum albumin as the standard. Samples containing equal amounts of protein were loaded onto 10–20% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat skim milk in Tris-buffered saline containing 0.1% Tween20 and then incubated with an anti-rat GLT-1 polyclonal antibody (1:500) overnight at 4 °C. Immunoreactivity was detected by sequential incubation of horseradish peroxidase-conjugated secondary antibody (1:1500) and ECL reagents (Amersham Bioscience). The membranes were stripped and incubated with anti-α-tubulin antibody. The densities of the corresponding immunoreactive bands were quantified with a laser scanning densitometer (CS-9600, Shimazu, Kyoto, Japan). The amount of GLT-1 in each sample was normalized relative to α-tubulin and expressed as a percentage of cells cultured at 33 °C.

2.6. Uptake of D-[^{3}H]aspartate

The uptake of excitatory amino acids was determined using radiolabeled D-aspartate, a non-metabolizable marker for the intracellular glutamate and aspartate pools. Both of these amino acids are transported on the same carrier protein and label the nonvesicular pool of excitatory amino acids (Barbour et al., 1993). HEK293/EcR cells (6×10^4 cells/100 μl/well) grown on 96-well culture plates were rinsed with HBSS and incubated in HBSS containing 0.5 μCi/well D-[^{3}H]aspartate and 5 μM D-aspartate at 37 °C for 30 min, the time in which aspartate uptake was found to proceed linearly with time. On the other hand, uptake in immortalized astrocytes ($7–8 \times 10^3$ cells/100 μl/well) was performed in the absence of D-aspartate. Na^+-independent uptake was performed in Na^+-free HBSS, where Na^+ had been replaced with choline chloride. At the end of the incubation, the uptake was terminated by washing with ice-cold HBSS followed by immediate cell lysis in 0.5 N NaOH. The cell lysate was mixed with liquid scintillation cocktail (Mscint-40, PerkinElmer Inc., Boston, MA, USA), and the radioactivity was measured with a TopCount-HTS liquid scintillation analyzer (PerkinElmer Inc.). In order to study the effects of bromocriptine and glutamate transporter inhibitors, including DHK and TBOA, on excitatory amino acid uptake, these compounds were added to HBSS containing D-[^{3}H]aspartate. The D-[^{3}H]aspartate uptake was calculated by subtracting the Na^+-independent uptake from the Na^+-dependent uptake and then expressed as the percentage of radioactivity in vehicle-treated cells.