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The role of inversely operating glutamate transporter in the paradoxical analgesia produced by glutamate transporter inhibitors

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ABSTRACT

Controlling extracellular glutamate level in a physiological range is important to maintain normal sensory transmission. Here, we investigated the paradoxical action of glutamate transporters in the rat formalin test to elucidate a possible role of inversely operating transporters in its analgesic mechanism. The effects of glutamate transporter inhibitor on formalin-induced pain behavior were examined. Then we performed a microdialysis study to clarify the differential change in extracellular glutamate concentration by intrathecal administration of transportable and non-transportable blockers. And we further investigated the mechanism pharmacologically via pretreatment with antagonists of various receptors and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining. Intrathecally-injected glutamate transporter inhibitors, non-transportable DL-threo-β-benzyloxyaspartat (TBOA) and transportable trans-pyrrolidine-2,4-dicarboxylic acid (*t*-PDC), produced paradoxical antinociception in the formalin test. In normal rats, inhibition of the glutamate transporter increased extracellular glutamate. In the formalin model rats, TBOA suppressed while t-PDC enhanced glutamate release. When tPDC was pretreated 30 min prior to formalin injection, glutamate release was blocked. Blocking α-2 adrenergic receptors reversed the tPDC analgesia. Increased apoptosis was not apparent in the spinal dorsal horn of tPDC-treated rats compared to the control group. These data suggest that glutamate transporters in a formalin-induced pain state work in a reverse mode and can be blocked from releasing glutamate by TBOA and preloaded tPDC. The analgesic mechanism of TBOA may be related to the blockade of inversely operating transporter, and that of tPDC may be associated with the activation of noradrenergic neurotransmission but not with dorsal horn neurotoxicity.

1. Introduction

Maintaining a physiological level of extracellular glutamate, a major excitatory amino acid neurotransmitter in the spinal dorsal horn, is key to preventing excitotoxicity and neurotoxicity produced by excessive activation of its receptors, which can occur under many pathological conditions (Danbolt, 2001). Regulation of the glutamate concentration in the synaptic cleft is carried out primarily by an efficient, high-affinity glutamate transporter localized in the cell membranes of glia and neurons (Mao, 2007). Thus, spinal glutamate transporter inhibition can lead to decreased uptake of and then excessive accumulation of glutamate in the synaptic cleft, which may, in turn, result in over-activation of glutamate receptors, producing a pronociceptive effect under normal conditions (Liaw et al., 2005).

Unexpectedly, under pathological pain conditions, inhibition of spinal glutamate transporter activity can produce antinociceptive effects. For example, intrathecal injection of the transportable inhibitor trans-pyrrolidine-2,4-dicarboxylic acid (*t*-PDC) or antisense oligonucleotides reduced nociceptive behavior in the rat formalin test (Niederberger et al., 2003). A non-transportable blocker, DL-threo-β-benzyloxyaspartate (TBOA), resulted in significant reductions in complete Freund's adjuvant (CFA)- and formalin-induced inflammatory pain (Yaster et al., 2011). However, current knowledge about this paradoxical action of glutamate transporter inhibitors is limited.

Mechanisms regarding the activation of inhibitory presynaptic group III metabotropic glutamate receptors (Yaster et al., 2011) and

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the modulation of glutamate release from presynaptic terminals (Yang et al., 2015) have been reported. However, other potential mechanisms, including postsynaptic desensitization of dorsal horn glutamate receptors, depletion of the glutamate-glutamine cycle in spinal glial cells, and inhibition of glutamate release by blocking inverse operation of the transporter, have not been investigated.

Above all, as suggested by Tao et al., (Tao et al., 2005) determining the extracellular levels of glutamate in the spinal cord after glutamate transporter inhibition during pathological pain status, and thus clarifying the possible role of reversed transporter, may be an essential step in investigating the mechanism(s) of this phenomenon. In a previous report that studied glutamate release through reverse transport induced by ATP depletion, a transportable inhibitor enhanced, while a non-transportable inhibitor blocked, uptake reversal of glutamate (Anderson et al., 2001). Therefore the glutamate transporter in a reversed mode may be differentially affected by transportable and non-transportable inhibitors (Anderson et al., 2001; Roettger and Lipton, 1996).

The purpose of the current study was to examine the mechanism of the antinociceptive effect of the glutamate transporter inhibitor. We first examined the effects of glutamate transporter inhibitors on inflammatory pain induced by intraplantar injection of formalin. Then, we performed a microdialysis study to clarify the differential change in the extracellular glutamate concentration by intrathecal administration of transportable and non-transportable blockers under normal and formalin-induced pain states. We further investigated the mechanism pharmacologically via pretreatment with antagonists of various receptors.

2. Materials and methods

2.1. Animal preparation

The study protocol was approved by the Institutional Animal Care and Use Committee of Chonnam National University. Experiments were conducted in male Sprague Dawley rats weighing 250–300 g. The rats were housed in a temperature-controlled room (22 ± 0.5 °C) under an alternating 12/12-h light/dark cycle in individual cages. Water and food were freely available. This manuscript adheres to the ARRIVE Guidelines for reporting animal research (Kilkenny et al., 2010).

2.2. Construction and implantation of an intrathecal catheter and microdialysis probe

For intrathecal drug administration, an 8.5 cm 32-gauge polyurethane catheter (RecathCo, Allison Park, PA, USA) was connected to a polyethylene catheter (PE-10) and inserted intrathecally using previously described methods (Yaksh and Rudy, 1976). Briefly, the rats were anesthetized with sevoflurane and placed in a stereotaxic head holder. The catheter (polyurethane tubing) was inserted through an incision in the atlanto-occipital membrane and advanced caudally 8.5 cm from the incision site for positioning at the lumbar enlargement of the spinal cord. The external end of the PE-10 catheter was plugged with a piece of steel wire and tunneled subcutaneously to exit at the head. The skin was closed using 3.0 silk. Rats exhibiting motor deficits after catheterization were euthanized immediately with an overdose of volatile anesthetic. Postoperatively, 7 days were allowed for recovery.

An intrathecal microdialysis probe was constructed using a technique modified and adapted from previous reports (Marsala et al., 1995; Tai et al., 2006). The probe was constructed using two 6 cm polyurethane tubes (178 μm inner diameter, 356 μm outer diameter) and a 4.2 cm cellulose hollow fiber (18 kDa molecular weight cut-off; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). A Nichrome-Formvar wire (66.04 μm diameter; A-M Systems, Inc., Sequim, WA, USA) was passed through the hollow fiber (active dialysis region), and its middle portion was bent to form a U-shaped loop. Both ends of the dialysis loop were connected with polyurethane tubes using cyclohexanone. The other ends of the two polyurethane tubes were attached to 4 cm PE-10 (280 μm inner diameter, 640 μm outer diameter) catheters. For collecting the cerebrospinal fluid (CSF) sample after drug treatment, both the constructed microdialysis probe and intrathecal catheter were placed intrathecally as described above.

2.3. Nociceptive test

The formalin test was performed by injecting 50 μl 5% formalin solution subcutaneously into the plantar surface of the rat hind paw using a 30-gauge needle, as described previously (Kim do et al., 2016). The formalin injection evoked a characteristic spontaneous flinching behavior which was observed as drawing the paw under the body and rapidly vibrating it. This behavior was quantified by periodically counting the number of flinches of the injected paw. The flinches were counted for 1-min periods from 1 to 2 min, 5–6 min, and every 5 min thereafter, up to 60 min. The observed responses appeared biphasically and were divided into phases I (0–9 min) and II (10–60 min). Because different drug effects may arise between early and late phase II, phase II was further divided into phase IIA (10–40 min) and IIB (41–60 min) (Malmberg et al., 1994). After the entire observation period, the rats were sacrificed with an overdose of volatile anesthetic. The formalin test was performed by an investigator blinded to the experimental drug and dose.

2.4. Drug treatment

On the day of experiments, the rats were randomly allocated into one of the following experimental or control (vehicle) groups. To assess the effects of glutamate transporter inhibition on formalin-induced pain behavior, trans-pyrrolidine-2,4-dicarboxylic acid (tPDC, 1 μmol, Tocris Cookson Ltd., Bristol, UK) or DL-threo-β-benzyloxyaspartate (TBOA, 10 μg, Tocris), transportable and non-transportable inhibitors of the glutamate transporter, respectively, were injected intrathecally immediately after formalin injection (n=5 in each group).

To determine the pharmacological mechanisms of the paradoxical action of glutamate transporter inhibitors, the following drugs were administered 10 min before the delivery of the glutamate transporter inhibitor, and the formalin test was performed (n=5 in each group): bicuculline 0.3 μg ($GABA_A$ receptor antagonist, Sigma Aldrich Co., St. Louis, MO, USA), saclofen 30 μg ($GABA_B$ receptor antagonist, Sigma Aldrich), (RS)-α-methylserine-O-phosphate 10 μg (MSOP, group III metabotropic glutamate receptor antagonist, Tocris), dihydroergocristine mesylate 3 μg (serotonin receptor antagonist, Research Biochemical Internationals, RBI, Natick, MA, USA.), yohimbine hydrochloride 10 μg (α-2 adrenergic receptor antagonist, Sigma Aldrich), naloxone 0.3 μg (opioid receptor antagonist, Sigma Aldrich), and CGS 15943 0.03 μg (adenosine receptor antagonist, RBI). Dihydroergocristine, yohimbine, and CGS 15943 were dissolved in 100% dimethyl sulfoxide (DMSO). All other drugs were dissolved in normal saline. All drugs were delivered in a volume of 10 μl, followed by an additional 10 μl saline to flush the catheter. Based on data from previous reports (Jeong et al., 2014; Niederberger et al., 2003; Yoon et al., 2003) and our pilot study, we determined the maximum doses that did not affect the formalin-induced flinch and assumed that the basal nociceptive thresholds were not changed.

2.5. Intrathecal microdialysis

One of the externalized PE-10 tubes (inflow) was connected to a microsyringe pump (ESP-64; Eicom Co., Kyoto, Japan) and perfused with Ringer's solution (147.0 mmol/l NaCl, 4.0 mmol/l KCl, and 2.3 mmol/l $CaCl_2$) at a constant flow rate (5 μl/min). The other was connected to a tube that served as an outflow. Microdialysis procedures were preceded by a 30 min washout period prior to the baseline sample

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