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Nicotine decreases the activity of glutamate transporter type 3

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HIGHLIGHTS

- Nicotine (0.001–1 μM) resulted in a time- and dose-dependent decrease in EAAT3 activity in a *Xenopus* oocyte expression system.
- Nicotine decreases EAAT3 activity, but seems to be dependent on PKC and PI3K.
- Our results may provide an additional mechanism for nicotine-induced seizure.

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ABSTRACT

Nicotine, the main ingredient of tobacco, elicits seizures in animal models and cigarette smoking is regarded as a behavioral risk factor associated with epilepsy or seizures. In the hippocampus, the origin of nicotine-induced seizures, most glutamate uptake could be performed primarily by excitatory amino acid transporter type 3 (EAAT3). An association between temporal lobe epilepsy and EAAT3 downregulation has been reported. Therefore, we hypothesized that nicotine may elicit seizures through the attenuation of EAAT3 activity. We investigated chronic nicotine exposure (72 h) cause reduction of the activity of EAAT3 in a *Xenopus* oocyte expression system using a two-electrode voltage clamp. The roles of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) were also determined. Nicotine (0.001–1 μM) resulted in a time- and dose-dependent decrease in EAAT3 activity with maximal inhibition at nicotine concentrations of 0.03 μM or higher and at an exposure time of 72 h. V_{max} on the glutamate response was significantly reduced in the nicotine group (0.03 μM for 72 h), but the K_m value of EAAT3 for glutamate was not altered. When nicotine-exposed oocytes (0.03 μM for 72 h) were pretreated with phorbol-12-myristate-13-acetate (PMA, a PKC activator), the nicotine-induced reduction in EAAT3 activity was abolished. PKC inhibitors (staurosporine, chelerythrine, and calphostin C) significantly reduced basal EAAT3 activity, but there were no significant differences among the PKC inhibitors, nicotine, and PKC inhibitors + nicotine groups. Similar response patterns were observed among PI3K inhibitors (wortmannin and LY294002), nicotine, and PI3K inhibitors + nicotine. In conclusion, this study suggests that nicotine decreases EAAT3 activity, and that this inhibition seems to be dependent on PKC and PI3K. Our results may provide an additional mechanism for nicotine-induced seizure.

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1. Introduction

Tobacco is a popular product world-wide despite the fact that nicotine causes toxicity and dependency. Pharmacologically, nicotine (methylpyridylpyrrolidine) is a water-soluble liquid alkaloid (Lavoie and Harris, 1991) which causes diverse central nervous system (CNS) effects, ranging from reduction of anxiety to seizure and coma (Picciotto et al., 2002). Smoking is regarded as a behavioral risk factor associated with epilepsy or seizure in epidemiological studies. People with epilepsy were found to smoke cigarettes more often than those without epilepsy (38.8% vs. 24.9%) (Kobau et al., 2004). In addition, participants reporting current cigarette smoking have an increased risk of seizure in comparison with those who never smoke (relative risk 2.60, 95% confidence interval 1.53–4.42) (Dworetzky et al., 2010). Moreover, accidental nicotine ingestion has been shown to cause seizures in children and adults (Lavoie and Harris, 1991; Smolinske et al., 1988).

Glutamate transporters, also known as excitatory amino acid transporters (EAATs), are a family of high-affinity,

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sodium-dependent transporters that span the plasma membrane of glia and neurons and contribute to the clearance of glutamate from the extracellular space in order to maintain synaptic glutamate concentrations and prevent excitotoxicity (Danbolt, 2001). Substantial alterations in glutamate transport may be implicated in a wide spectrum of neurologic disorders, such as amyotrophic lateral sclerosis, epilepsy, Huntington's disease, Alzheimer's disease, ischemic stroke injury, white matter injury, and schizophrenia (Beart and O'Shea, 2007; Maragakis and Rothstein, 2001; Sheldon and Robinson, 2007).

Five different types of EAATs have been cloned. EAAT1 and EAAT2 are localized mainly in glial cells, EAAT3 and EAAT4 in neurons, and EAAT5 in the retina. In the hippocampus, abundantly expressed EAAT3 (Danbolt, 2001) may play a major role as glutamate transporter because many synapses are not encompassed by astrocytes (Bergles et al., 1999). Temporal lobe seizures are related to hippocampal sclerosis, a characteristic feature of hippocampal pathology (Bouilleret et al., 1999; Wieser, 2004). In addition, dysfunction of EAAT3 has been reported to be related to temporal lobe epilepsy (Crino et al., 2002; Mathern et al., 1999), and in vivo electrophysiological studies have revealed that nicotine-induced seizures originate in the hippocampus (Cohen et al., 1981; Floris et al., 1964). Therefore, nicotine may elicit seizures through attenuation of EAAT3 activity.

In this study, we examined the effects of nicotine on EAAT3 activity expressed in *Xenopus* oocytes and investigated the roles of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), two intracellular signaling molecules, in the effect of nicotine on EAAT3.

2. Experimental procedures

The study protocol was approved by the Institutional Animal Care and Use Committee at Seoul National University College of Medicine (protocol number: 12-0175). Mature female *Xenopus laevis* frogs were purchased from Xenopus I (Dexter, MI). Molecular biology reagents were obtained from Ambion (Austin, TX). Nicotine and other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.1. Oocyte preparation

Xenopus oocytes were harvested and microinjected as previously described (Do et al., 2002a). We anesthetized frogs in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO, USA) in water. Frogs underwent an operation on ice after checking for unresponsiveness to toe pinching. Following a 5 mm incision in the lower lateral abdominal region, an ovarian lobule, containing about 150–200 oocytes, was removed. Oocytes were instantly immersed in calcium-free OR-2 solution (NaCl 82.5 mM, KCl 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, and 0.1% collagenase type Ia; pH = 7.5) to remove the vitelline membrane. Oocytes were defolliculated by gentle shaking for nearly 2 h, and then incubated for one day in modified Barth's solution (NaCl 88 mM, KCl 1 mM, $NaHCO_2$ 2.4 mM, $CaCl_2$ 0.41 mM, $MgSO_4$ 0.82 mM, $Ca(NO_3)_2$ 0.3 mM, gentamicin 0.1 mM, and HEPES 15 mM; pH = 7.6) at 18 °C. Fully grown stage V or VI *Xenopus* oocytes were selected for the following experiments (Hollmann et al., 2000).

2.2. Expression of EAAT3

The complementary DNA (cDNA) of rat EAAT3 was provided by Dr. M.A. Hediger (Brigham and Women's Hospital, Harvard Institute of Medicine, Boston, MA, USA). The cDNA was subcloned into a commercial vector (Bluescript-SKm), and plasmid DNA was linearized using a restriction enzyme (Not I). We synthesized the messenger RNA (mRNA) in vitro using a transcription kit (Ambion, Austin, TX, USA). EAAT3 mRNA was quantified spectrophotometrically and diluted in sterile water. Thirty nanoliters of this mRNA (1 ng/nl) was injected into the cytoplasm of oocytes with an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA, USA). The prepared oocytes were subsequently incubated at 18 °C for 3–4 days to express EAAT3 before electrophysiologic recording.

2.3. Electrophysiological recording

We measured electrophysiological changes at room temperature (21–23 °C). Microelectrodes were prepared with a micropipette puller and 10 μl glass capillary tubes (Drummond Scientific Co.). The diameter of the microelectrode tips was adjusted to approximately 10 μm by breaking the tip and the resistance of the microelectrode was estimated at 1–5 MΩ when filled with 3 M KCl. Oocytes were perfused with Tyrode's solution (NaCl 150 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, dextrose 10 mM, and HEPES 10 mM; pH = 7.5) at a flow rate of 3 ml/min before measuring the currents. A single oocyte was voltage-clamped using a two-electrode oocyte voltage clamp amplifier (OC725-C: Warner Co., New Haven, CT, USA), with a holding potential of −70 mV and the evoked currents were analyzed with the Ooclamp software program. Data from oocytes that did not show a stable holding current of less than 0.6 μA were discarded. L-Glutamate was diluted in Tyrode's solution and perfused over an oocyte for 20 s at a rate of 3 ml/min. Inward currents yielded by superfusion of L-glutamate were recorded at 125 Hz for 1 min (baseline: 5 s, L-glutamate application: 20 s, washing with Tyrode's solution: 35 s). We measured the response induced by L-glutamate, which was calculated by integration of the inward currents and described as microCoulombs (μC). The response should reflect the sum of the total amount of transported glutamate. At least three different frogs were used for analysis in all of the experiments.

2.4. Chemicals

Nicotine ((−)-nicotine hydrogen tartrate), phorbol-12-myristate-13-acetate (PMA), staurosporine, chelerythrine, calphostin C, LY294002, and wortmannin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.5. Experimental chemical treatment

To evaluate the dose–response effect of nicotine on EAAT3 activity, oocytes were incubated in nicotine at serial concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM) by dilution in modified Barth's solution for 72 h. In the control group, oocytes were incubated in modified Barth's solution alone. In our previous study, the median effective concentration of glutamate that induced EAAT3 activity was 27.2 μM (Do et al., 2002b), so we used 30 μM glutamate as an agonist in this study. To study the effects of nicotine on the V_{max} and K_m of EAAT3 with L-glutamate, we used serial concentrations of L-glutamate (3, 10, 30, 100, and 300 μM).

To investigate PKC involvement in the effects of nicotine on EAAT3 activity, PKC activator (100 nM PMA) or PKC inhibitor (100 μM chelerythrine, 2 μM staurosporine, or 9 μM calphostin C) was applied to the oocytes for 10 min or 1 h, respectively, before recording the currents. Oocytes were exposed to the PI3K inhibitors (10 μM wortmannin and 50 μM LY294002) for 1 h to investigate the effect of PI3K inhibition on EAAT3 activity. To evaluate time course of the effects of nicotine exposure on the activity of EAAT3, oocytes were incubated in nicotine for 24, 48, and 72 h. The reversibility of the nicotine effect on EAAT3 activity was investigated by washing

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