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Dapoxetine induces neuroprotective effects against glutamate-induced neuronal cell death by inhibiting calcium signaling and mitochondrial depolarization in cultured rat hippocampal neurons

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) have an inhibitory effect on various ion channels including Ca^{2+} channels. We used fluorescent dye-based digital imaging, whole-cell patch clamping and cytotoxicity assays to examine whether dapoxetine, a novel rapid-acting SSRI, affect glutamate-induced calcium signaling, mitochondrial depolarization and neuronal cell death in cultured rat hippocampal neurons. Pretreatment with dapoxetine for 10 min inhibited glutamate-induced intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in a concentration-dependent manner (Half maximal inhibitory concentration=4.79 μM). Dapoxetine (5 μM) markedly inhibited glutamate-induced $[Ca^{2+}]_i$ increases, whereas other SSRIs such as fluoxetine and citalopram only slightly inhibited them. Dapoxetine significantly inhibited the glutamate-induced $[Ca^{2+}]_i$ responses following depletion of intracellular Ca^{2+} stores by treatment with thapsigargin. Dapoxetine markedly inhibited the metabotropic glutamate receptor agonist, (S)–3,5-dihydroxyphenylglycine-induced $[Ca^{2+}]_i$ increases. Dapoxetine significantly inhibited the glutamate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-induced $[Ca^{2+}]_i$ responses in either the presence or absence of nimodipine. Dapoxetine also significantly inhibited AMPA-evoked currents. However, dapoxetine slightly inhibited N-methyl-D-aspartate (NMDA)-induced $[Ca^{2+}]_i$ increases. Dapoxetine markedly inhibited 50 mM K^+-induced $[Ca^{2+}]_i$ increases. Dapoxetine significantly inhibited glutamate-induced mitochondrial depolarization. In addition, dapoxetine significantly inhibited glutamate-induced neuronal cell death and its neuroprotective effect was significantly greater than fluoxetine. These data suggest that dapoxetine reduces glutamate-induced $[Ca^{2+}]_i$ increases by inhibiting multiple pathways mainly through AMPA receptors, voltage-gated L-type Ca^{2+} channels and metabotropic glutamate receptors, which are involved in neuroprotection against glutamate-induced cell death through mitochondrial depolarization.

1. Introduction

Glutamate induces intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in neurons by activating glutamate receptors including ionotropic N-methyl-D-aspartate (NMDA) and non-NMDA receptors, and metabotropic glutamate receptors. Excessive glutamate-induced $[Ca^{2+}]_i$ increase induces mitochondrial matrix Ca^{2+} overload, which can lead to an enhanced formation of reactive oxygen species that triggers neuronal cell death (Nicholls, 2004). Glutamate-induced neurotoxicity is thought to be involved in pathological conditions such as brain ischemia, epilepsy and chronic neurodegenerative disorders (Arundine and Tymianski, 2003; Choi, 1992; Lipton and Rosenberg, 1994).

The major action of Selective serotonin reuptake inhibitors (SSRIs) is to prevent the uptake of serotonin by membrane serotonin transporter in the synaptic cleft (Wong et al., 1974), which enhances endogenous serotonin levels in the central nervous system (Carrasco and Sandner, 2005). Fluoxetine is known to inhibit various ion channels such as Ca^{2+}-permeable ion channels (Fan, 1994; Hahn et al., 1999; Kim et al., 2005, 2013; Kiss et al., 2012; Maertens et al.,

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1999; Pancrazio et al., 1998; Szasz et al., 2007; Wang et al., 2003) and to attenuate neuronal cell death (Jin et al., 2009; Kim et al., 2013; Li et al., 2006; Lim et al., 2009).

Dapoxetine, a novel rapid-acting SSRI, was developed to treat premature ejaculation (Feret, 2005). Although recent reports have shown that dapoxetine blocks K^+ channels (Jeong et al., 2012a, 2012b), no reports have established whether dapoxetine affects glutamate-induced Ca^{2+} signaling and neuronal cell death in neurons. We investigated whether dapoxetine affects glutamate-induced calcium signaling and mitochondrial depolarization in cultured rat hippocampal neurons. We also examined how dapoxetine affects glutamate-induced neuronal cell death.

Our results indicate that dapoxetine markedly reduces glutamate-induced $[Ca^{2+}]_i$ increases by inhibiting multiple pathways mainly through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, voltage-gated L-type Ca^{2+} channels, and metabotropic glutamate receptors that are involved in the neuroprotective effects against glutamate-induced cell death through mitochondrial depolarization.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle media, fetal bovine serum and horse serum were obtained from Gibco-BRL (Grand island, NY, USA). Fura-2 acetoxymethyl ester and rhodamine 123 were purchased from Molecular Probes (Eugene, OR, USA). Dapoxetine hydrochloride was obtained from AK Scientific Inc. (Union City, CA, USA). (*S*)-AMPA was obtained from A. G. Scientific, Inc. (San Diego, CA, USA). 3,5-dihydroxyphenylglycine (DHPG) and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Culture of primary rat hippocampal cells

Rat hippocampal cells were grown in primary co-culture as previously described (Kim et al., 2013) with minor modifications. Primary cells were obtained from the hippocampus of embryonic day 17 adult maternal Sprague-Dawley rats (250–300g). All procedures of animal research were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Experiment provided by the Institutional Animal Care and Use Committee in the College of Medicine, The Catholic University of Korea. Fetuses were removed on embryonic day 17 from rats that had been anesthetized with urethane (1.3g/kg body weight, i.p.). Hippocampi were dissected and placed in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution adjusted to pH 7.4 with NaOH. Cells were dissociated by trituration through a 5-ml pipette and a flame-narrowed Pasteur pipette. The cell suspension was centrifuged at 201g for 3 min, and the cells were resuspended in Dulbecco's modified eagle media without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Dissociated cells were plated in six-well culture plates at a density of 50,000 cells/well, onto 25-mm-round cover glasses (Fisher Scientific, Pittsburgh, PA, USA) that were coated with poly-D-lysine (0.1 mg/ml) and then washed with H_2O. Neurons and glial cells were grown in a humidified atmosphere of 10% CO_2/90% air (pH 7.4) at 37 °C. The medium was replaced 72–90 h after plating with Dulbecco's modified eagle media supplemented with 10% horse serum and penicillin/streptomycin and fed by an exchange of 25% of the medium after 7 days. The cells were cultured without mitotic inhibitors for a minimum of 12 days, and were used between 10 and 12 days in culture for fluorescent dye-based digital imaging methods and whole cell patch clamping.

2.3. Culture of primary rat pure hippocampal neurons

Isolation of hippocampal neurons was performed using the same procedures mentioned above. Dissociated cells were then plated at a density of 40,000 cells per well onto 96 wells previously coated with matrigel (0.2 mg/ml) (BD Bioscience, San Jose, CA, USA). Cells were grown in Neurobasal medium (Gibco/Life Technologies, St. Petersburg, FL, USA) supplemented with 2% B27, 1% penicillin/streptomycin, 0.5 mM glutamine, and 25 μM glutamate at 37 °C in 10% CO_2. One-half of the culture medium was changed every 3–4 days without glutamate (Yang et al., 2015). For cytotoxicity assays, cells were used between 11 and 12 days in culture.

2.4. Calcium imaging

Calcium imaging was performed as described by Kim et al. (2013). The hippocampal cells were loaded with 12 μM fura-2 acetoxymethylester in HEPES-buffered Hank's balanced salt solution containing 0.5% bovine serum albumin for 45 min at 37 °C. The HEPES-buffered Hank's balanced salt solution was composed of the following: 20 mM HEPES; 137 mM NaCl; 1.26 mM $CaCl_2$; 0.4 mM $MgSO_4$; 0.5 mM $MgCl_2$; 5 mM KCl; 0.4 mM KH_2PO_4; 0.6 mM Na_2HPO_4; 3 mM $NaHCO_3$; and 5.6 mM glucose. Fura-2-loaded cells were alternately excited at 340 and 380 nm. Digital fluorescence images (510 nm) were collected with a computer-controlled, cooled, charge-coupled device camera (1280×1035 binned to 256×207 pixels, Quantix, Photometrics, Tucson, AZ, USA). A ratio of 340/380 nm was calculated from the background-subtracted digital images. Background images were collected at the beginning of each of the experiments after removing cells from another area to the coverslip.

2.5. Whole-cell voltage clamp recordings

Whole-cell currents were recorded with an EPC-8 amplifier (HEKA Elektronik, Lambrecht, Germany) in conjunction with a 3-barrel pipette with a fast-step solution switcher (SF-77B; Warner Instruments, Hamden, CT, USA) at room temperature (20–24 °C). Control and drug solutions were perfused through adjacent square glass capillaries placed within 100 μm of the neuronal cell body. Patch electrodes were pulled from borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL, USA) using a programmable horizontal microelectrode puller (P-97; Sutter Instrument Co., Novata, CA, USA). Patch pipettes had a resistance of 3–6 MΩ when filled with internal solution. Recordings were acquired using a Digidata 1322 A and pCLAMP 9.2 Software (Axon Instruments, Inc., CA, USA), filtered at 1 kHz, and digitized at 2–5 kHz. The holding potential was set at −50 mV. The external solution contained the following: 140 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM glucose, pH 7.3 with NaOH. The patch electrode was filled with a solution containing the following: 140 mM CsCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′* -tetra-acetic acid (EGTA), and 10 mM HEPES, pH 7.3 with CsOH.

2.6. Measurement of mitochondrial membrane potential

To monitor mitochondrial membrane potential, cells were loaded with 10 μM rhodamine 123 for 15 min. The fluorescence of rhodamine 123 was detected at 535 ± 25 nm following excitation of cells at 480 ± 20 nm using the same system that was used for calcium imaging, as mentioned above. Change in mitochondrial membrane potential was shown as a percentage of the initial intensity of rhodamine 123. Each measure of fluorescence was background-subtracted and normalized to starting values as F/F_0 (Yang et al., 2015).