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HYDROGEN PEROXIDE MODULATES NEURONAL EXCITABILITY AND MEMBRANE PROPERTIES IN VENTRAL HORN NEURONS OF THE RAT SPINAL CORD

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Abstract—Hydrogen peroxide (H_2O_2), a reactive oxygen species, is an important signaling molecule for synaptic and neuronal activity in the central nervous system; it is produced excessively in brain ischemia and spinal cord injury. Although H_2O_2-mediated modulations of synaptic transmission have been reported in ventral horn (VH) neurons of the rat spinal cord, the effects of H_2O_2 on neuronal excitability and membrane properties remain poorly understood. Accordingly, the present study investigated such effects using a whole-cell patch-clamp technique. The bath-application of H_2O_2 decreased neuronal excitability accompanied by decreased input resistance, firing frequency, and action potential amplitude and by increased rheobase. These H_2O_2-mediated changes were induced by activation of extrasynaptic, but not synaptic, $GABA_A$ receptors. Indeed, GABAergic tonic currents were enhanced by H_2O_2. On the other hand, the amplitude of medium and slow afterhyperpolarization (mAHP and sAHP), which plays important roles in controlling neuronal excitability and is mediated by small-conductance calcium-activated potassium (SK) channels, was significantly decreased by H_2O_2. When extrasynaptic $GABA_A$ receptors were completely blocked, these decreases of mAHP and sAHP persisted, and H_2O_2 increased excitability, suggesting that H_2O_2 per se might have the potential to increase neuronal excitability via decreased SK channel conductance. These findings indicate that activating extrasynaptic $GABA_A$ receptors or SK channels may attenuate acute neuronal damage caused by H_2O_2-induced hyperexcitability and therefore represent a novel therapeutic target for the prevention and treatment of H_2O_2-induced motor neuron disorders.

Key words: tonic current, extrasynaptic $GABA_A$ receptor, afterhyperpolarization, small-conductance calcium-activated potassium channel, patch clamp.

INTRODUCTION

Hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), is a relatively stable and lipid-soluble molecule that diffuses into the cytoplasm (Halliwell, 1992; Ward and Giles, 1997), where it is an important signaling molecule in synaptic and neuronal activity in the central nervous system (CNS) (Giorgio et al., 2007; Rice, 2011). Under physiological conditions, H_2O_2 production is balanced by enzymes such as catalase and glutathione peroxidase, which reduce H_2O_2 to H_2O and O_2 (Halliwell, 1992; Hou et al., 2010). However, excessive production of H_2O_2 has been implicated in ischemia-reperfusion lesions of the brain (Hyslop et al., 1995) and in spinal cord injury (Liu et al., 1999). In the CNS, H_2O_2 modulates neuronal functions, including synaptic transmission (Chen et al., 2001; Takahashi et al., 2007; Nani et al., 2010; Ohashi et al., 2016) and cellular excitability (Avshalumov et al., 2005; Pouokam et al., 2009; Nani et al., 2010; Garcia et al., 2011; Ostrowski et al., 2014).

Concerning spinal ventral horn (VH) neurons, which are vulnerable to oxidative stress (Carri et al., 2003) and ischemia (Sakurai et al., 1997; Nohda et al., 2007), we have recently reported an increase in glutamate release from excitatory presynaptic terminals following the exogenous application of H_2O_2, and a corresponding increase in γ-aminobutyric acid (GABA) from inhibitory presynaptic terminals (Ohashi et al., 2016). However, little is known regarding the effects of H_2O_2 on VH neuron excitability and cellular membrane properties, despite evidence for H_2O_2 in other CNS regions, including brainstem (Nani et al., 2010; Ostrowski et al., 2014), hippocampus (Garcia et al., 2011), and midbrain (Avshalumov et al., 2005). Therefore, using a whole-cell patch-clamp method, the present study investigated the mechanisms by which excessive levels of H_2O_2 influence neuronal excitability and membrane properties in rat VH neurons.

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Abbreviations: ACSF, artificial cerebrospinal fluid; AP, action potential; AHP, afterhyperpolarization; BIC, bicuculline; CNS, central nervous system; fAHP, fast afterhyperpolarization; GABA, γ-aminobutyric acid; IPSC, inhibitory postsynaptic current; IR-DIC, infrared-differential interference contrast; mAHP, medium afterhyperpolarization; mIPSC, miniature inhibitory postsynaptic current; R_{in}, input resistance; RMP, resting membrane potential; ROS, reactive oxygen species; sAHP, slow afterhyperpolarization; sIPSC, spontaneous inhibitory postsynaptic current; SK channel, small conductance potassium channel; STR, strychnine; TTX, tetrodotoxin; VH, ventral horn.

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EXPERIMENTAL PROCEDURES

Study approval

The Animal Care and Use Committee at Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan) approved all experimental procedures that involved the use of animals. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of spinal cord slices

Rat spinal cord slices were prepared as described in a previous study (Ohashi et al., 2016). In brief, neonatal Wistar rats of either sex (7–15 days old) were anesthetized using urethane (1.5 g/kg by intraperitoneal injection), and the lumbosacral segment (L1–S3) of the spinal cord was subsequently removed. Spinal cord specimens were stored in pre-oxygenated, ice-cold (2–4 °C) artificial cerebrospinal fluid (ACSF), and transverse spinal cord slices (500 μm) were obtained using a microslicer (Linear Slicer PRO 7; Dosaka, Kyoto, Japan). The ACSF was composed of (in mM) 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11.5 D-glucose. Spinal cord slices were then transferred to a recording chamber and set on the stage of an upright microscope featuring an infrared-differential interference contrast (IR-DIC) system (E600FN; Nikon, Tokyo, Japan). Each slice was fixed with an anchor, and superfused with ACSF (gas mixture of 95% O_2 and 5% CO_2 (pH = 7.4)) at 5–6 ml/min, with the temperature maintained at 36 °C ± 0.5 °C using a temperature control system (TC-324B; Warner Instruments, Hamden, CT, USA). Slices were used for recording after a recovery period of ⩾40 min.

Patch-clamp recordings from spinal VH neurons

As reported previously (Ohashi et al., 2016), whole-cell patch-clamp recordings were obtained from visually identified large VH neurons in Rexed lamina IX (size: >25 μm) using an IR-DIC microscope combined with a charge-coupled device camera (C2400-79H; Hamamatsu Photonics, Hamamatsu, Japan). A somal size > 20 μm was used as the cut-off point in previous studies of postnatal rat lumbar motor neurons (Takahashi, 1990); however, large interneurons (>20 μm) have been detected in the ventral half of the rat lumbar spinal cord (Thurbon et al., 1998a,b). Therefore, putative motor neurons were identified as the largest cells in the VH (somal diameter > 25 μm). Borosilicate glass capillaries were used to construct whole-cell patch-clamp pipettes (1.5-mm outer diameter; World Precision Instruments, Sarasota, FL, USA). Pipettes typically demonstrated a resistance of 4–8 M when filled with an internal solution (see below for recipes). Signals were amplified using an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA), filtered, and digitized at 2 kHz and 5 kHz respectively. Data were derived and analyzed using the pCLAMP 10.3 data acquisition program (Molecular Devices).

Current-clamp protocols

To test neuronal excitability and membrane properties, current-clamp recordings were performed using a potassium gluconate-based pipette solution consisting of (in mM) 135 potassium gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, and 5 ATP-Mg (pH 7.2). Resting membrane potential (RMP) was measured under $I = 0$ condition prior to current injection, and neurons with RMP higher than −50 mV were excluded. Once stable access was established, 1-s hyper- and depolarizing currents (−80 to 220 pA, 20 pA incremental steps) were injected.

Voltage-clamp protocols

A cesium sulfate-based solution composed of (in mM) 110 Cs_2SO_4, 5 tetraethylammonium, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, and 5 ATP-Mg (pH 7.2) was used to establish the whole-cell configuration. Next, neurons were voltage-clamped at 0 mV in order to record inhibitory postsynaptic currents (IPSCs). Since the reversal potentials of excitatory postsynaptic currents were 0 mV under the above experimental conditions (Aoyama et al., 2010; Honda et al., 2012; Yamamoto et al., 2012), IPSCs were specifically recorded as upward deflections at 0 mV. To isolate $GABA_A$ receptor-mediated IPSCs, strychnine (STR, 2 μM) was added as previously reported (Baba et al., 2000; Ataka and Gu, 2006; Ohashi et al., 2016). To identify tonic $GABA_A$ receptor-mediated currents, the selective $GABA_A$ receptor antagonist, bicuculline (BIC, 20–200 μM), was added, and the change in holding current was measured.

Drug application

The following pharmacological agents were purchased from Wako (Osaka, Japan): H_2O_2 (1 mM), tetrodotoxin (TTX; 1 μM), CGP35348 (20 μM). BIC (20–200 μM), STR (2 μM), and GABA (1–100 μM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Immediately prior to each experiment, H_2O_2 was prepared by dissolving in ACSF. TTX, STR, and CGP35348 were dissolved at 1000 times the working concentration in distilled water. Dimethyl sulfoxide was used to dilute BIC solutions to 1000 times their storage concentration. These were then diluted to their working concentration in ACSF immediately prior to use. Solutions were administered to the whole slice via perfusion using a three-way stopcock at a constant perfusion rate and temperature. The volume of the recording chamber was approximately 1.0 ml. Within 15 s of opening the stopcock, the solutions reached the recording chamber, and within 90 s of its being closed, each drug was completely washed out. To study the acute effects of ROS, H_2O_2 was applied for 5 min at a concentration of 1 mM, which was previously shown to induce neurotoxicity in spinal neurons (Taccola et al., 2008) and hypoglossal motoneurons (Nani et al., 2010) *in vitro*. Slices were discarded after each H_2O_2 application experiment.