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REACTIVE OXYGEN SPECIES ENHANCE EXCITATORY SYNAPTIC TRANSMISSION IN RAT SPINAL DORSAL HORN NEURONS BY ACTIVATING TRPA1 AND TRPV1 CHANNELS

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Abstract—Central neuropathic pain (CNP) in the spinal cord, such as chronic pain after spinal cord injury (SCI), is an incurable ailment. However, little is known about the spinal cord mechanisms underlying CNP. Recently, reactive oxygen species (ROS) have been recognized to play an important role in CNP of the spinal cord. However, it is unclear how ROS affect synaptic transmission in the dorsal horn of the spinal cord. To clarify how ROS impact on synaptic transmission, we investigated the effects of ROS on synaptic transmission in rat spinal cord substantia gelatinosa (SG) neurons using whole-cell patch-clamp recordings. Administration of tert-butyl hydroperoxide (t-BOOH), an ROS donor, into the spinal cord markedly increased the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) in SG neurons. This t-BOOH-induced enhancement was not suppressed by the Na^+ channel blocker tetrodotoxin. However, in the presence of a non-N-methyl-D-aspartate glutamate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione, t-BOOH did not generate any sEPSCs. Furthermore, in the presence of a transient receptor potential ankyrin 1 (TRPA1) channel antagonist (HC-030031) or a transient receptor potential vanilloid 1 (TRPV1) channel antagonist (capsazepine or AMG9810), the t-BOOH-induced increase in the frequency of sEPSCs was inhibited. These results indicate that ROS enhance the spontaneous release of glutamate from presynaptic terminals onto SG neurons through TRPA1 and TRPV1 channel activation. Excessive activation of these ion channels by ROS may induce central sensitization in the spinal cord and result in chronic pain such as that following SCI.

Key words: ROS, spinal cord, pain, TRPV1, TRPA1, central neuropathic pain.

INTRODUCTION

Central neuropathic pain (CNP) in the spinal cord, such as chronic pain after spinal cord injury (SCI), is an incurable disease for which the underlying molecular mechanisms have not been elucidated. As there is no effective treatment for CNP, a large number of individuals suffer from this form of severe chronic pain. A major feature of CNP in the spinal cord is central sensitization induced by neuronal plasticity in substantia gelatinosa (SG) neurons. However, neuronal plasticity in the spinal cord is a complex process impacted by numerous factors.

Several studies suggest that reactive oxygen species (ROS) can cause central sensitization in the spinal cord, and are involved in persistent pain (Wang et al., 2004; Salvemini et al., 2011). ROS are highly reactive molecules derived from O_2, and include free radicals [e.g. superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$)] and other reactive species [e.g. hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$)]. A major function of ROS is immunological host defense. However, while ROS are essential for health, high levels of ROS can cause various disorders, such as cancer, arteriosclerosis, hypertension and neurodegenerative diseases (Dröge, 2002; Valko et al., 2007). At excess levels, the normal physiological roles of ROS in cellular metabolism and signal transduction are supplanted by toxicity (Lander, 1997). Recently, ROS have been implicated in the etiology of chronic pain, including neuropathic and inflammatory pain (Salvemini et al., 2011). For example, it has been reported that ROS are involved in long-term potentiation (LTP) in the dorsal horn (Lee et al., 2010) and in capsaicin-induced secondary hyperalgesia (Schwartz et al., 2008). Therefore, ROS may mediate nociceptive signaling in the dorsal horn of the spinal cord, in addition to functioning as neuromodulators. It is well known that in spinal cord trauma, the release of ROS often induces second injury; therefore, it is thought that ROS are involved in the pathogenesis of post-SCI pain (Hulsebosch et al., 2009). However, the cellular

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Abbreviations: AITC, allyl isothiocyanate; AMPA, α-amino-3-hydroxy-5-methyl-4-isozazole propionate; CNP, central neuropathic pain; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DRG, dorsal root ganglion; EPSC, excitatory postsynaptic currents; IPSC, inhibitory postsynaptic currents; LTP, long-term potentiation; mEPSCs, miniature EPSCs; NAC, N-acetylcysteine; NMDA, N-methyl-D-aspartate; PBN, phenyl-N-tert-butylnitrone; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCI, spinal cord injury; sEPSC, spontaneous excitatory postsynaptic currents; SG, substantia gelatinosa; t-BOOH, tert-butyl hydroperoxide; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; TTX, tetrodotoxin.

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mechanisms underlying the effects of ROS in the spinal cord are still unclear.

Moreover, recent reports have suggested that transient receptor potential (TRP) channels are involved in CNP in the spinal cord (Kanai et al., 2005; Patapoutian et al., 2009; Patwardhan et al., 2009; Kim et al., 2012). TRP channels belong to a family of ion channels that are activated by temperature and which are expressed in primary sensory nerve terminals, where they provide information about thermal changes in the environment (Tominaga, 2007; Vay et al., 2012). There are six thermosensitive ion channels in mammals (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1), all of which belong to the TRP superfamily. These channels are involved in chemical, mechanical and thermal nociception. TRP channels are drug targets for the relief of pain, including neuropathic pain (Levine and Alessandri-Haber, 2007; Patapoutian et al., 2009; Stucky et al., 2009; Holzer, 2011; Wei et al., 2011b). In particular, TRPV1 (TRP vanilloid 1) and TRPA1 (TRP ankyrin 1) have been a major focus of research into the mechanisms of inflammatory and neuropathic pain. Therefore, to clarify the mechanisms underlying neuropathic pain, we investigated the effects of ROS on glutamatergic excitatory synaptic transmission in SG neurons in adult rat spinal cord slices using the whole-cell patch-clamp recording technique, and we analyzed the role of TRPV1 and TRPA1 channels in these ROS-mediated effects.

EXPERIMENTAL PROCEDURES

All of the experimental procedures involving the use of animals were approved by the Ethics Committee on Animal Experiments, Kansai University of Health Sciences, and were in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and associated guidelines.

Spinal cord slice preparation

The methods used for obtaining adult rat spinal cord slice preparations have been described previously (Nakatsuka et al., 1999). In brief, male adult Sprague–Dawley rats (5–6 weeks of age, 170–200 g) were deeply anesthetized with urethane (1.2 g/kg, IP), and then lumbosacral laminectomy was performed. The lumbosacral spinal cord (L1–S3) was removed and placed in pre-oxygenated Krebs solution at 1–3 °C. Immediately after the removal of the spinal cord, the rats were given an overdose of urethane and were then killed by exsanguination. The pia-arachnoid membrane was removed after cutting all of the ventral and dorsal roots near the root entry zone. The spinal cord was mounted on a microslicer and a 600-μm-thick transverse slice was cut from the lumbar region containing the L4 or L5 dorsal root entry zone. The slice was placed on a nylon mesh in the recording chamber, which had a volume of 0.5 ml, and then perfused at a rate of 10–15 ml/min with Krebs solution saturated with 95% O_2 and 5% CO_2, and maintained at 36 ± 1 °C. The Krebs solution contained the following (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 glucose, pH 7.4.

Patch-clamp recordings from SG neurons

Blind whole-cell patch-clamp recordings were made from SG neurons with patch-pipette electrodes having a resistance of 5–10 MΩ. The patch-pipette solution used to record excitatory postsynaptic currents (EPSCs) was composed of the following (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. Membrane potentials were held at −70 mV in voltage-clamp mode. After making a gigaohm seal, the membrane patch was ruptured by a brief period of more negative pressure, thus resulting in a whole cell configuration. Signals were acquired with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA). Data were digitized with an analog-to-digital converter (Digidata 1440A; Molecular Devices) and stored on a personal computer using the pCLAMP 10 data acquisition program (Molecular Devices). They were analyzed using Mini Analysis 6.0 software (Synaptosoft, Fort Lee, NJ, USA) and the pCLAMP 10 data acquisition program. SG neurons were viable for up to 24 h in slices perfused with pre-oxygenated Krebs solution. However, all of the recordings described here were obtained within 12 h. Whole-cell patch-clamp recordings were stable for up to 4 h. The membrane potentials were not corrected for the liquid junction potential between the Krebs and patch-pipette solutions.

Application of drug

Drugs were dissolved in Krebs solution and applied by perfusion via a three-way stopcock without any change in the perfusion rate or the temperature. The time necessary for the solution to flow from the stopcock to the surface of the spinal cord was ~30 s. The drugs used in this study were tert-butyl hydroperoxide (t-BOOH), phenyl-N-tert-butylnitrone (PBN), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), N-acetylcysteine (NAC), HC-030031, capsazepine, AMG9810 (Sigma, St. Louis, MO, USA), tetrodotoxin (TTX) (Latoxan, Valence, France) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (TOCRIS, Bristol, UK). CNQX, TEMPOL, HC-030031, capsazepine and AMG9810 were dissolved in dimethyl sulfoxide as 1000× stock solutions. TTX, PBN and NAC were dissolved in distilled water as 1000× stock solutions. These drugs were diluted to the final concentration in Krebs solution immediately before use. The osmotic pressure of nominally Ca^{2+}-free, high-Mg^{2+} (5 mm) Krebs solution was adjusted by lowering the Na^+ concentration.

Statistical analysis

All numerical data were expressed as the mean ± S.E.M. Paired Student's *t* test or Welch's *t* test was used to determine the statistical significance between means, and the Kolmogorov–Smirnov test was used to compare