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Research Paper

Constitutive activity of transient receptor potential vanilloid type 1 triggers spontaneous firing in nerve growth factor-treated dorsal root ganglion neurons of rats

Naoki Kitamura*, Erika Nagami, Yumi Matsushita, Tomohiko Kayano, Izumi Shibuya

Laboratory of Veterinary Physiology, Joint Department of Veterinary Medicine, Faculty of Agriculture, Tottori University, 4-101, Koyama-cho Minami, Tottori 680-8553, Japan

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ABSTRACT

Dorsal root ganglion (DRG) neurons cultured in the presence of nerve growth factor (NGF, 100 ng/ml) often show a spontaneous action potential. Underlying mechanisms of this spontaneous firing were examined using the patch clamp technique. The spontaneous firing in the on-cell configuration was abolished by a decrease in the Na^+ concentration and by the TRPV1 antagonists capsazepine (10 μM) and BCTC (1 μM). These responses were accompanied by hyperpolarization of the resting potential. The holding current observed in neurons voltage clamped at –60 mV in the whole-cell configuration was significantly larger in the neurons that fired spontaneously, indicating that these neurons had an additional cation conductance that caused depolarization and triggered action potentials. The holding current in the firing neurons was decreased by extracellular Na^+ reduction, capsazepine and BCTC. The amplitudes of the capsazepine- or BCTC-sensitive component of the holding current in the spontaneously firing neurons were ten times as large as those recorded in the other neurons showing no spontaneous firing. However, the amplitudes of the current responses to capsaicin (1 μM) were not different regardless of the presence of spontaneous firing or treatment with NGF. These results indicate that chronic NGF treatment of cultured DRG neurons in rats induces a constitutively active cation conductance through TRPV1, which depolarizes the neurons and triggers spontaneous action potentials in the absence of any stimuli. Since NGF in the DRG is reported to increase after nerve injury, this NGF-mediated regulation of TRPV1 may be a cause of the pathogenesis of neuropathic pain.

1. Introduction

Peripheral nerve injury often causes an abnormal pain that continues after relief of the injury itself. Patients feel pain in response to weak stimuli that usually cause no pain (allodynia, hyperalgesia) or in the absence of any stimuli (chronic pain). This condition is called neuropathic pain, and it is well known that neuropathic pain is poorly responsive to usual analgesic treatments, including the administration of narcotic drugs such as morphine. At present, effective therapy for neuropathic pain is lacking, and neuropathic pain spoils the quality of life of many patients. The pathogenic mechanisms of neuropathic pain are complicated and not well understood, which may be a reason that we have no effective treatment for neuropathic pain. It is reported that artificial injury of peripheral nerves in rats induces neuropathic symptoms (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Decosterd and Woolf, 2000; Winkelstein et al., 2001; Barrot, 2012), and abnormal spontaneous firing has been observed in sensory neurons of some animal models with nerve injury, both *in vivo* (Burchiel, 1984; Devor et al., 1992; Eide, 1998; Liu et al., 2000; Sun et al., 2005) and *in vitro* (Petersen et al., 1996; Study and Kral, 1996; Amir et al., 1999; Devor, 1999; Liu et al., 1999). Such abnormal firing is considered to be a cause of spontaneous pain.

Nerve growth factor (NGF) is known as one of the mediators that cause neuropathic pain because NGF induces hyperalgesia in rats (Lewin et al., 1993; Woolf et al., 1994; Andreev et al., 1995) and because the expression level of NGF in the dorsal root ganglion (DRG) rises after nerve injury (Herzberg et al., 1997; Shen et al., 1999). Therefore, trials using anti-NGF agents to cure neuropathic pain conditions have been conducted (Cattaneo, 2010; Ossipov, 2011; McKelvey et al., 2013). Actions of NGF in the pathogenesis of neuropathic pain are complicated: NGF seems to have effects on both peripheral tissues and the central nervous system (Lewin et al., 1994; Hao et al., 2000). It

* Corresponding author.
E-mail address: nakit@tottori-u.ac.jp (N. Kitamura).

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is also reported that increased NGF in the DRG causes an extension of sympathetic nerves that make synapses onto DRG neurons and transmit excitatory signals by releasing noradrenaline (Zhang and Tan, 2011). On the other hand, it was reported that DRG neurons that were isolated from adult rats and cultured in the presence of NGF generated action potentials (APs) spontaneously (Kitamura et al., 2005); from these neurons, spontaneous APs were recorded in the on-cell configuration without intracellular dialysis with an artificial solution, and spontaneous action currents (named "I_{sp}") were recorded even under the voltage-clamped condition in the whole-cell configuration (Kayano et al., 2013). Based on the evidence that I_{sp} was blocked by tetrodotoxin (a blocker of the voltage-gated Na^+ channel), it is concluded that I_{sp} reflects spontaneous discharges occurring in loosely voltage-clamped areas of the cell membrane. Chronic treatment of DRG neurons with NGF seemed to activate an intrinsic mechanism, which caused the hyperexcitability, within the membrane of the soma of DRG neurons because the I_{sp} was also recorded from the outside-out patch membranes excised from the soma (Kayano et al., 2013).

The essential factors for neurons to generate an AP are (1) a resting membrane potential that is polarized below the threshold potential for the generation of the AP and (2) an ion conductance that drives membrane potentials to a potential above the threshold of the AP. We hypothesized that NGF induces some additional ionic conductance, which is constitutively active in the absence of any stimuli, in cultured DRG neurons and that this constitutively active conductance makes neurons hyperexcitable. One of the typical ion channels that confers such conductance to neurons is a non-selective cation channel belonging to the transient receptor potential (TRP) superfamily. Among these channels, TRP vanilloid 1 (TRPV1) plays very important roles in nociception (Caterina et al., 1997). It is reported that NGF increases the expression level and activity of TRPV1 in trigeminal neurons (Price et al., 2005), DRG neurons (Ji et al., 2002; Stein et al., 2006; Eskander et al., 2015) and the heterologous expression system (Zhang et al., 2005; Stein et al., 2006). Therefore, we examined the role of TRPV1 in the generation of spontaneous APs in NGF-treated cultured DRG neurons of rats in the present study and found that chronic treatment with NGF induces an additional cation conductance through TRPV1, which triggers spontaneous firing.

2. Experimental procedures

2.1. Cell isolation and culture

All animal experiments were performed in accordance with the guidelines of Tottori University, and this study was approved by the Institutional Animal Care and Use Committee, Tottori University. DRG neurons were isolated from male Wistar rats (7–12 weeks old) using a conventional enzymatic procedure. The rats were sacrificed by decapitation under anesthesia with isoflurane, and all efforts were made to minimize the suffering of the rats. Ganglia were dissected from the entire length of the vertebral column. The collected ganglia were incubated at 37 °C in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing collagenase type IV (200–400 U/ml; Worthington Biochemicals, Lakewood, NJ, USA), DNase I (1–50 μg/ml; Sigma, St Louis, MO, USA) and bovine serum albumin (BSA, 1 mg/ml, Sigma) for 2 h and then rinsed with PBS to remove collagenase. Next, ganglia were incubated in PBS containing trypsin (Gibco trypsin 1:250; 0.25%; Life Technologies, Carlsbad, CA, USA) and BSA (1 mg/ml) for 10 min. After the enzymatic digestion, tissues were gently agitated with a silicon-coated Pasteur pipette and centrifuged to remove the enzymes. The isolated cells were suspended in Dulbecco's-modified Eagle medium (DMEM, Life Technologies) containing 4.5 g/l of glucose and cultured on coverslips coated with poly-D-lysine (Sigma). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 until use. DMEM was supplemented with 10% fetal bovine serum (MP Biochemicals, Irvine, CA, USA), 100 U/ml penicillin (Sigma), 100 ng/ml streptomycin (Sigma), and 5 μM cytosine arabinoside (Sigma). The culture medium was changed every 2–3 days. NGF-7S (100 ng/ml, Sigma) was added to the medium 3–5 days after the isolation of neurons. Neurons were used in the electrophysiological experiments after 4–8 days of culture.

2.2. Electrophysiology

The on-cell and whole-cell recording was made at room temperature (22–24 °C). Heat-polished glass electrodes with a tip resistance of 2.5–4 MΩ were used. DRG neurons with a small diameter (10–30 μm), which had been reported to be largely C-neurons (Harper and Lawson, 1985a, 1985b), were used to record membrane potentials and currents. Neurons with these sizes are well known to express TRPV1 (Luo et al., 2004; Yu et al., 2008) and we confirmed TRPV1 expression by recording capsaicin-evoked current responses in this study. A standard bath solution consisted of 150 NaCl, 6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 D-glucose and 10 HEPES (in mM), and the pH was adjusted to 7.4 with Tris. When an effect of a decrease in the Na^+ concentration was examined, Na^+ was replaced with N-methyl-D-glucamine chloride (NMDG-Cl; Merck, Darmstadt, Germany). The pipette solution consisted of 130 K-gluconate, 10 Na-gluconate, 4.5 $MgCl_2$, 0.74 $CaCl_2$, 10 EGTA-2K and 10 HEPES (in mM), and the pH was adjusted to 7.3 with Tris or gluconate. The liquid junction potential between the pipette solution and the bath solution was not corrected because it was recorded to be smaller than 2 mV. Neurons were continuously perfused with the bath solution at a flow rate of 1 ml/min throughout the experiments. Current responses under the voltage-clamped condition were measured with Axopatch 200 A (Molecular Devices, Sunnyvale, CA, USA) or EPC-10 (HEKA, Lambrecht/Pfalz, Germany), and potential changes under the current-clamp condition were measured by EPC-10 (HEKA). Cell capacitances were determined by integrating the area under a capacity transient current elicited by a 5-mV voltage step from the holding potential, and a series resistance was also calculated from the capacity transient and the cell capacitance. Voltage errors at the holding potential caused by a series resistance were smaller than 1 mV and were not corrected. To estimate basal levels of resting membrane potentials or holding currents between spontaneous spikes, we calculated a moving average during 10 s, in which the influence of rapid responses such as APs was minimized. In the figures, the moving average during 10 s is shown as the basal level of the membrane potentials and holding currents.

2.3. Drugs

A concentrated stock solution of NGF-7S at 10 μg/ml was made by dissolution in DMEM and stored at –30 °C until use. Concentrated stock solutions of 4-(3-chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl) phenyl]-1- piperazinecarboxamide (BCTC, 10 mM, Sigma), 5'-iodoresiniferatoxin (5'-IRTX; 200 μM; Alomone Labs, Jerusalem, Israel), capsaicin (10 mM, Sigma), capsazepine (10 mM, Sigma), icilin (50 mM, Sigma) and SB366791 (30 mM, Sigma) were made by dissolution in DMSO and stored at –30 °C until use. All other chemicals used were of analytical grade.

2.4. Data analysis and statistics

Data acquisition was performed at a sampling frequency of 40–100 kHz throughout the experiments by a personal computer (Macintosh; Apple, Cupertino, CA, USA) in conjunction with an analog/digital converter (Power Lab; AD Instruments, Castle Hill, NSW, Australia). Data were analyzed with AxoGraph (AxoGraph Scientific, Sydney, NSW, Australia), Patch Master (HEKA), Lab Chart (AD Instruments), IGOR Pro (WaveMetrics, Lake Oswego, OR, USA) and Excel (Microsoft, Redmond, WA, USA). Data are presented as the mean values ± SEM (n = the number of observations). Statistical