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pH-dependent inhibition of tetrodotoxin-resistant Na^+ channels by diclofenac in rat nociceptive neurons

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of inflammatory pain. It is well established that NSAIDs exert their analgesic effects by inhibiting cyclooxygenase to prevent the production of prostaglandins; however, several NSAIDs including diclofenac also modulate other ion channels expressed in nociceptive neurons. In this study, we investigated the pH-dependent effects of diclofenac on tetrodotoxin-resistant (TTX-R) Na^+ channels in rat trigeminal sensory neurons by using the whole-cell patch clamp technique. Diclofenac decreased the peak amplitude of TTX-R Na^+ currents (I_{Na}) in a concentration dependent manner. While diclofenac had little effect on the voltage-activation relationship, it significantly shifted the steady-state fast inactivation relationship toward hyperpolarized potentials. Diclofenac increased the extent of use-dependent inhibition of TTX-R Na^+ currents. Diclofenac also significantly accelerated the development of inactivation and retarded the recovery from inactivation of TTX-R Na^+ channels. The effects of diclofenac on TTX-R Na^+ channels were stronger at pH 6.0 than at pH 7.4 for most of the parameters tested. Considering that the extracellular pH falls in inflamed tissues, and that TTX-R Na^+ channels expressed on nociceptive neurons are implicated in the prostaglandin-mediated development and maintenance of inflammatory hyperalgesia, our findings could provide an additional analgesic effect of diclofenac under acidic pH conditions.

1. Introduction

Sensory neurons express multiple types of voltage-gated Na^+ channels, which can be divided into tetrodotoxin-sensitive (TTX-S; $Na_V1.1$, $Na_V1.2$, $Na_V1.6$, and $Na_V1.7$) and TTX-resistant (TTX-R) Na^+ channels ($Na_V1.8$ and $Na_V1.9$) (Akopian et al., 1999; Chahine and O'Leary, 2014; Waxman and Zamponi, 2014). Functionally, about half of TTX-S Na^+ channels are inactivated at resting membrane potentials of nociceptive neurons, but TTX-R Na^+ channels can still be activated by depolarization from resting membrane potentials (Elliott and Elliott, 1993; Gold and Gebhart, 2010; Ogata and Tatebayashi, 1993). Therefore, TTX-R Na^+ channels might be suitable for the generation of action potential even at certain conditions that TTX-S Na^+ channels are inactivated, by the elevation of resting membrane potential (Renganathan et al., 2001; Rush et al., 1998; Pinto et al., 2008). TTX-R Na^+ channels can be potentiated by several inflammatory mediators, such as PGE_2 and serotonin (England et al., 1996; Gold et al., 1996, 1998; Lai et al., 2004). In inflamed tissues, on the other hand, extracellular pH significantly falls as low as 5.4 (Reeh and Steen, 1996), and the resultant local tissue acidosis is involved in the inflammatory excitation and sensitization of nociceptive neurons (Steen et al., 1992, 1995a, 1995b). While acidic pH directly activates acid-sensitive ion channels, such as acid-sensing ion channels (ASICs) and transient receptor potential vanilloid 1, to excite nociceptive neurons (Holzer, 2009; Krishtal, 2003; Wemmie et al., 2006), the acid modulation of several ion channels related to action potentials would be also considered because the nociceptive signals are conducted as action potentials along sensory afferents.

Prostaglandins (PGs), which are generated from arachidonic acid by cyclooxygenases (COXs), are representative inflammatory mediators that elicit a number of pathological results including pain. Of them, PGE_2 plays pivotal roles in inflammatory hyperalgesia by inducing the peripheral and central sensitization, e.g., phosphorylation of $Na_V1.8$ in nociceptive neurons (England et al., 1996) and inhibition of glycine receptors in dorsal horn neurons (Harvey et al., 2004). Therefore, non-steroidal anti-inflammatory drugs (NSAIDs), which block COXs to prevent the production of PGs, are useful drugs for the treatment of inflammatory pain (Simmons et al., 2004). However, COX inhibition might not be the only mechanism for NSAID analgesia, as several NSAIDs modulate many ion channels in nociceptive neurons (for review, Gwanyanya et al., 2012). For example, aspirin and diclofenac are known to block ASICs expressed on peripheral nociceptive neurons (Voilley et al., 2001). Diclofenac and meclofenamic acid potentiate some types of voltage-gated K^+ channels including KCNQ (Peretz et al., 2005).

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Furthermore, diclofenac blocks voltage-gated Na^+ channels in sensory neurons (Lee et al., 2003). Such direct effects on ion channels expressed on nociceptive neurons are likely to also contribute to the analgesic effects mediated by several NSAIDs. However, the direct effects of NSAIDs on ion channels might vary according to the extracellular pH, because extracellular pH falls in inflamed tissues, and NSAIDs themselves are weak acids. In the present study, therefore, we examined whether the inhibitory effect of diclofenac on TTX-R Na^+ channels depends on the extracellular pH.

2. Methods

2.1. Preparation

All experiments complied with the guiding principles for the care and use of animals approved by the Kyungpook National University and Council of the Physiological Society of Korea, and every effort was made to minimize both the number of animals used and their suffering.

Sprague Dawley rats (5–6-weeks-old, either sex) were decapitated under ketamine anesthesia (100 mg/kg, i.p.). The trigeminal ganglia (TG) were dissected and treated with an external solution (in mM; 150 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4 with Tris-base) containing 0.3% collagenase (type I) and 0.3% trypsin (type I) for 50–60 min at 37 °C. Thereafter, TG neurons were dissociated mechanically by triturating with fire-polished Pasteur pipettes in a culture dish (Primaria 3801, Becton Dickinson, Rutherford, NJ, USA). The isolated TG neurons were used for electrophysiological experiments 1–6 h after preparation.

2.2. Electrophysiology

All electrical measurements were performed using conventional whole-cell patch recordings and a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Union City, CA, USA) with a K^+-free external solution (in mM; 130 NaCl, 20 TEA-Cl, 3 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, and was adjusted to a pH with Tris-base). In a subset of experiments using low Na^+ external solution, 100 mM NaCl was replaced with equimolar *N*-methyl-D-glucamine-Cl. Neurons were voltage clamped at a holding potential (V_H) of −80 mV. Patch pipettes were made from borosilicate capillary glass (G-1.5; Narishige, Tokyo, Japan) using a pipette puller (P-97; Sutter Instrument Co., Novato, CA, USA). The resistance of the recording pipettes filled with the internal solution (in mM; 140 CsF, 10 CsCl, 2 EGTA, 2 ATP-Na_2, and 10 HEPES with the pH adjusted to 7.2 with Tris-base) was 0.8–1.5 M Ω. Membrane potentials were corrected for the liquid junction potential (~ −11 mV, measured by exchanging the bath solution from internal solution to standard external solution), and the pipette capacitance and series resistance (40–70%) were compensated for. Neurons were viewed under phase contrast on an inverted microscope (TE-2000; Nikon, Tokyo, Japan). Membrane currents were filtered at 5 kHz, digitized at 20 kHz, and stored on a computer equipped with pCLAMP 10.3 (Molecular Devices). Capacitative and leakage currents were subtracted by the P/4 protocol using pCLAMP program. During recordings, 10 mV hyperpolarizing step pulses (30 ms in duration) were periodically applied to monitor the access resistance, and recordings were discontinued if access resistance changed by more than 10%. All experiments were performed at room temperature (22–25 °C). To record TTX-R Na^+ currents (I_{Na}), the K^+-free external solution routinely contained 300 nM TTX and 100 μM Cd^{2+} to block TTX-sensitive (TTX-S) Na^+ channels and voltage-gated Ca^{2+} channels, respectively. Depolarizing step pulses to evoke the TTX-R I_{Na} was applied with an interval of 10 s (except where indicated), which was sufficiently long to recover from the inactivation of TTX-R Na^+ channels.

2.3. Data analysis

The amplitude of TTX-R Na^+ currents (I_{Na}) was measured by subtracting the baseline from the peak amplitude of TTX-R I_{Na} by using Clampfit program (Molecular Devices). The continuous curves for the concentration–response relationship were fitted using a least-squares fit to the following equation, $I = 1 - [C^n / (C^n + IC_{50}^n)]$, where I is the relative amplitude of TTX-R I_{Na} changed by diclofenac, C is the diclofenac concentration, IC_{50} is the diclofenac concentration for the half-maximal response, and n is the Hill coefficient. The amplitude of TTX-R I_{Na}, was transformed into conductance (G) using the following equation; $G = I / (V - E_{Na})$, where E_{Na} is the Na^+ equilibrium potential calculated by the Nernst equation. The voltage-activation and -inactivation relationships of TTX-R Na^+ channels were fitted to the Boltzmann equations, respectively; $G/G_{max} = 1 / \{1 + \exp[(V_{50,act} - V) / k]\}$ and $I/I_{max} = 1 - 1 / \{1 + \exp[(V_{50,inact} - V) / k]\}$, where G_{max} and I_{max} are the maximum conductance and current amplitude, respectively, $V_{50,\,act}$ and $V_{50,inact}$ are half-maximum potentials for activation and fast inactivation, respectively, and k is the slope factor. The fast (τ_{fast}) and slow time constants (τ_{slow}) of the decay of single TTX-R I_{Na} and the kinetic data for the recovery from inactivation were fitted to the following equation; $I(t) = A_0 + A_{fast} \times [1 - \exp(-t / \tau_{fast})] + A_{slow} \times [1 - \exp(-t / \tau_{slow})]$, and the kinetic data for the development of inactivation were fitted to the following equation; $I(t) = A_0 + A_{fast} \times [\exp(-t / \tau_{fast})] + A_{slow} \times [\exp(-t / \tau_{slow})]$, where $I(t)$ is the amplitude of TTX-R I_{Na} at time t, and A_{fast} and A_{slow} are the amplitude fraction of τ_{fast} and τ_{slow}, respectively. The weighted time constant (τ_{WD} or $\tau_{weighted}$) was calculated by using the following equation: τ_{WD} or $\tau_{weighted} = [(\tau_{fast} \times A_{fast}) + (\tau_{slow} \times A_{slow})] / (A_{fast} + A_{slow})$. Numerical values are provided as the mean ± SEM using values normalized to the control. Significant differences in the mean amplitude were tested using Student's paired two-tailed t-test, except where indicated, with absolute values rather than normalized ones. Values of $p < 0.05$ were considered significantly different.

2.4. Drugs

The drugs and chemicals used in the present study were collagenase, trypsin, diclofenac-Na, $CdCl_2$, EGTA, HEPES, MES, and ATP-Na_2 (from Sigma, St. Louis, MO, USA). Diclofenac was dissolved in distilled water to obtain a stock solution of 100 mM. The pH of the pH-6.0 solution containing ≥30 μM diclofenac was finely adjusted with Tris-base. In another set of experiments, pH 6.0 solution was prepared using MES instead of HEPES. All solutions containing drugs were applied using the 'Y-tube system' for rapid solution exchange (Murase et al., 1989).

3. Results

3.1. Effect of diclofenac on TTX-R Na^+ channels

The application of pH 6.0 solution inhibited TTX-R I_{Na} as described below, but could also directly induce an inward current. This inward current was seen in a subset of small TG neurons (25/163), was blocked by amiloride (300 μM, nonselective ASIC blocker) and was transient, disappearing within 5 s of application of pH 6.0 solution. These currents represent ASIC currents, as we have also recently described (Nakamura and Jang, 2015). In all the following experiments, the effects of acidic pH on TTX-R I_{Na} were examined at least 20 s after application of pH 6.0 solution, when the ASIC currents had long desensitized and disappeared. We examined the effects of diclofenac on TTX-R I_{Na} at normal (pH 7.4) and acidic (pH 6.0) pH. Diclofenac reversibly decreased the amplitude of TTX-R I_{Na} in a concentration-dependent manner (Fig. 1A,B). However, the inhibitory effects of diclofenac on TTX-R Na^+ channels were stronger at pH 6.0 than pH 7.4 (Fig. 1B). The IC_{50} values of diclofenac were >1000 μM and 124.7 ± 10.8 μM at pH 7.4 and pH 6.0, respectively (n = 9, Fig. 1B). While diclofenac (100 μM) had