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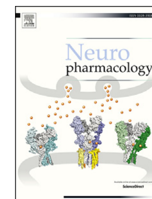

Loperamide inhibits sodium channels to alleviate inflammatory hyperalgesia

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ABSTRACT

Previous studies demonstrated that Loperamide, originally known as an anti-diarrheal drug, is a promising analgesic agent primarily targeting mu-opioid receptors. However some evidences suggested that non-opioid mechanisms may be contributing to its analgesic effect. In the present study, Loperamide was identified as a Nav1.7 blocker in a pilot screen. In HEK293 cells expressing Nav1.7 sodium channels, Loperamide blocked the resting state of Nav1.7 channels ($IC_{50} = 1.86 \pm 0.11$ μM) dose-dependently and reversibly. Loperamide produced a 10.4 mV of hyperpolarizing shift for the steady-state inactivation of Nav1.7 channels without apparent effect on the voltage-dependent activation. The drug displayed a mild use- and state-dependent inhibition on Nav1.7 channels, which was removed by the local anesthetic-insensitive construct Nav1.7-F1737A. Inhibition of Nav1.7 at resting state was not altered significantly by the F1737A mutation. Compared to its effects on Nav1.7, Loperamide exhibited higher potency on recombinant Nav1.8 channels in ND7/23 cells ($IC_{50} = 0.60 \pm 0.10$ μM) and weaker potency on Nav1.9 channels (3.48 ± 0.33 μM). Notably more pronounced inhibition was observed in the native Nav1.8 channels (0.11 ± 0.08 μM) in DRG neurons. Once mu-opioid receptor was antagonized by Naloxone in DRG neurons, potency of Loperamide on Nav1.8 was identical to that of recombinant Nav1.8 channels. The inhibition on Nav channels may be the main mechanism of Loperamide for pain relief beyond mu-opioid receptor. In the meanwhile, the opioid receptor pathway may also influence the blocking effect of Loperamide on sodium channels, implying a cross-talk between sodium channels and opioid receptors in pain processing.

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1. Introduction

As an anti-diarrheal medication approved for the treatment of non-bacterial diarrhea, Loperamide, a mu-opioid receptor agonist, has been demonstrated as a peripherally selective opiate anti-hyperalgesic agent in multiple pain models, including inflammatory pain (DeHaven-Hudkins et al., 1999; Nozaki-Taguchi and Yaksh, 1999; Sevostianova et al., 2005), neuropathic pain (Guan et al., 2008; Shinoda et al., 2007) and cancer pain (Menendez et al., 2005) et al. The drug does not penetrate the blood-brain barrier and therefore lacks the side effects generally associated with administration of centrally acting opiates. Thus Loperamide has become a promising analgesic agent. Studies have shown that the antihyperalgesic effect of Loperamide cannot be completely

Abbreviations: MOR, mu-opioid receptor; VGSCs, Voltage-gated sodium channels; HEK, human embryo kidney; KO, knock-out; PWF, paw withdrawal frequency; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; PWL, paw withdrawal latency; TTX, tetrodotoxin.

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antagonized by mu-receptor antagonist Naloxone (Sevostianova et al., 2005; Shannon and Lutz, 2002). We also found that Loperamide was still able to relieve the inflammatory pain induced by thermal and mechanical stimuli even if mu-opioid receptor (MOR) has been knocked-out. Those results indicated that other targets might be involved in Loperamide-induced analgesia beyond its primary target.

It has been demonstrated that sodium channels including Nav1.7 and Nav1.8 were up-regulated in several inflammatory pain models (Black et al., 2004; Huang et al., 2013; Liang et al., 2013). If Nav1.7 was deleted in nociceptors, inflammatory pain responses were reduced or abolished in mice (Nassar et al., 2004). Consistently, loss-of-function mutations cause inability to experience pain in humans (Cox et al., 2006; Weiss et al., 2011) and gain-of-function mutations are responsible for extreme pain syndrome (Drenth and Waxman, 2007; Fertleman et al., 2006). Similar to what has been found in Nav1.7$^{-/-}$ mice, responses to inflammatory pain were diminished in the Nav1.8$^{-/-}$ mice (Akopian et al., 1999) and Nav1.9$^{-/-}$ mice. These knockout mice exhibited reduced or absent thermal and mechanical inflammatory hyperalgesia (Amaya et al., 2006; Priest et al., 2005).

Several opioid agonists have been reported to cause opioid-receptor independent inhibition of sodium channel, including Morphine, Meperidine and Buprenophine et al. (Haeseler et al., 2006; Leffler et al., 2012; Wagner et al., 1999). Although some papers indicated that Loperamide has blockade activity on sodium channels in the airway and heart (Ghosal et al., 1996, 2000; Kang et al., 2016), there were no reports elaborating the effect of Loperamide on neuronal sodium channels. Thus considering the important role of sodium channels in pathological pain, we hypothesized that sodium channels may be playing an important role for Loperamide-induced antihyperalgesia in the inflammatory pain. Therefore we determined to characterize the effect of Loperamide on sodium channels in the recombinant cells and neuronal cells in Dorsal root ganglion.

2. Materials and methods

2.1. Cell culture

Stable cell line expressing human Nav1.7 was generated in HEK-293 cells. These cells were routinely cultured in high glucose DMEM (Invitrogen, Carlsbad, CA) with 10% (vol/vol) FBS (Gemini Bio Products), 2 mM L-glutamine (Invitrogen), supplemented with 300 μg/ml hygromycin for Nav1.7. Cells were maintained and passaged when reaching 80% confluency. Stable cell line expressing rat KCNQ2 potassium channels in CHO–K1 cells was cultured in DMEM medium with 10% FBS, supplemented with 2 mM L-glutamine, and 500 μg/mL Geneticin. Nav1.7-F1737A and human Nav1.8 plasmids were transiently expressed in HEK-293 cells and ND7/23 cells, respectively. Transfections with 2 μg sodium channel plasmids and 0.2 μg green fluorescent protein plasmid(GFP) were performed in 35-mm dishes according to the protocol provided with Lipofectamine LTX plus reagent (Invitrogen, Carlsbad, CA). Cells were seeded on the glass cover slips for electrophysiological recording 24–48 h post transfection.

2.2. Dorsal root ganglion neuronal culture

Adult male Sprague-Dawley rats were put to death with isoflurane. Dorsal root ganglions (DRGs) were collected in cold DH10 [90% DMEM/F-12 (Gibco, Grand Island, NY), 10% FBS (Gibco, Grand Island, NY), 1% penicillin-streptomycin] and treated with enzyme solution [3.5 mg/ml dispase, 1.6 mg/ml collagenase type I and DNAse 1 unit/ml in HBSS without Ca^{2+} and Mg^{2+} (Gibco, Grand Island, NY)] at 37 °C. After centrifugation, dissociated cells were resuspended in DH10 and plated at a density of 1.5×10^5 to 4×10^5 cells on glass coverslips coated with poly-L-lysine (0.5 mg/ml, Sigma, St. Louis, MO) and laminin (10 μg/ml, Invitrogen). The cells were cultured in Neuronal Basal Medium, supplemented with B27, NGF, GlutaMAX and used for electrophysiological recording after dissociation for 2 h.

2.3. Manual patch-clamp recording in cell lines and DRG neurons

Traditional whole cell voltage clamp recording was performed at room temperature (22–25 °C) to record Nav1.7 currents expressing in HEK-293 cells and sodium currents in DRG neurons. Cells with stable expression of Nav1.7 sodium channels were seeded on poly-L-lysine coated glass coverslips one day before recording. Recording pipettes were pulled with borosilicate glass (World Precision Instruments Inc., Sarasota, FL) to 3–5 MΩ when filled with an internal solution and placed in a bath solution. For total sodium currents and TTX-resistant currents in DRG neurons, the bath solution contains 35 mM NaCl, 105 mM Choline Cl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Tetraethylammonium, 0.1 Cadmium Cl, 10 mM glucose and 10 mM HEPES at pH 7.4 adjusted with NaOH. For Nav1.8 and Nav1.9 channels in DRG neurons and recombinant sodium channels, bath solution contains 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose,10 mM HEPES, 20 mM Tetraethylammonium, 0.1 Cadmium Cl at pH 7.4 adjusted with NaOH. For TTX-resistant sodium channels (including Nav1.8 and Nav1.9), TTX 300 nM was including in the bath solution. Internal solution for sodium channels contains 140 mM CsF, 10 mM NaCl, 1 mM EGTA, 10 mM HEPES 10 at pH 7.3 adjusted with CsOH. For KCNQ2 stable cell line, bath solution contains 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES at pH 7.4 adjusted with NaOH. The internal buffer contains 145 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES and 5 mM MgATP, at pH 7.3 adjusted with KOH. Isolated cells were voltage-clamped in whole-cell mode with an EPC-9 amplifier (HEKA, Germany), and currents were sampled at 10 kHz. Series resistance was compensated by 60–80%. Cells were continuously perfused with bath solution through a gravity-driven perfusion system (ALA Scientific, Farmingdale, NY). Stock solutions of all chemicals were made with DMSO. Immediately before each experiment, drugs were diluted in external solutions to desired concentrations and applied by perfusion.

2.4. Animal experiments

2.4.1. Animal care

Adult male wild type C57BL/6J mice and μ-Opioid Receptor (MOR, mu-receptor) KO mice were purchased from the Jackson Laboratory. The MOR KO strain of mice has been well-characterized by Dr Brigitte Kieffer lab (Martin et al., 2003; Matthes et al., 1996). The animal protocols were approved by the Animal Care and Use Committee at the Johns Hopkins University. Animal procedures were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain and the ethical guidelines to investigate experimental pain in a conscious animal. Behavioral tests were performed after animals were acclimatized to the facilities for 1 week. To minimize variability of the behavioral outcome measures, animals were trained for 3–5 days before baseline measurement and were habituated to the test environment for ≥30 min before testing.

2.4.2. Inflammatory pain induced by complete Freund's adjuvant (CFA)

As described by Chu et al. (2005), inflammatory pain was

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