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Removing TRPV1-expressing primary afferent neurons potentiates the spinal analgesic effect of δ-opioid agonists on mechano-nociception

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ABSTRACT

Most δ-opioid receptors are located on the presynaptic terminals of primary afferent neurons in the spinal cord. However, their presence in different phenotypes of primary afferent neurons and their contribution to the analgesic effect of δ-opioid agonists are not fully known. Resiniferatoxin (RTX) is an ultra-potent transient receptor potential vanilloid type 1 channel (TRPV1) agonist and can selectively remove TRPV1-expressing primary afferent neurons. In this study, we determined the role of δ-opioid receptors expressed on TRPV1 sensory neurons in the antinociceptive effect of the δ-opioid receptor agonists [D-Pen^2,D-Pen^5]-enkephalin and [D-Ala^2,Glu^4]-deltorphin. Nociception was measured by testing the mechanical withdrawal threshold in the hindpaw of rats. Changes in the δ-opioid receptors were assessed using immunocytochemistry and the [^{3}H]-naltrindole radioligand binding. In RTX-treated rats, the δ-opioid receptor on TRPV1-immunoreactive dorsal root ganglion neurons and afferent terminals in the spinal cord was diminished. RTX treatment also significantly reduced the maximal specific binding sites (31%) of the δ-opioid receptors in the dorsal spinal cord. Interestingly, intrathecal injection of [D-Pen^2,D-Pen^5]-enkephalin or [D-Ala^2,Glu^4]-deltorphin produced a large and prolonged increase in the nociceptive threshold in RTX-treated rats. These findings indicate that loss of TRPV1-expressing afferent neurons leads to a substantial reduction in presynaptic δ-opioid receptors in the spinal dorsal horn. However, the effect of δ-opioid agonists on mechano-nociception is paradoxically potentiated in the absence of TRPV1-expressing sensory neurons. This information is important to our understanding of the cellular sites and mechanisms underlying the spinal analgesic effect of δ-opioid agonists.

1. Introduction

The spinal cord dorsal horn is a critical site for the nociceptive transmission and analgesic actions of μ- and δ-opioid receptor agonists. Similar to μ-opioid receptors, the δ-opioid receptors are distributed in the dorsal root ganglion (DRG) neurons and their central terminals in the superficial dorsal horn of the spinal cord (Abbadie et al., 2002; Wang and Wessendorf, 2001; Zhang et al., 1998). Activation of δ-opioid receptors inhibits nociceptive transmission at the spinal level. For example, intrathecal administration of the δ-opioid receptor agonist produces a potent analgesic effect in acute and chronic pain models (Holdridge and Cahill, 2006; Hurley et al., 1999; Malmberg and Yaksh, 1992). Furthermore, spinally administered δ-opioid agonists inhibit the evoked response of spinal dorsal horn neurons (Khan et al., 2002). The δ-opioid receptors are located both presynaptically on the central terminals of primary afferents and postsynaptically on the spinal dorsal horn neurons (Abbadie et al., 2002; Besse et al., 1990). However, the presence of δ-opioid receptors in different phenotypes of nociceptive afferent neurons and their relative contribution to the analgesic effect produced by spinally administered δ-opioid agonists are not fully known.

The capsaicin receptor, transient receptor potential vanilloid type 1 channel (TRPV1), is essential for the detection of thermal nociception. Mice deficient in TRPV1 show an impaired response to noxious heat (Caterina et al., 2000). Resiniferatoxin (RTX), originally extracted from the cactus-like plant *Euphorbia resinifera*, is an ultra-potent TRPV1 agonist (Szallasi and Blumberg, 1999). RTX treatment induces a long-lasting impairment of thermal nociception (Chen and Pan, 2006b; Pan et al., 2003). However, mechano-nociception remains largely intact in the absence of TRPV1-expressing sensory neurons (Chen and Pan, 2006b; Chen et al., 2007b). Unlike capsaicin, which only removes certain primary

Abbreviations: DELT, [D-Ala^2,Glu^4]-deltorphin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DRG, dorsal root ganglion; IB$_4$, *Griffonia simplicifolia* isolectin B$_4$; RTX, resiniferatoxin; TRPV1, transient receptor potential vanilloid type 1 channel.

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sensory neurons in neonatal rats, RTX can remove TRPV1-expressing sensory neurons in adult rats (Chen and Pan, 2006b; Pan et al., 2003). The use of RTX is a preferred approach since it circumvents the developmental plasticity induced by capsaicin treatment in neonatal animals. Thus, RTX has become a valuable tool for studying mechanical pain transmission through non-TRPV1 sensory neurons and for studying the functional importance of presynaptic opioid receptors in the spinal cord.

We have shown that RTX treatment leads to a substantial reduction in the number of presynaptic μ-opioid receptors in the spinal dorsal horn (Chen and Pan, 2006b). Paradoxically, the analgesic effect of μ-opioid receptor agonists is significantly potentiated in RTX-treated rats (Chen and Pan, 2006b). In the spinal cord, the ratio of pre- to postsynaptic δ-opioid receptors is greater than that of μ-opioid receptors (2:1 vs. 1:1, respectively) (Abbadie et al., 2002). Because many δ-opioid receptors may be located on TRPV1-expressing afferent neurons, RTX treatment could remove many presynaptic δ-opioid receptors in the spinal dorsal horn. In the present study, we determined the relative contribution of presynaptic δ-opioid receptors to the analgesic effect of spinally administered δ-opioid agonists. We found that RTX treatment caused a substantial reduction in presynaptic δ-opioid receptors in the spinal cord but it failed to attenuate the effect of δ-opioid agonists on mechano-nociception. This study provides new evidence that the δ-opioid receptors on non-TRPV1-expressing primary afferents and spinal dorsal horn neurons play an important role in the modulation of mechano-nociception.

2. Methods

2.1. Animals

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 200–220 g were used in this study. Rats received a single intraperitoneal injection of RTX (200 μg/kg, LC Laboratories, Woburn, MA) under isoflurane (2% in O_2) anesthesia. RTX was dissolved in a mixture of 10% Tween-80 and 10% ethanol in normal saline (Chen and Pan, 2006b; Pan et al., 2003). We have shown that systemic RTX treatment deletes primary afferent neurons without any evident effect on spinal dorsal horn neurons that express TRPV1 in adult rats (Chen and Pan, 2006b). Rats in the control group received intraperitoneal injection of the vehicle. The experiments were conducted 4–5 weeks after RTX and vehicle injections unless stated otherwise. The surgical preparation and experimental protocols were approved by the University of Texas M. D. Anderson Cancer Center and conformed to the National Institutes of Health guidelines on the ethical use of animals.

Intrathecal catheters (PE-10 polyethylene tubing) were inserted in RTX- and vehicle-treated rats during isoflurane-induced anesthesia. In each case, the catheter was advanced 8 cm caudally through an incision in the cisternal membrane and secured to the musculature at the incision site. The rats were allowed to recover for 5–7 days before being used to test the antinociceptive effect of the specific δ-opioid agonists [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) and [D-Ala^2,Glu^4]-deltorphin (DELT). Only animals with no evidence of neurological deficits after catheter insertion were studied. Drugs for intrathecal injection were dissolved in normal saline and administered in a volume of 5 μl followed by a 10 μl flush with normal saline. Repeated intrathecal injections in the same animals were separated by 4–5 days. DPDPE and DELT were obtained from Tocris (Ellisville, MO).

2.2. Behavioral assessment of nociception

To quantitatively assess the thermal sensitivity, rats were placed on the glass surface of a thermal testing apparatus (IITC, Woodland Hills, CA). The rats were allowed to acclimate for 30 min before testing. The temperature of the glass surface was maintained constant at 30 °C. A mobile radiant heat source located under the glass was focused onto the hindpaw of each rat. The paw withdrawal latency was recorded by a timer, and the mean value from both hindpaws was determined. The cutoff time point of 30 s was used to prevent tissue damage (Chen and Pan, 2003; Pan et al., 2003).

The nociceptive mechanical threshold was measured using an Ugo Basil Analgesimeter (Varese, Italy). The test was performed by applying a noxious pressure to the hindpaw. By pressing a pedal that activated a motor, the force increased at a constant rate on the linear scale. When the animal responded by withdrawal of the paw or vocalization, the pedal was immediately released and the nociceptive threshold reads on a scale. The cutoff weight of 400 g was used to avoid tissue injury (Chen and Pan, 2006b; Chen et al., 2007b). Both hindpaws were tested in each rat, and the mean value was used as the nociceptive withdrawal threshold.

2.3. Double fluorescence labeling of δ-opioid receptors and IB_4 in the DRGs and spinal cord

To determine the effect of RTX treatment on the δ-opioid receptors and *Griffonia simplicifolia* isolectin B_4 (IB_4)-positive DRG neurons and primary afferent terminals in the spinal dorsal horn, double fluorescence labeling of δ-opioid receptors and IB_4 (a marker for unmyelinated primary neurons and afferent fibers; Kitchener et al., 1994, 1993) was performed in the lumbar DRGs and spinal cords from three RTX-treated and three vehicle-treated rats 4 weeks after treatment. As described previously (Chen and Pan, 2006b; Chen et al., 2007b), each rat was intracardially perfused with 4% paraformaldehyde and 10% sucrose in 0.1 M PBS (pH 7.4) while under deep anesthesia with pentobarbital sodium (60 mg/kg, i.p.). The lumbar spinal cord and the DRGs were quickly removed and postfixed in the same fixative solution and cryoprotected in 30% sucrose in PBS for 48 h at 4 °C. The spinal cord and DRG tissues were cut into 25- and 30-μm-thick sections, respectively. The sections were collected free-floating in 0.1 M PBS and blocked in 4% normal goat serum in PBS for 1 h. Then sections were incubated with the primary antibody (rabbit anti-δ-opioid receptors, dilution 1:1000, Neuromics; Minneapolis, MN) diluted in PBS solution containing 4% normal goat serum and 0.1% TX-100 for 2 h at room temperature and overnight at 4 °C. Subsequently, sections were rinsed in PBS and incubated with the secondary antibody (Alexa Fluor-488 conjugated to goat anti-rabbit IgG, dilution: 5 μg/ml; Molecular Probes, Eugene, OR) for 1.5 h. The sections were rinsed again and incubated with Alexa Fluor-594 conjugated to IB_4 (dilution: 2 μg/ml, Molecular Probes) for 2 h at room temperature. Finally, the sections were rinsed and mounted on slides, dried, and coverslipped. Omission of the primary antibody resulted in negative labeling in all the sections examined.

2.4. Double immunofluorescence labeling of TRPV1- and δ-opioid receptors in the DRG and spinal cord

Double immunofluorescence labeling of δ-opioid receptors and TRPV1 was performed in L_4 and L_5 DRGs and the lumbar spinal cord from three RTX-treated and three vehicle-treated rats. The tissue preparation techniques were the same as described above. The DRG and spinal cord sections were incubated with a mixture of primary antibodies (guinea pig anti-TRPV1, dilution 1:1,000; and rabbit anti-δ-opioid receptors, dilution 1:1000, Neuromics) diluted in PBS solution containing 4% normal goat serum and 0.1% TX-100 for 2 h at room temperature and overnight at 4 °C. Subsequently, spinal sections were rinsed in PBS and incubated with the mixture of secondary antibodies (Alexa Fluor-488 conjugated to goat anti-guinea pig IgG; and Alexa Fluor-594 conjugated to goat anti-rabbit IgG, dilution: 5 μg/ml; Molecular Probes) for 1.5 h. For the DRG sections, a different mixture of secondary antibodies (Alexa Fluor-488 conjugated to goat anti-rabbit IgG; and Alexa Fluor-594 conjugated to goat anti-guinea pig IgG, dilution: 5 μg/ml; Molecular Probes) was used. The sections were then rinsed in PBS for 30 min, mounted on slides, dried, and coverslipped. Omission of the primary antibodies resulted in negative labeling in the DRG and spinal cord.

The sections were examined on a laser scanning confocal microscope (Zeiss LSM 510, Germany), and areas of interest were photodocumented. Alexa Fluor-488 and Alexa Fluor-594 fluorochromes were excited at 488- and 543-nm wavelengths, respectively. Immunoreactivity was examined under the conditions of optimal resolution (small pinhole, thin optical slice, and high-numerical-aperture water- or oil-immersion objective). The pinhole diameter was set to 1 airy unit (which corresponds to 1.2 μm depth of field). To completely rule out crosstalk between the fluorescent detection channels, the multitracking configuration was used.

2.5. [^{3}H]-naltrindole membrane bindings in the spinal cord

To determine if RTX treatment would alter the binding number and affinity of the δ-opioid receptors in the spinal cord, saturation binding of [^{3}H]-naltrindole, a specific radioligand for δ-opioid receptors (Contreras et al., 1993), was carried out using the dorsal spinal cord tissue membranes. Four RTX- and four vehicle-treated rats were decapitated after being anesthetized with 2–3% isoflurane. The whole spinal cord was quickly harvested, and the dorsal half was dissected and used for the binding experiment. The tissue was homogenized in ice-cold 50 mM Tris–HCl buffer containing 3 mM $MgCl_2$ and 1 mM EGTA (pH 7.4) and disrupted by sonication. The homogenate was then centrifuged at 500*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 48,000*g* for 20 min at 4 °C. The pellet was resuspended in Tris–HCl buffer and was centrifuged again as described in the preceding text. The final pellet was resuspended in 50 mM Tris–HCl buffer containing (in mM) 3 $MgCl_2$, 100 NaCl, and 0.2 EGTA (pH 7.7) and disrupted by sonication for 5 s. The protein content was measured by means of the method of Bradford using the bovine serum albumin as the standard (Protein Assay Kit II, Bio-Rad Laboratories, Hercules, CA). Saturation radioligand binding experiments were performed using 200-μl aliquots of tissues and increasing concentrations of [^{3}H]-naltrindole (35 Ci/mmol, PerkinElmer Life Sciences, Inc., Boston, MA) from 10 to 1200 pM. Nonspecific binding was determined with 1 μM naloxone (Sigma, St. Louis, MO). Incubation was performed in duplicate in Tris–HCl buffer at 25 °C for 60 min. The reaction was terminated by filtration through Whatman GF/B filters on a cell harvester with ice-cold Tris–HCl buffer (pH 7.4). Radioactivity was measured by