Loss of TRPV1-Expressing Sensory Neurons Reduces Spinal μ Opioid Receptors But Paradoxically Potentiates Opioid Analgesia

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Chen, Shao-Rui and Hui-Lin Pan. Loss of TRPV1-expressing sensory neurons reduces spinal μ opioid receptors but paradoxically potentiates opioid analgesia. J Neurophysiol 95: 3086–3096, 2006. First published February 8, 2006; doi:10.1152/jn.01343.2005. Systemic administration of resiniferatoxin (RTX), an ultrapotent capsaicin analogue, removes transient receptor potential vanilloid type 1 (TRPV1)-expressing afferent neurons and impairs thermal but not mechanical nociception in adult animals. In this study, we determined how loss of TRPV1-expressing sensory neurons alters the antinociceptive effect of μ opioids and opioid receptors in the spinal cord. The effect of morphine and (−)αⅡL,αβL-Gly-ol)-enkephalin (DAMGO) was measured by testing the paw mechanical withdrawal threshold in rats treated with RTX or vehicle. RTX treatment deleted TRPV1-immunoreactive dorsal root ganglion neurons and nerve terminals in the spinal dorsal horn. Also the μ opioid receptor immunoreactivity was markedly reduced in the superficial dorsal horn of RTX-treated rats. However, RTX treatment did not affect the dorsal horn neurons labeled with both TRPV1- and μ opioid receptor-immunoreactivity. Surprisingly, intrathecal morphine or DAMGO produced a greater increase in the withdrawal threshold in RTX- than in vehicle-treated rats. The duration of the effect of intrathecal morphine and DAMGO in RTX-treated rats was also profoundly increased. Furthermore, the antinociceptive effect of systemic morphine was significantly potentiated in RTX-treated rats. The BMAX (but not Kp) of [3H]-DAMGO binding and DAMGO-stimulated [35S]GTPγS activity in the dorsal spinal cord were significantly reduced in the RTX group. This study provides novel information that although destruction of TRPV1-expressing afferent neurons eliminates presynaptic μ opioid receptors present on TRPV1-expressing afferent neurons but paradoxically potentiates the analgesic effect of μ opioid agonists. Mechanonociception, transmitted through non-TRPV1 sensory neurons, is subject to potent modulation by μ opioid agonists.

INTRODUCTION

Nociception is transduced by different populations of primary sensory neurons and appears modality specific. The small-diameter dorsal root ganglion (DRG) neurons and associated Aδ- and C-fiber afferents are critical for detecting noxious stimuli and initiating pain sensation (Burgess and Perl 1967; Harper and Lawson 1985; Slugg et al. 2000). These DRG neurons are functionally and phenotypically diverse and express various receptors and ion channels. The capsaicin receptor, transient receptor potential vanilloid type 1 (TRPV1), plays an essential role in detecting thermal nociception. In this regard, mice deficient of TRPV1 show impaired pain response to heat (Caterina et al. 2000). Resiniferotoxin (RTX), originally isolated from the cactus-like plant Euphorbia resinifera, is an ultrapotent TRPV1 agonist (Szallasi and Blumberg 1999). Systemic injection of RTX destroys TRPV1-expressing sensory neurons and induces a long-lasting impairment of thermal nociception in adult rats (Pan et al. 2003; Xu et al. 1997). Interestingly, mechano-nociception is largely intact in RTX-treated rats (Pan et al. 2003; Xu et al. 1997). Thus RTX is a valuable tool to study the nociceptive pathway transmitted through non-TRPV1 afferent neurons and its modulation.

The μ opioid receptor agonists, such as morphine, remain the most potent analgesics to treat patients with moderate to severe pain. Opioids have a distinct effect on nociception with little effect on other sensory modalities. The spinal cord dorsal horn is critically involved in pain transmission and modulation and is a major site for the analgesic action of μ opioid agonists (Chen et al. 2005; Magnuson and Dickenson 1991; Yaksh and Noueihed 1985). The μ opioid receptors are located presynaptically on central terminals of primary afferent neurons as well as postsynaptic dorsal horn neurons (Fields et al. 1980; Gamse et al. 1979; Yoshimura and North 1983). However, the relative role of pre- and postsynaptic μ opioid receptors in the spinal opioid analgesia is not fully known.

In addition to the loss of TRPV1-expressing afferent neurons, RTX may eliminate μ opioid receptors located on the TRPV1-expressing presynaptic terminals in the spinal dorsal horn. The influence of destruction of TRPV1-expressing afferent neurons on the antinociceptive effect of opioids has not been studied previously. Therefore we used RTX-treated animals to determine the relative importance of presynaptic μ opioid receptors in spinal opioid analgesia. Unexpectedly, we found that although destruction of TRPV1-expressing afferent neurons eliminates presynaptic μ opioid receptors present on TRPV1-expressing afferent neurons, it paradoxically potentiates the antinociceptive effect produced by intrathecal and systemic μ opioid agonists. This study reveals an important function of μ opioid receptors in modulation of mechanonociception transmitted by non-TRPV1 afferent neurons and extends our current knowledge of the pain pathways and mechanisms underlying opioid analgesia.

METHODS

Animals

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 200–220 g were used in this study. Rats received a single intraperi-
toneal injection of RTX (200 µg/kg, LC Laboratories, Woburn, MA) under halothane (2% in O₂) anesthesia. RTX was dissolved in a mixture of 10% Tween-80 and 10% ethanol in normal saline (Pan et al. 2003). Rats in the control group received intraperitoneal injection of the vehicle. The experiments were conducted 4–5 wk after RTX and vehicle injections unless stated otherwise. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine and conformed to 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 halothane-induced anesthesia. The catheters were advanced 8 cm caudal through an incision in the cisternal membrane and secured to the musculature at the incision site. The catheters were allowed to recover for 3–7 days before being used to test the antinociceptive effect of morphine or (D-Ala²,N-Me-Phe⁴,Gly-ol⁵)-enkephalin (DAMGO). 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. Repeat intrathecal injections in the same animals were separated by 4–5 days. Morphine was obtained from Astra Pharmaceuticals (Westborough, MA). DAMGO and (D-Phe⁴,Cys⁷-Tyr⁸-D-Trp⁹-Arg¹⁰-Preⁱ-NH₂ (CTAP) were purchased from Sigma (St. Louis, MO).

Behavioral assessment of thermal nociception

To quantitatively assess the thermal sensitivity of the normal rats, rats were placed on the glass surface of a thermal testing apparatus (IRT, 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 used. The cut-off of 30 s was used to prevent potential tissue damage (Chen and Pan 2003a; Pan et al. 2003).

Behavioral testing of mechanical nociception

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 displayed pain by withdrawal of the paw or vocalization, the pedal was immediately released and the nociceptive threshold read on a scale. The cut-off of 400 g was used to avoid potential tissue injury (Chen and Pan 2003b). Both hindpaws were tested in each rat, and the mean value was used as the nociceptive withdrawal threshold.

Double fluorescence labeling of TRPV1 and IB₄ in DRGs and spinal dorsal horn

To determine the effect of RTX treatment on TRPV1-positive and \textit{Griffonia simplicifolia} isolecitin B₄ (IB₄)-positive DRG neurons and central terminals, double labeling of TRPV1 and IB₄ [a marker for unmyelinated afferent fibers (Kitchener et al. 1993, 1994)] in DRG neurons and the spinal dorsal horn was performed on three vehicle- and four RTX-treated rats 4 wk after treatment. Under deep anesthesia with pentobarbital sodium (60 mg/ml ip), each rat was intracardially perfused with 250 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and 200 ml of 10% sucrose in 0.1 M PBS (pH 7.4). The lumbar spinal cord and L₄ and L₅ DRGs were removed quickly and postfixed in the same fixative solution and cryoprotected in 30% sucrose in PBS for 48 h at 4°C. The tissues were cut to 30 µm in thickness and collected free-floating in 0.1 M PBS. For TRPV1 immunofluorescent labeling, the sections were rinsed in 0.1 M PBS and blocked in 4% normal goat serum in PBS for 1 h. The sections were then incubated with the primary antibody (rabbit anti-TRPV1 N-terminus, dilution 1:1000, Neuronics, Minneapolis, MN) diluted in PBS containing 2% normal goat serum and 0.3% 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). The sections then were rinsed in PBS for 30 min and incubated with Alexa Fluor 594 conjugated to IB₄ (Molecular Probes, dilution: 2 µg/ml) for 2 h at room temperature. Finally, the sections were rinsed and mounted on slides, dried, and coverslipped. The sections were examined on a laser scanning confocal microscope (Leica, Germany), and areas of interest were photodocumented.

Double immunofluorescence labeling of TRPV1 and µ opioid receptors in the DRG and spinal cord

To examine the effect of RTX treatment on the distribution of µ opioid receptors in the DRGs and spinal dorsal horn, double immunofluorescence labeling of µ opioid receptors and TRPV1 was performed in L₄ and L₅ DRGs and the lumbar spinal cord from three RTX- and three vehicle-treated rats. As described previously (Chen and Pan 2003a; Wu et al. 2004, 2005), the tissue sections were rinsed in 0.1 M PBS and blocked in 4% normal goat serum in PBS for 1 h. Then sections were incubated with the mixture of primary antibodies (rabbit anti-µ opioid receptors, dilution 1:1000, Neuronics; guinea pig anti-TRPV1, dilution 1:1,000, Neuromics) diluted in PBS solution containing 2% normal goat serum and 0.3% 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 mixture (Alexa Fluor 488 conjugated to goat anti-rabbit IgG, Alexa Fluor 594 conjugated to goat anti-guinea pig IgG, Molecular Probes, dilution: 5 µg/ml) for 1.5 h. The sections then were rinsed in PBS for 30 min, mounted on slides, dried, and coverslipped. The sections were examined on a laser scanning confocal microscope. Omission of the primary antibody and preabsorption of the anti-µ opioid receptor antibody with the blocking peptide resulted in negative labeling in the DRGs and the spinal cord. The cell counting of DRG neurons was performed by an investigator who was blinded to the experimental condition.

[^3H]-DAMGO membrane bindings in the spinal cord

To determine if RTX treatment alters the binding number and affinity of the µ opioid receptors in the spinal cord, saturation binding of [^3H]-DAMGO, a specific radioligand for µ opioid receptors, was carried out using the spinal cord tissue membranes (Chen and Pan 2003a). Six RTX- and six vehicle-treated rats were decapitated after being anesthetized with halothane. 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 buffer containing 3 mM MgCl₂ 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 fresh Tris buffer and was centrifuged again as described in the preceding text. The final pellet was resuspended in 50 mM Tris buffer containing (in mM) 3 MgCl₂, 100 NaCl, and 0.2 EGTA (pH 7.4) and disrupted by sonication for 5 s. Saturation radioiodide binding experiments were performed using 200 µl aliquots of tissue and increasing concentrations of [^3H]-DAMGO (76 Ci/mmol, Amersham Biosciences, Piscataway, NJ) from 0.3 to 11 nM, as we described previously (Chen and Pan 2003a). Nonspecific binding was determined with 1 µM naloxone (Sigma, St. Louis, MO). Incubation was performed in duplicate in Tris buffer at 25°C for 60 min. The reaction was terminated by filtration through Whatman GF/B filters on a cell harvester with cold Tris buffer (pH 7.4). Radioactivity was quantified.
by immersion of filters in the scintillation fluid, then incubated overnight at room temperature, and measured the next day using a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA). The protein content was measured based on the method of Bradford using the bovine serum albumin as standards (Protein Assay Kit II, Bio-Rad Laboratories, Hercules, CA).

**DAMGO [35S]GTPγS activity in spinal cord membranes**

Additional experiments were performed to further determine if the functional coupling of μ opioid receptors to G proteins is altered by RTX treatment. The [35S]GTPγS binding assay is based on the principle that the inactive state of the G protein α subunit has a relatively high affinity for GDP over GTP, whereas activation of a G-protein-coupled receptor by its agonist shifts the α subunit into a higher affinity for GTP versus GDP. The [35S]GTPγS is a hydrolysissensitive form of GTP, and the degree to which an agonist stimulates [35S]GTPγS activity can be quantified in tissue membranes. Thus the agonist-stimulated [35S]GTPγS binding can provide functional information about the receptor (i.e., coupling of G proteins to activated receptors). As described previously (Chen et al. 2002), the DAMGO-stimulated [35S]GTPγS binding was performed using isolated membranes. The dorsal spinal cord tissue was homogenized in cold membrane buffer [containing (in mM) 50 Tris-HCl, 3 MgCl₂, and 1 EGTA, pH 7.4] and centrifuged at 500 g for 10 min at 4°C. The supernatant was centrifuged at 48,000 g for 30 min at 4°C. The pellet was resuspended in assay buffer [containing (in mM) 50 Tris-HCl, 3 MgCl₂, 0.2 EGTA, and 100 NaCl, pH 7.7] and measured for protein content. Concentration-effect curves of the DAMGO-stimulated [35S]GTPγS binding included 0.1–10 μM DAMGO with and without 1 μM o-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP, a specific μ opioid receptor antagonist), 30 μM GDP, 0.05 nM [35S]GTPγS, 4 mU/ml adenosine deaminase, 20 μg protein, and assay buffer in a final volume of 1 ml. The basal binding was determined in the presence of GDP and absence of agonists, and the nonspecific binding was assessed in the presence of 10 μM GTPγS. After incubation for 1 h at 30°C, reactions were terminated by rapid filtration (Brandel, Gaithersburg, MD) through Whatman GF/B glass fiber filters followed by three washes with 3 ml cold Tris buffer (50 mM Tris-HCl, pH 7.7). Bound radioactivity was determined by the liquid scintillation counter after overnight extraction of the filter in 7 ml scintillation fluid.

**Statistical analysis**

Data are presented as means ± SE. Paw-withdrawal thresholds in response to thermal and mechanical stimulation before and after RTX treatment were compared using a paired Student’s t-test or repeated-measures ANOVA. The effect of morphine and DAMGO on the mechanical withdrawal threshold was determined by repeated measures followed by Tukey’s post hoc test. Because the strongest component of potentiation of opioid action in this study was the extended duration of opioids, the ED₅₀ value was calculated by integrating the area under the time-effect curve for each rat. The [³H]-DAMGO saturation binding data were processed using nonlinear regression analysis (Prism, GraphPad Software, San Diego, CA) to calculate maximal specific binding (Bₘₐₓ) and dissociation constant (Kᵦ). For DAMGO-stimulated [³H]-DAMGO binding, nonlinear regression analyses of concentration-effect curves were also performed using Prism. The percent of stimulation of [³H]-DAMGO binding activity was calculated as: (net stimulated binding/basal binding) X 100%. Differences were considered to be statistically significant when P < 0.05.

**RESULTS**

**Effect of RTX on the withdrawal threshold in response to noxious heat and pressure stimuli**

Systemic injection of RTX caused a large increase in the paw-withdrawal threshold in response to the heat stimulus (Fig. 1A). This diminished thermal sensitivity occurred within 3 days after RTX injection and lasted for the duration (5 wk) of the experiments. Furthermore, RTX produced a small, but significant, decrease in the paw-withdrawal threshold in response to noxious pressure applied to the hindpaw (Fig. 1B). This slight increase in mechanical sensitivity also appeared within 3 days after RTX injection and remained unchanged for ≥5 wk. By contrast, systemic injection of the vehicle did not significantly alter the paw-withdrawal threshold in response to the noxious heat and pressure stimuli during the entire period of the experiment (Fig. 1, A and B).

**Effect of RTX on IB₄-labeling and TRPV1-immunoreactive DRG neurons and afferent fibers in the spinal dorsal horn**

To examine the effect of RTX treatment on TRPV1- and IB₄-positive primary afferent neurons, double fluorescent labeling was conducted in the L₄ and L₅ DRG from vehicle- and RTX-treated rats 4 wk after treatment. TRPV1 immunoreactivity and IB₄-labeling were present in small- and medium-
sized DRG neurons in vehicle-treated rats (Fig. 2A). IB₄-labeling was present in most TRPV1-immunoreactive neurons. In contrast, the TRPV1-immunoreactive cell bodies were largely absent in DRGs removed from RTX-treated rats (Fig. 2A). IB₄-positive neurons were also substantially reduced in DRGs of RTX-treated rats. To quantify the reduction of TRPV1- and IB₄-positive DRG neurons by RTX, six confocal images were randomly selected from each DRG (two DRGs/rat) in three RTX- and three vehicle-treated rats, and the total number of TRPV1-immunoreactive and IB₄-positive cell bodies was counted from each section. This analysis showed that ~97% (1034 in vehicle control vs. 31 in RTX group) TRPV1-immunoreactive DRG neurons were lost in RTX-treated rats. Furthermore, there was a 68% (1189 in vehicle control vs. 381 in RTX group) reduction in IB₄-positive DRG neurons in RTX-treated animals.

**FIG. 2.** Confocal images showing the effect of RTX on transient receptor potential vanilloid type 1 (TRPV1)- and isolectin B₄ (IB₄)-positive dorsal root ganglion (DRG) neurons and afferent terminals in the spinal cord. **A:** representative confocal images showing TRPV1 (green)-immunoreactive and IB₄ positive (red)-DRG neurons from 1 vehicle- and 1 RTX-treated rat. Scale bar, 40 μm. **B:** confocal images showing TRPV1 (green)-immunoreactive and IB₄ positive (red)-afferent terminals in the spinal dorsal horn of 1 vehicle- and 1 RTX-treated rat. Scale bar, 80 μm. Co-localization of TRPV1-immunoreactivity and IB₄ labeling is indicated in yellow when 2 images are digitally merged. All images are single confocal optical sections.
In vehicle-treated rats, TRPV1-immunoreactive- and IB₄-positive terminals were present in the superficial dorsal horn of the spinal cord (Fig. 2B). As shown in Fig. 2B, dense TRPV1 immunoreactivity was seen both at the dorsal root entry zone and in laminae I and II. TRPV1 immunoreactivity appeared largely co-localized on the same terminals labeled with IB₄ in the laminae I and II of rats treated with the vehicle. By comparison, the TRPV1-immunoreactive terminals were completely abolished in the spinal cord dorsal horn in RTX-treated rats (Fig. 2B). Also, IB₄-positive terminals were markedly decreased in the spinal dorsal horn of RTX-treated rats. This decrease was especially prominent in the more medial region of the dorsal horn (Fig. 2B). The reduction in IB₄-positive afferent terminals and the depletion of TRPV1 immunoreactivity in the superficial dorsal horn of RTX-treated rats both reflect the cytotoxic effect of RTX on a subpopulation of sensory neurons. These findings indicate that RTX treatment destroys TRPV1-expressing afferent neurons and their central terminals in the spinal dorsal horn.

**Effect of RTX treatment on the immunoreactivity of μ opioid receptors and TRPV1 in DRG neurons and the spinal dorsal horn**

As shown in Fig. 3A, the μ opioid receptor immunoreactivity was present in different sizes of DRG neurons in vehicle-treated rats. Also, small-diameter DRG neurons contained immunoreactivity for both TRPV1 and μ opioid receptors (Fig. 3A). Furthermore, to quantify the reduction of the μ opioid receptor immunoreactive DRG neurons by RTX, the total number of μ opioid receptor-immunoreactive cell bodies was counted from six confocal images randomly selected from each DRG (2 DRGs/rat) in three RTX- and three vehicle-treated rats. This analysis showed that ~27% (1,548 in vehicle control vs. 1,130 in RTX group) decrease in the number of cell bodies immunoreactive to the μ opioid receptors in RTX-treated rats (Fig. 3A).

The immunoreactivity of μ opioid receptors was mainly concentrated in the superficial laminae of the spinal dorsal horn in vehicle-treated rats (Fig. 3B). The μ opioid receptor immunoreactivity appeared co-localized on some terminals labeled with TRPV1 in laminae I and II of the spinal cord (Fig. 3B). In RTX-treated rats, TRPV1 immunoreactivity was largely eliminated in the superficial dorsal horn. There was also a substantial reduction in the density of μ opioid receptor immunoreactivity in the laminae I and II of the spinal cord of RTX-treated rats (Fig. 3B). Furthermore, a subpopulation of dorsal horn neurons containing TRPV1 immunoreactivity was present in the superficial dorsal horn, and these neurons were also immunoreactive to μ opioid receptors (Fig. 4A). Interestingly, the dorsal horn neurons that contained both μ opioid receptor and TRPV1 immunoreactivities were not affected by RTX treatment (Fig. 4B).

**Effect of morphine and DAMGO on mechanical nociception in RTX- and vehicle-treated rats**

Because RTX treatment removed μ opioid receptor immunoreactivity present in TRPV1-expressing DRG neurons and afferent terminals in the spinal cord, we initially tested the effect of intrathecal morphine on the hindpaw-withdrawal threshold in response to the noxious pressure in RTX- and vehicle-treated rats 4 wk after treatment. Intrathecal injection of 2–10 μg morphine in the vehicle group dose-dependently increased the withdrawal threshold in response to the pressure stimulus (Fig. 5A). The maximal effect of morphine appeared within 30 min after intrathecal injection, and the effect gradually diminished within 2–3 h. By comparison, intrathecal injection of 2–10 μg morphine produced a large effect on the pressure-evoked paw-withdrawal threshold in RTX-treated rats (Fig. 5B). Notably, 5 and 10 μg of morphine increased the withdrawal threshold to the cut-off values, and this effect lasted for 5–6 h after a single intrathecal injection in RTX-treated rats (Fig. 5B). The estimated ED₅₀ value (95% confidence limits) in vehicle- and RTX-treated groups was 4.2 (2.3–12.6) and 1.1 (0.06–1.9) μg, respectively (P < 0.05). Similar potentiated and prolonged effect of intrathecal morphine was also observed in seven separate rats tested 10–14 days after RTX injection (data not shown). Intrathecal pretreatment with a highly specific μ opioid receptor antagonist, CTAP (3 μg), 15 min before morphine administration abolished the effect of intrathecal injection of 10 μg morphine on the paw-withdrawal threshold (Fig. 5B). Intrathecal injection of 3 μg CTAP alone had no significant effect on the withdrawal threshold in all rats tested (data not shown).

We next compared the effect of a highly specific μ opioid peptide agonist, DAMGO, on the paw-withdrawal threshold in RTX- and vehicle-treated rats. Intrathecal injection of 0.1 and 0.3 μg DAMGO significantly increased the withdrawal threshold in response to the pressure stimulus, and this effect lasted ~45 min in the vehicle group (n = 8, Fig. 6). By comparison, the same doses of DAMGO produced a significantly larger increase in the withdrawal threshold in the RTX group (Fig. 6). Intrathecal injection of 0.3 μg DAMGO elevated the withdrawal threshold to the cut-off for ~45 min after injection in RTX-treated rats.

To determine if loss of TRPV1-expressing afferent neurons alters the antinociceptive effect of the μ opioid given systemically, we further tested the effect of intraperitoneal administration of 2.5 and 5 mg/kg morphine in additional RTX- and vehicle-treated rats. In the vehicle group, intraperitoneal injection of 2.5 and 5 mg/kg morphine significantly increased the hindpaw-withdrawal threshold in response to the pressure stimulus (Fig. 7). Interestingly, the same doses of morphine given intraperitoneally produced a larger and longer increase in the paw-withdrawal threshold in the RTX than in the vehicle group (Fig. 7).

**[^3]H-DAMGO saturation binding in the spinal cord of RTX- and vehicle-treated rats**

To determine if RTX treatment changes the number of binding sites and affinity of μ opioid receptors in the spinal cord, we analyzed [^3]H-DAMGO saturation binding using membranes of the dorsal spinal cord from RTX- and vehicle-treated rats. In these experiments, membrane homogenates were prepared from individual rats (n = 6 rats in each group), and six replicate experiments were performed. We observed that [^3]H-DAMGO had a single and saturable high-affinity binding site in the dorsal spinal cord tissue in both the RTX and vehicle groups (Fig. 8A). The maximal specific binding (Bₘₜₐₓ) of [^3]H-DAMGO binding in RTX-treated rats was significantly
FIG. 3. Confocal images showing the effect of RTX on μ opioid receptor- and TRPV1-immunoreactive DRG neurons and afferent terminals in the spinal cord. A: representative confocal images showing μ opioid receptor (green) and TRPV1 (red) immunoreactivities in DRG neurons of one vehicle- and one RTX-treated rat. Scale bar, 40 μm. B: confocal images showing μ opioid receptor (green) and TRPV1 (red) immunoreactivities in afferent terminals in the spinal dorsal horn of 1 vehicle- and 1 RTX-treated rat. Scale bar, 80 μm. Inset: high-magnification images (scale bar = 5 μm) showing co-localization of μ opioid receptor and TRPV1 immunoreactivity in the lamina I. Co-localization of the μ opioid receptor and TRPV1 immunoreactivity is indicated in yellow when 2 images are digitally merged. All images are single confocal optical sections.
reduced compared with that in control rats (178.6 ± 5.2 vs. 237.6 ± 5.4 fmoI/mg protein, \(P < 0.05\)). However, the dissociation constant (\(K_D\)) value was similar in the vehicle (1.26 ± 0.14 nM)- and RTX (1.23 ± 0.18 nM)-treated groups.

**DAMGO \(^{35}\)S\(\text{GTP}\gamma\text{S}\) activity in the spinal cord membranes from RTX- and vehicle-treated rats**

Additionally, to further examine if RTX treatment induced potential changes in the \(\mu\) opioid receptor-G protein coupling in the spinal cord, the \(^{35}\)S\(\text{GTP}\gamma\text{S}\) activity stimulated by DAMGO was performed using isolated membranes from the dorsal spinal cord of RTX- and vehicle-treated rats. In these experiments, membrane homogenates were prepared from individual rats (\(n = 8\) rats in each group), and eight replicate experiments were run. The concentration-effect curve for the DAMGO-stimulated \(^{35}\)S\(\text{GTP}\gamma\text{S}\) binding in spinal cord membranes is shown in Fig. 8B. The DAMGO-stimulated \(^{35}\)S\(\text{GTP}\gamma\text{S}\) binding was completely blocked by 1 \(\mu\)M CTAP (data not shown). Compared with that in vehicle-treated rats, the maximal DAMGO-stimulated \(^{35}\)S\(\text{GTP}\gamma\text{S}\) activity in the dorsal spinal cord was significantly reduced in RTX-treated rats (63.7 ± 2.5 vs. 48.5 ± 1.4\% \(P < 0.05\), Fig. 8B). When \(^{35}\)S\(\text{GTP}\gamma\text{S}\) activity stimulated by 10 \(\mu\)M DAMGO was considered as the maximal response in each group, the estimated EC\(_{50}\) value (95\% confidence limits) of DAMGO was 0.44 (0.14–2.45) and 0.46 (0.16–2.69) \(\mu\)M in the control and RTX group, respectively. The basal \(^{35}\)S\(\text{GTP}\gamma\text{S}\) binding was not significantly different between RTX- and vehicle-treated rats (56.6 ± 2.2 vs. 59.2 ± 2.4 fmoI/mg protein, \(P > 0.05\)).

**DISCUSSION**

This is the first study to determine the influence of loss of TRPV1 sensory neurons and reduction of presynaptic \(\mu\) opioid receptors on the spinal opioid analgesic effect. Because RTX deletes TRPV1-expressing primary afferent nerves, this will likely reduce the presynaptic \(\mu\) opioid receptors localized on the central terminals of these neurons. This offers a unique opportunity to determine the relative role of presynaptic \(\mu\) opioid receptors in analgesia produced by spinal \(\mu\) opioids. In the present study, we demonstrated that removal of TRPV1-expressing afferent neurons by RTX not only failed to attenuate the antinociceptive effect of \(\mu\) opioid agonists but unexpectedly caused a large potentiation of the antinociceptive effect of intrathecal and systemic \(\mu\) opioid agonists. To our knowledge, this augmented analgesic effect of spinal \(\mu\) opioid agonists after reduction of spinal presynaptic \(\mu\) opioid receptors has not been reported previously.

The spinal cord dorsal horn is a critical site for the processing of nociceptive information and for the opioid analgesic effect (Dickenson 1995; Light and Willcockson 1999; Saeki and Yaksh 1993; Yaksh and Noueihed 1985). Opioids likely inhibit synaptic transmission through both pre- and postsynaptic mechanisms in the spinal dorsal horn. About 75\% of \(\mu\) opioid receptors in the spinal cord are located presynaptically on primary afferent terminals (Dickenson 1995). Activation of presynaptic \(\mu\) opioid receptors reduces the release of excitatory neurotransmitters from the central terminals of primary afferent neurons and inhibits nociceptive inputs into the spinal cord dorsal horn (Chen et al. 2005; Kohno et al. 1999; Light and Willcockson 1999; Schneider et al. 1998). Thus the presynaptic site of action of \(\mu\) opioid agonists on nociceptive afferent terminals in the spinal cord has been considered an important mechanism underlying spinal opioid analgesia. Because RTX selectively removes TRPV1-expressing afferent neurons and the associated presynaptic \(\mu\) opioid receptors in the spinal cord, this study was originally designed to examine to what extent...
extent the analgesic effect of spinally administered μ opioid agonists would be attenuated in rats treated with RTX. There is no evidence suggesting that RTX acts through TRPV2 channels. RTX is a well-known capsaicin analogue, and its binding is competitively inhibited by TRPV1 antagonist capsazepine (Szallasi et al. 1999). Furthermore, in contrast to TRPV1, TRPV2 is expressed in medium- to large-sized DRG neurons with myelinated fibers and does not respond to capsaicin (Lewinter et al. 2004; Tamura et al. 2005). It is important to

FIG. 5. Effect of intrathecal morphine on the nociceptive withdrawal threshold in RTX- and vehicle-treated rats. A: time course of the effect of intrathecal injection of 2 (n = 9), 5 (n = 8), and 10 (n = 8) μg morphine on the nociceptive withdrawal threshold in vehicle control rats. B: time course of the effect of intrathecal injection of 2 (n = 9), 5 (n = 9), and 10 (n = 8) μg morphine on the nociceptive withdrawal threshold in RTX-treated rats. Note that intrathecal injection of 10 μg morphine failed to alter significantly the withdrawal threshold in the presence of 3 μg D-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP). The nociceptive threshold was determined by the withdrawal response of the hindpaw to a noxious pressure stimulus. Data presented as means ± SE. *, P < 0.05 compared with the respective baseline control.

FIG. 6. Effect of intrathecal injection of 0.1 and 0.3 μg of (d-Ala²,N-Me-Phe⁷,Gly-ol³)enkephalin (DAMGO) on the nociceptive withdrawal threshold in 7 vehicle- and 8 RTX-treated rats. The nociceptive threshold was determined by the withdrawal response of the hindpaw to a noxious pressure stimulus. Data presented as means ± SE. *, P < 0.05 compared with the respective baseline control.

FIG. 7. Effect of intraperitoneal injection of 2.5 and 5 mg/kg of morphine on the nociceptive withdrawal threshold in 8 vehicle- and 8 RTX-treated rats. The nociceptive threshold was determined by the withdrawal response of the hindpaw to a noxious pressure stimulus. Data presented as means ± SE. *, P < 0.05 compared with the respective baseline control.

FIG. 8. Effect of RTX on the μ opioid receptor binding in the spinal cord dorsal horn. A: comparison of specific [³⁵S]-DAMGO binding to rat dorsal spinal cord membranes from rats treated with vehicle and RTX (n = 6 replicates in each group). B: concentration-effect curve for the DAMGO-stimulated [³⁵S]-GTPγS activity in the dorsal spinal cord membrane obtained from control and RTX-treated rats (n = 8 replicates in each group). The data are expressed as the percentage of basal [³⁵S]-GTPγS binding.
note that the effect of RTX is not to specifically eliminate the TRPV1 channels but rather to destroy the primary afferent neurons expressing TRPV1 channels. Consequently, it is likely that any ion channels and receptors that are present on TRPV1-expressing DRG neurons are also eliminated by RTX treatment. Destruction of TRPV1-expressing DRG neurons by the cytotoxicity of RTX is clearly supported by our finding showing that the number of µ opioid receptor-immunoreactive and IB4-positive DRG neurons was markedly reduced by RTX treatment. Furthermore, it has been documented that both RTX and capsaicin can kill primary sensory neurons through calcium influx and overloading (Karai et al. 2004; Ohia et al. 2001; Wu et al. 2005). Our immunocytochemistry data confirmed that RTX eliminated presynaptic µ opioid receptors on TRPV1-immunoreactive afferent terminals in the spinal dorsal horn. The radioligand binding experiments also demonstrated ~30% reduction of the specific binding site of µ opioid receptors in the spinal cord of RTX-treated rats. It has been shown that TRPV1 is expressed by a subpopulation of dorsal horn neurons in lamina I and II (Doly et al. 2004; Valtchanoff et al. 2001). Interestingly, we found that systemic RTX treatment had no effect on the superficial dorsal horn neurons that express both TRPV1 and µ opioid receptors. Previous studies have shown that neonatal capsaicin treatment reduces µ opioid receptors in the spinal cord (Abbadie et al. 2002) but does not affect morphine analgesia in rats (Jancso and Jancso-Gabor 1980). In contrast to our hypothesis, we found that loss of TRPV1-expressing afferent neurons paradoxically potentiates the antinociceptive effect of µ opioid agonists. In this regard, intrathecal injection of two structurally dissimilar µ opioid receptor agonists, morphine and DAMGO, produced a profound effect on nociception measured with a noxious pressure stimulus in RTX-treated rats. Additionally, the analgesic effect produced by intraperitoneal morphine was also significantly potentiated in rats treated with RTX, suggesting that the µ opioid agonist administered systemically also is more efficacious in suppressing the mechano-nociception in animals lacking TRPV1-expressing afferent neurons.

RTX-induced paradoxical changes in the reduction of spinal µ opioid receptors and potentiation of the potency of opioid analgesia are completely unexpected. Changes in the µ opioid receptor number/binding affinity, redistribution, desensitization, and G protein coupling and downstream signaling may explain increased opioid efficacy in RTX-treated rats. Our immunocytochemistry and radioligand binding data do not support the possibility that the potentiated analgesic effect of spinal µ opioid agonists is due to the compensatory upregulation of µ opioid receptors in the spinal cord of RTX-treated rats. Both immunocytochemistry and radioligand-binding experiments showed a consistent reduction of µ opioid receptors in the spinal cord of RTX-treated rats. We also found that the Kd value of the specific µ opioid receptor binding in the spinal cord was similar in the vehicle and RTX groups. Furthermore, the DAMGO-stimulated [35S]GTPγS activity in the dorsal spinal cord was substantially reduced in RTX-treated rats. Although the proportion of µ opioid receptors on TRPV1- and non-TRPV1-sensory neurons may be altered by RTX treatment, it is less likely that the potentiated effect of spinal µ opioid agonists in RTX-treated rats is due to increased binding affinity of the µ opioid agonists or increased µ opioid receptor coupling to G proteins.

The mechanisms underlying this puzzling RTX-induced paradoxical change in opioid potency and µ opioid receptors in the spinal cord are currently unknown. One of the possibilities is that more functional µ opioid receptors may be distributed in non-TRPV1- than TRPV1-expressing afferent neurons. DRG neurons subserving specific sensory modalities are supported by different neurotrophins (Snider and McMahon 1998). In adulthood, approximately one-half of nociceptors are dependent on nerve growth factor (NGF) for trophic support; these neurons express the TrkA receptor for NGF and contain neuropeptides such as substance P and calcitonin-gene related peptide (Averill et al. 1995; McMahon et al. 1994; Molliver et al. 1995). The other half are sensitive to glial-cell-line-derived neurotrophic factor (GDNF); most of these DRG neurons possess receptors for GDNF, express TRPV1, and bind to IB4 (Bennett et al. 1998; Guo et al. 1999; Wang et al. 1994). DRG neurons expressing NGF receptors (mostly IB4- and TRPV1-negative) are important for normal pain perception. In this regard, rats and mice deprived of NGF during embryonic development by antibodies or gene targeting are unable to respond to painful stimuli (Crowley et al. 1994; Johnson et al. 1980; Smeyne et al. 1994). Furthermore, humans with mutations of NGF TrkA receptors or NGF beta gene are unable to detect pain (Einarsdottir et al. 2004; Mardy et al. 2001). It is known how analgesics such as opioids modulate nociception transmitted by different phenotypes of primary afferent neurons. We have shown that the density of µ opioid receptors is ~18% higher in IB4-negative than in IB4-positive (mostly expressing TRPV1) DRG neurons (Wu et al. 2004). Also the µ opioid agonists produce a greater inhibition of voltage-gated Ca2+ channels, especially the N- and P/Q-type, in IB4-negative than in IB4-positive DRG neurons (Wu et al. 2004). The present data, combined with the prior cellular study, lead to the prediction that the density of µ opioid receptors in TRPV1-negative sensory neurons may increase after RTX treatment.

Another possibility for this paradoxical finding is that RTX may alter the spinal cord circuitry so that the synaptic connection/interaction is reorganized somehow to facilitate the inhibitory effect of µ opioid agonists. We have demonstrated a small degree of sprouting of myelinated afferent terminals in the spinal lamina II after RTX treatment (Pan et al. 2003). It has been shown that morphine increases the activity of lamina II neurons but suppresses the firing of deeper dorsal horn neurons (Chen et al. 2005; Sastry and Goh 1983; Woolf and Fitzgerald 1981). Furthermore, the inhibitory action of morphine on deep dorsal horn projection neurons depends on its effect on GABAergic and glycinergic inputs to lamina II neurons (Chen et al. 2005). In RTX-treated rats, C fiber termination in the lamina II is largely removed, and sprouting of TRPV1-negative primary afferent terminals could re-target different lamina II neurons (Pan et al. 2003). This reorganization of the spinal dorsal horn circuitry may constitute a neuronal plasticity for the potent inhibition of µ opioid agonists of nociceptive inputs transmitted through non-TRPV1 afferent neurons. It would be interesting to examine if TRPV1 and non-TRPV1 afferent terminals form synapses with different types of inhibitory and excitatory interneurons in the spinal dorsal horn after RTX treatment. Additionally, the absence of TRPV1-expressing afferent fibers may result in less nociceptive input to spinal dorsal horn neurons, resulting in enhanced response to µ opioid agonists. Hence further studies are war-
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ranted to determine how reorganization of the spinal dorsal horn circuitry after RTX treatment leads to increased analgesic effect of \( \mu \) opioid agonists.

The potential differences in biochemical pathways responsible for the termination (receptor desensitization, internalization, and sequestration) of the \( \mu \) opioid actions between TRPV1 and non-TRPV1 nociceptive afferent pathways should also be considered. The \( \mu \) opioid receptor can be desensitized after activation by \( \mu \) opioid agonists through phosphorylation by G-protein-coupled receptor kinases (GRKs) (Schulz et al. 2002). Phosphorylated \( \mu \) opioid receptors are then bound by arrestins (Bohn et al. 1999; Whistler and von Zastrow 1998), which prevent further stimulation of G proteins and downstream signaling pathways by \( \mu \) opioid agonists. The \( \mu \) opioid receptor also can be phosphorylated by protein kinase C (mostly TRPV1-expressing) DRG neurons (Molliver et al. 1995), and activation of protein kinase C reduces the inhibitory effect of \( \mu \) opioid agonists on voltage-gated Ca\(^{2+} \) channels (King et al. 1999). This difference may contribute to a greater effect of \( \mu \) opioids on voltage-gated Ca\(^{2+} \) channels in IB\(_4\)-negative than in IB\(_4\)-positive DRG neurons (Wu et al. 2004).

Additional studies are necessary to determine if opioid desensitization and tolerance are altered in RTX-treated animals. Although TRPV1 is well known for its critical role in thermal nociception, pain elicited by mechanical stimuli is far more clinically important. Thermal and mechanical nociception may be mediated by different (and overlapping to certain degree) subpopulations of distinct phenotypic nociceptors. In RTX-treated rats, mechano-nociception remains largely intact (Pan et al. 2003; Xu et al. 1997). Thus non-TRPV1 primary afferents appear important in the transduction and transmission of mechano-nociception. Nevertheless the phenotypes of primary afferent neurons specifically involved in mechano-nociception have not been identified. RTX-treated rats can be used to study the physiological function and pharmacological modulation of mechano-nociception transmitted by non-TRPV1 primary afferent neurons. Further studies on the differences in the opioid actions and opioid receptor desensitization between different phenotypic nociceptive afferent neurons will provide additional new information for the differential opioid modulation of modality-specific nociception. This new information will be important for a better understanding of the analgesic mechanisms of \( \mu \) opioid agonists and provides a rationale for improved opioid therapies to treat different painful conditions.

GRANTS

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