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Resistance to Morphine Analgesic Tolerance in Rats Deleted of TRPV1-Expressing Sensory Neurons

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Abstract

Deletion of TRPV1-expressing afferent neurons reduces presynaptic μ opioid receptors but paradoxically potentiates the analgesic efficacy of μ opioid agonists. In this study, we determined if removal of TRPV1-expressing afferent neurons by resiniferatoxin (RTX), an ultrapotent capsaicin analogue, influences the development of opioid analgesic tolerance. Morphine tolerance was induced by daily intrathecal injections of 10 μ g of morphine for 14 consecutive days or by daily intraperitoneal injections of 10 mg/kg of morphine for 10 days. In vehicle-treated rats, the effect of intrathecal or systemic morphine on the mechanical withdrawal threshold was gradually diminished within 7 days. However, the analgesic effect of intrathecal and systemic morphine was sustained in RTX-treated rats at the time the morphine effect was lost in the vehicle group. Furthermore, the μ opioid receptor-G protein coupling in the spinal cord was significantly decreased (~22%) in vehicle-treated morphine tolerant rats, but was not significantly altered in RTX-treated rats receiving the same treatment with morphine. Additionally, there was a large reduction in protein kinase C γ -immunoreactive afferent terminals in the spinal dorsal horn of RTX-treated rats. These findings suggest that loss of TRPV1-expressing sensory neurons attenuates the development of morphine analgesic tolerance possibly by reducing μ opioid receptor desensitization through protein kinase C γ in the spinal cord. These data also suggest that the function of presynaptic μ opioid receptors on TRPV1-expressing sensory neurons is particularly sensitive to down-regulation by μ opioid agonists during opioid tolerance development.

Keywords

nociceptors; analgesia; dorsal root ganglia; opioids; receptor binding; pain

INTRODUCTION

Pain is typically initiated by activation of different populations of nociceptors and appears to be modality-specific. For example, the transient receptor potential vanilloid type 1 (TRPV1) ion channel, plays an essential role in detecting thermal nociception. It has been shown that thermal but not mechanical nociception is reduced in mice deficient of TRPV1 receptors (Caterina et al., 2000). The capsaicin analogue, resiniferatoxin (RTX), is an ultrapotent TRPV1 agonist (Szallasi and Blumberg, 1999). Systemic administration of RTX in adult rats deletes

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TRPV1-expressing sensory neurons and substantially impairs thermal but not mechanical nociception (Pan et al., 2003, Chen and Pan, 2006b). Clinically, pain elicited by mechanical stimuli is far more important than that elicited by thermal stimuli. Use of RTX is an important pharmacological approach to study mechanical nociception transduced by non-TRPV1-sensory neurons.

It remains unclear if the modality-specific nociceptive information is differentially modulated by different classes of analgesics. The μ opioid receptor agonists remain the most widely used class of analgesics to treat patients with moderate and severe pain. At clinically relevant doses, morphine produces its analgesic effect primarily through μ opioid receptors. In particular, the μ opioid receptors located in the superficial dorsal horn of the spinal cord (Gamse et al., 1979, Yoshimura and North, 1983) play an important role in the analgesic action of μ opioids. Intrathecal injection of μ opioid agonists produces potent analgesia in animals and humans (Yaksh and Noueihed, 1985). Furthermore, intrathecal injection of μ opioid antagonists reduces or completely blocks the analgesic effect produced by systemic administration of morphine (Yaksh and Noueihed, 1985, Chen and Pan, 2006a). Because administration of RTX induces a substantial reduction in presynaptic μ opioid receptors (Chen and Pan, 2006b), we have used RTX to study the role of presynaptic μ opioid receptors in opioid analgesia. Interestingly, removal of presynaptic μ opioid receptors present on TRPV1-expressing afferent neurons unexpectedly potentiates the analgesic effect of systemic or intrathecal injection of μ opioid agonists (Chen and Pan, 2006b). However, the molecular mechanism underlying the paradoxical reduction in presynaptic μ opioid receptors and the concomitant increase in opioid efficacy is not clear.

Because the duration of the opioid analgesic effect is profoundly increased in RTX-treated rats (Chen and Pan, 2006b), we have postulated that the biochemical pathways responsible for termination of the μ opioid actions and desensitization of functional μ opioid receptors differ between TRPV1-expressing and non-TRPV1-expressing sensory neurons. If this is the case, the time course of μ opioid receptor desensitization and tolerance development can be influenced when the presynaptic μ opioid receptors on TRPV1-expressing primary afferents are deleted by RTX. Therefore, to investigate this possibility, we determined if the development of the analgesic tolerance of morphine was affected by removal of TRPV1-expressing sensory afferents. We found that the development of tolerance to morphine was substantially attenuated in RTX-treated rats and that the μ opioid receptor-G protein coupling was reduced by chronic morphine in vehicle-, but not RTX-, treated rats. Also, RTX produced a substantial reduction in protein kinase C γ (PKC γ)-containing primary afferent terminals in the spinal cord. These findings are important for our understanding of the complex molecular mechanisms underlying the signaling and desensitization of μ opioid receptors expressed in heterogeneous populations of sensory neurons.

MATERIALS AND METHODS

Animals and RTX treatment

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighting 280–320 g were used in this study. Rats in the treatment group received a single intraperitoneal injection of RTX (200 μ g/kg, LC Laboratories, Woburn, MA) while under isoflurane-induced anesthesia for 4–5 hr. RTX was dissolved in a mixture of 10% Tween-80 and 10% ethanol in normal saline (Chen and Pan, 2006b). Rats in the control group received a single intraperitoneal injection of the vehicle. The morphine tolerance experiments were conducted 2–3 weeks after the RTX and vehicle injections. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center and of Pennsylvania State University College of Medicine and conformed to the National Institutes of Health guidelines for the ethical use of animals.

Intrathecal catheter implantation

Intrathecal catheters were implanted in RTX- and vehicle-treated rats during isoflurane-induced anesthesia. Briefly, each animal was placed prone on a stereotaxic frame and a small incision was made at the back of the animal's neck. Then, a small puncture was made in the atlanto-occipital membrane of the cisterna magna, and a polyethylene catheter (PE-10, 8.0 cm) was inserted such that the caudal tip reached the lumbar enlargement of the spinal cord. The rostral end of the catheter was exteriorized and the wound was closed with sutures. The animals were allowed to recover for 4–5 days after the surgery. Animals displaying signs of motor or neurological dysfunction were excluded from the study. Drugs for intrathecal injection were dissolved in normal saline and administered in a volume of 5 μ l, followed by a 10 μ l flush with normal saline.

Behavioral assessment of mechanical nociception

The paw pressure test was used to measure mechanical nociception using the Ugo Basil Analgesimeter (Varese, Italy). Noxious pressure was gradually applied to the hindpaws of the animals. To activate the device, a foot pedal was pressed which activated a motor that applied a constant increasing force on a linear scale. When the animal displayed pain by either withdrawing of the paw or vocalization, the pedal was immediately released, and the animal's nociceptive threshold was read on the scale. A maximum of 400 g was used as a cut-off to avoid potential tissue injury to the animals (Chen and Pan, 2003, 2006b). Both of the hindpaws in each animal were tested, and the mean value was used as the nociceptive withdrawal threshold.

Behavioral assessment of thermal nociception

To quantitatively assess the thermal nociceptive threshold, rats were placed on the glass surface of a thermal testing apparatus (IITC Inc., 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. The paw withdrawal latency was recorded using a timer. A cut-off of 30 s was used to prevent potential tissue damage (Chen et al., 2002, Chen and Pan, 2006b).

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

In three vehicle- and three RTX-treated rats, the effect of RTX treatment on TRPV1- and *Griffonia simplicifolia* isolectin B₄ (IB₄)-positive primary afferent terminals in the spinal dorsal horn was assessed using double labeling of TRPV1 and IB₄ (Guo et al., 1999, Pan et al., 2003, Chen and Pan, 2006b) 3 weeks after treatment. Under deep anesthesia with sodium pentobarbital (60 mg/kg, i.p.), 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 was removed quickly and cut to 30 μ m in thickness. For TRPV1 immunofluorescent labeling (Pan et al., 2003, Chen and Pan, 2006b), the sections were incubated with the primary antibody (rabbit anti-TRPV1 N-terminus, dilution 1:1000, Neuromics Inc, Minneapolis, MN) for 2 hr 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 μ g/ml, Molecular Probes, Eugene, OR). The sections then were incubated with Alexa Fluor-594 conjugated to IB₄ (Molecular Probes, dilution: 2 μ g/ml) for 2 hr at room temperature. Tissue sections were examined on a laser scanning confocal microscope (Zeiss, Germany), and areas of interest were photodocumented.

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

To examine the effect of RTX treatment on the μ opioid receptors in the spinal dorsal horn, double immunofluorescence labeling of μ opioid receptors and TRPV1 (Chen and Pan, 2006b) was performed in the lumbar spinal cord from 3 RTX- and 3 vehicle-treated rats. Briefly, tissue sections were first incubated with the mixture of primary antibodies (rabbit anti- μ opioid receptors, dilution 1:1000, Neuromics; and guinea pig anti-TRPV1, dilution 1:1000, Neuromics) for 2 hr at room temperature and overnight at 4°C. Subsequently, sections were incubated with the secondary antibody mixture (Alexa Fluor-488 conjugated to goat anti-rabbit IgG; and Alexa Fluor-594 conjugated to goat anti-guinea pig IgG, Molecular Probes, dilution: 5 μ g/ml) for 1.5 hr. The sections then were rinsed in PBS for 30 min, mounted on slides, dried, and coverslipped.

Double immunofluorescence labeling of PKC γ and μ opioid receptors in the spinal cord

To determine the effect of RTX treatment on PKC γ -immunoreactivity in the spinal dorsal horn, double immunofluorescence labeling of μ opioid receptors and PKC γ was performed in the lumbar spinal cord from 4 RTX- and 3 vehicle-treated rats. Tissue sections were first incubated with the mixture of primary antibodies (guinea pig anti- μ opioid receptors, dilution 1:1000, Neuromics; and rabbit anti-PKC γ , dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hr at room temperature and overnight at 4°C. The specificity of the anti-PKC γ antibody has been determined using tissues from PKC γ -knockout mice (Zeititz et al., 2001). Subsequently, sections were incubated with the secondary antibody mixture (Alexa Fluor-594 conjugated to goat anti-rabbit IgG; and biotin conjugated to goat anti-guinea pig IgG) for 1.5 hr. After wash, the sections were incubated with Alexa Fluor-488 conjugated streptavidin for 1 hr. Omission of the primary antibodies in the above experiments resulted in negative labeling in the spinal cord.

To estimate the extent of changes in μ opioid receptor- and PKC γ -immunoreactivity in the spinal cord by RTX, imaging analysis was performed on 8–10 sections from each animal. A threshold intensity at which most of the background labeling was suppressed was set for each image (Chen and Pan, 2003). The same region of the superficial dorsal horn (i.e., lamina I and outer lamina II) was outlined in the sections from vehicle- and RTX-treated rats. The percentage of pixels within the area with an intensity above the threshold was measured by a computer-based imaging analysis program (AIS; Imaging Research, Inc., St. Catharines, Ontario, Canada). The quantification of PKC γ -immunoreactivity was performed by an investigator who was blinded to the experimental intervention. Results are expressed as the percentage change in the fluorescence density in RTX-treated rats compared to that in vehicle-treated animals.

Agonist-stimulated [35 S]GTP γ S activity in spinal cord membranes

Additional experiments were performed to further determine if the functional μ opioid receptor is altered by RTX treatment. The [35 S]GTP γ S binding assay is used for this protocol because the agonist-stimulated [35 S]GTP γ S binding can provide functional information about the receptor (i.e., coupling of G proteins to activated receptors). The [35 S]GTP γ S binding stimulated by (D-Ala², N-Me-Phe⁴, Gly-ol⁵)-enkephalin (DAMGO), a highly specific μ opioid agonist, was performed using isolated membranes. The dorsal spinal cord tissue was homogenized in cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EGTA, pH 7.4) and centrifuged at 500 $\times$ g for 10 min at 4°C. The supernatant was then centrifuged at 48,000 $\times$ g for 30 min at 4°C. The pellet was resuspended in an assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, pH 7.7) and measured for protein content. Concentration-effect curves of the DAMGO-stimulated [35 S]GTP γ S binding included 0.1–10.0 μ M DAMGO with and without 1 μ M D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP, a specific μ opioid receptor antagonist), 30 μ M GDP, 0.05 nM [35 S]GTP γ S, 4 mU/ml adenosine deaminase, 20 μ g protein, and assay buffer in a final volume of 1 ml. The basal level

of [^{35}S]GTP γ S binding was determined in the presence of GDP and absence of agonists, and the nonspecific [^{35}S]GTP γ S binding was assessed in the presence of 10 μM GTP γ S (Chen and Pan, 2006b). After incubation for 1 hr at 30°C, reactions were terminated using 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 using the liquid scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA).

Statistical analysis

Data were presented as mean $\pm$ S.E.M. Paw withdrawal thresholds in response to thermal stimulation before and after RTX treatment were compared using a paired Student's *t* test. The effect of morphine on the mechanical withdrawal threshold was determined using repeated measures ANOVA followed by Tukey's *post hoc* test. The difference in the peak analgesic effect of morphine between vehicle- and RTX-treated rats was compared using two-way ANOVA with Bonferroni's *post hoc* test. For DAMGO-stimulated [^{35}S]GTP γ S binding, nonlinear regression analyses of concentration-effect curves were performed. The percent of stimulation of [^{35}S]GTP γ S activity was calculated as: (net stimulated binding/basal binding) $\times$ 100%. $P < 0.05$ was deemed statistically significant.

RESULTS

In all of the RTX-treated rats, there was a large increase in the paw withdrawal threshold in response to the heat stimulus as tested 2 weeks after RTX injection (26.82 ± 2.64 vs. 9.42 ± 1.22 s pre-treatment, $n = 21$, $P < 0.05$). In contrast, systemic injection of the vehicle did not significantly alter the paw withdrawal threshold in response to the noxious heat (9.43 ± 1.28 vs. 9.52 ± 1.31 s pre-treatment, $n = 20$). For the tolerance induction experiments, intrathecal or systemic morphine treatment began three weeks after RTX and vehicle injections.

Effect of RTX on TRPV1- and μ opioid receptor-immunoreactivity in the spinal dorsal horn

TRPV1-immunoreactive- and IB $_4$ -positive-terminals were largely distributed in the superficial dorsal horn of the spinal cord in vehicle-treated rats (Fig. 1A). Also, TRPV1 immunoreactivity was largely co-localized on the same terminals labeled with IB $_4$ in the superficial dorsal horn. In contrast, the TRPV1-immunoreactive terminals were absent from the spinal dorsal horn of RTX-treated rats (Fig. 1A). Furthermore, IB $_4$ -positive terminals in the spinal dorsal horn were markedly reduced in RTX-treated rats (Fig. 1A).

In vehicle-treated rats, the immunoreactivity of μ opioid receptors was distributed largely in the superficial lamina of the spinal cord dorsal horn (Fig. 1B). Also, the μ opioid receptor-immunoreactivity was co-localized on some terminals labeled with TRPV1 in the superficial dorsal horn (Fig. 1B). In RTX-treated rats, TRPV1-immunoreactivity was eliminated in the superficial dorsal horn. Furthermore, the density of μ opioid receptor-immunoreactivity in the lamina I and lamina II of the spinal cord was substantially reduced in RTX-treated rats (Fig. 1B). Semi-quantitative imaging analysis showed a $57.3 \pm 7.8\%$ reduction in the density of μ opioid receptor-immunoreactivity in the lamina I and lamina II in RTX-treated rats compared with that in vehicle-treated rats.

Time course of development of spinal morphine tolerance in RTX-treated rats

Spinal morphine tolerance was induced by daily injection of a total amount of 10 μg of morphine through the intrathecal catheter for 14 consecutive days. To test the analgesic effect of morphine, we first injected 5 μg of morphine and measured the time course of its effect on the paw withdrawal threshold in response to application of the noxious pressure. Each day at the end of the behavioral testing, the remaining 5 μg of morphine was injected intrathecally.

A smaller dose of morphine (5 μ g) was chosen to determine the nociceptive threshold because intrathecal bolus injection of 10 μ g of morphine increased the withdrawal threshold to the cut-off value. Nociceptive testing was performed before and every 30–60 min after the first injection of 5 μ g of morphine for 6 hr.

Figure 2A shows the time course of the analgesic effect of 5 μ g of morphine administered intrathecally on the paw withdrawal threshold in 9 RTX-treated and 8 vehicle-treated groups. In the vehicle group, daily intrathecal injection of 10 μ g of morphine caused a gradual attenuation of the morphine effect on the paw withdrawal threshold. Within 1 week, this effect was completely lost in vehicle-treated rats. In contrast, the maximal effect of morphine on the paw withdrawal threshold was largely sustained in RTX-treated rats during the 14-day period of daily intrathecal injection of 10 μ g of morphine (Figure 2A). Even by day 14, the peak effect produced by intrathecal injection of 5 μ g of morphine was still evident in the RTX group. Furthermore, the magnitude and the duration of the morphine effect was significantly greater on both day 1 and day 14 in the RTX- than in vehicle-treated group (Figure 2B). Nevertheless, in RTX-treated rats, both the peak effect and the duration of action of 5 μ g of morphine were significantly attenuated on day 14 when compared with those on day 1 (Figure 2B).

Time course of tolerance development by systemic morphine in RTX-treated rats

In separate groups of RTX- and vehicle-treated rats, we also determined the time course of development of morphine tolerance by systemic administration. In this protocol, 10 mg/kg of morphine was injected daily into the intraperitoneal cavity of rats for 10 consecutive days. To test the antinociceptive effect of morphine, we first injected 5 mg/kg of morphine and measured the time course of its effect on the paw withdrawal threshold in response to application of noxious pressure. Each day at the end of the behavioral testing, the remaining 5 mg/kg of morphine was injected intraperitoneally.

Figure 3 illustrates the different time courses of the maximal effect on the paw withdrawal threshold produced by 5 mg/kg of morphine administered intraperitoneally between RTX- and vehicle-treated groups. The maximal effect of morphine appeared within 30 min following intraperitoneal injection in both groups. In 7 vehicle-treated rats, daily intraperitoneal injection of 10 mg/kg of morphine induced a gradual reduction in the effect of morphine on the paw withdrawal threshold (Figure 3). By day 6, intraperitoneal injection of 5 mg/kg of morphine failed to produce a significant effect in vehicle-treated rats. In comparison, the reduction in the morphine effect following daily injection of 10 mg/kg of morphine was much slower in the RTX-treated group ($n = 8$, Figure 3). Even one week after daily morphine treatment, intraperitoneal injection of 5 mg/kg of morphine still increased significantly the paw withdrawal threshold in RTX-treated rats. Nonetheless, daily injection of 10 mg/kg morphine for 10 days eventually diminished the morphine effect in the RTX-treated group (Figure 3). Furthermore, the baseline paw withdrawal threshold was significantly reduced in the vehicle-treated group (Figure 3).

DAMGO-stimulated [35 S]GTP γ S activity in the spinal cord membranes from morphine tolerant and RTX-treated rats

We also determined how chronic treatment of intrathecal morphine affects μ opioid receptor-G protein coupling in the spinal cord in vehicle- and RTX-treated rats. The [35 S]GTP γ S activity stimulated by DAMGO was performed using isolated membranes from the dorsal spinal cord of RTX- and vehicle-treated rats receiving intrathecal morphine. In these experiments, membrane homogenates were prepared from the dorsal half of the spinal cord from RTX- and vehicle-treated rats at the end of daily intrathecal morphine experiments (day 14). The dorsal spinal tissues obtained from RTX- and vehicle-treated rats that did not receive morphine were used as controls. As shown in Figure 4, DAMGO produced a concentration-dependent increase

in [35 S]GTP γ S binding in the spinal cord membranes in all four groups. In the control rats (without morphine treatment), the maximal DAMGO-stimulated [35 S]GTP γ S activity in the dorsal spinal cord was significantly lower in the RTX-treated group than in the vehicle group ($52.3 \pm 1.08\%$ vs. $62.45 \pm 1.32\%$, $n = 6$, $P < 0.05$, Fig. 4). Furthermore, the maximal DAMGO-stimulated [35 S]GTP γ S activity in the dorsal spinal cord was significantly reduced in vehicle-treated morphine-tolerant rats, when compared with that in vehicle-treated rats that did not receive morphine ($62.45 \pm 1.32\%$ vs. $48.59 \pm 1.54\%$, $P < 0.05$, Fig. 4). However, the DAMGO-stimulated [35 S]GTP γ S activity in the dorsal spinal cord did not differ significantly between rats treated with RTX alone and rats that received both RTX and morphine treatments ($52.03 \pm 1.08\%$ vs. $51.76 \pm 1.52\%$, $n = 6$, Fig. 4). The basal [35 S]GTP γ S binding was not significantly different among the vehicle, vehicle plus morphine, RTX, and RTX plus morphine groups (56.4 ± 2.6 , 56.8 ± 2.2 , 55.8 ± 2.6 , and 59.1 ± 2.4 fmol/mg protein, respectively).

Effect of RTX on protein kinase C γ and μ opioid receptor-immunoreactivity in the spinal dorsal horn

Because the protein kinase C (PKC), especially the PKC γ isoform, plays an important role in the development of opioid analgesic tolerance (Narita et al., 2001a, Narita et al., 2001b, Zeitz et al., 2001), additional experiments were performed to determine the effect of RTX treatment on PKC γ immunoreactivity in the spinal cord. In vehicle-treated rats, the immunoreactivity of PKC γ was distributed mainly in the superficial laminae of the spinal cord dorsal horn (Fig. 5). The PKC γ -immunoreactivity was present on the terminals in the lamina I and lamina II and was especially dense on neurons located in the inner lamina II and lamina III. The μ opioid receptor-immunoreactivity was co-localized on many terminals labeled with PKC γ in the superficial dorsal horn (Fig. 5). In RTX-treated rats, the PKC γ -immunoreactivity in the superficial dorsal horn was substantially decreased (Fig. 5). Because TRPV1-expressing primary afferents mainly terminate in the lamina I and outer lamina II, the density of PKC γ -immunoreactivity in this region was analyzed. The density of PKC γ -immunoreactive terminals in the lamina I and outer lamina II was reduced $48.1 \pm 6.5\%$ in RTX-treated rats. However, the neurons containing PKC γ -immunoreactivity were little affected in the inner lamina II and lamina III of RTX-treated rats.

DISCUSSION

The most salient finding of this study was that removal of TRPV1-expressing afferent neurons attenuates the development of morphine tolerance. Chronic administration of μ opioid agonists induces tolerance, which limits their use in treatment of chronic pain. Many patients taking opioids for pain control need increasing doses of the drug to maintain adequate analgesia. This dose escalation can eventually lead to inadequate pain relief. Although the μ opioid receptors in the spinal cord are critical for the analgesic effect of morphine (Yaksh and Noueihed, 1985, Dickenson, 1995, Chen and Pan, 2006a, b), little is known about the role of pre- and postsynaptic μ opioid receptors in the spinal cord in the development of the analgesic tolerance of μ opioid agonists. In this study, we found that development of the analgesic tolerance resulting from both intrathecal and systemic injections of morphine was largely attenuated in RTX-treated rats. Notably, at the outset of this study, we hypothesized that because opioid action is prolonged in RTX-treated rats, morphine tolerance would develop sooner in RTX- than in vehicle-treated rats. Although the analgesic effect of morphine is augmented in RTX-treated rats (Chen and Pan, 2006b), we used the same dose of morphine in the RTX and control groups. Had we lowered the morphine dose in RTX-treated rats to “normalize” the analgesic effect of morphine, it would have further diminished the development of opioid tolerance in this group. This study shows that morphine analgesic tolerance is delayed in the absence of TRPV1-expressing primary afferent neurons.

Another interesting finding of this study was that functional μ opioid receptors present on TRPV1-afferent terminals are particularly susceptible to down-regulation by morphine. Although the mechanisms of opioid tolerance are not fully known, studies have shown that the decreased coupling of the μ opioid receptor with inhibitory G proteins is an important mechanism (Sim et al., 1996, Maher et al., 2001, Narita et al., 2001a). Agonist-stimulated [35 S]GTP γ S binding to μ opioid receptors can be used to examine the effect of chronic opioid treatment on μ opioid receptor-G protein coupling (Traynor and Nahorski, 1995). In the previous study (Maher et al., 2001), reduction of μ opioid receptor-G protein coupling occurs in the spinal cord following chronic morphine treatment. However, it is unclear if the down-regulation of μ opioid receptors occurs to those on presynaptic terminals or on postsynaptic dorsal horn neurons (Gamse et al., 1979, Yoshimura and North, 1983). In the current study, we found that chronic morphine treatment significantly reduced (~22%) DAMGO-stimulated [35 S]GTP γ S binding in the spinal cord in vehicle-, but not in RTX-, treated rats. These data suggest that μ opioid receptor-G protein coupling on TRPV1-expressing sensory neurons and terminals is particularly sensitive to desensitization by chronic exposure to μ opioids. Therefore, the desensitization mechanisms for native μ opioid receptors in different populations of sensory neurons are likely heterogenous.

In the present study, we also demonstrated that the PKC γ -immunoreactivity in the spinal dorsal horn is substantially reduced in rats in which TRPV1-sensory neurons have been deleted. The signaling mechanisms involved in the termination of the μ opioid actions (receptor desensitization, internalization, and sequestration) are not fully understood. The μ opioid receptor can be desensitized by G protein-coupled receptor kinases (Schulz et al., 2002) and β -arrestins (Whistler and von Zastrow, 1998, Bohn et al., 2000, Bohn et al., 2002). Also, the μ opioid receptor can be phosphorylated by PKC (Mestek et al., 1995, Narita et al., 2001b). Stimulation of PKC reduces the inhibitory effect of μ opioid agonists on voltage-gated Ca $^{2+}$ channels (King et al., 1999). Thus, differential expression of PKC in TRPV1- and non-TRPV1-sensory neurons could profoundly influence the rate of μ opioid receptor desensitization. In this regard, PKC appears to be mainly expressed in IB $_4$ -positive (mostly TRPV1-expressing) DRG neurons (Molliver et al., 1995). This may explain why μ opioid agonists have a greater effect on voltage-gated Ca $^{2+}$ channels in IB $_4$ -negative than IB $_4$ -positive DRG neurons (Wu et al., 2004). The role of PKC in the analgesic tolerance of μ opioids has been implicated in previous studies. For instance, chronic opioid treatment increases PKC levels in the spinal cord (Granados-Soto et al., 2000). Also, intrathecal injection of PKC inhibitors attenuates opioid tolerance (Granados-Soto et al., 2000). A PKC isoenzyme, PKC γ , appears to play a critical role in opioid tolerance since the μ opioid analgesia and G protein activation are potentiated in PKC γ -knockout mice (Narita et al., 2001a, Narita et al., 2001b). Furthermore, attenuation of opioid tolerance has been shown in mice deficient in PKC γ (Zeitze et al., 2001). As shown in this study, the μ opioid receptors were co-expressed with PKC γ in some afferent terminals in the superficial dorsal horn. Reduction of PKC γ in the spinal cord may render the remaining μ opioid receptors in the spinal cord more resistant to desensitization in RTX-treated rats. Therefore, RTX probably induces a preferential loss of μ opioid receptors in TRPV1-expressing afferent terminals with a high level of PKC γ in the spinal cord, resulting in attenuation of opioid tolerance. This finding also could offer a plausible explanation for the paradoxical increase in μ opioid analgesia and a decrease in μ opioid receptors in the spinal cord in RTX-treated rats (Chen and Pan, 2006b).

Differential expression of PKC γ in neurons expressing μ opioid receptors may lead to a heterogenous desensitization of μ opioid receptors in different populations of sensory neurons in the dorsal root ganglia and spinal cord. Nevertheless, we observed that DAMGO still produced a large increase in [35 S]GTP γ S binding in the spinal cord in morphine-tolerant rats. This suggests that even in the tolerant state, the majority of μ opioid receptor-G protein coupling remains functional in the spinal cord. The compensatory responses that counteract the

inhibitory effect of μ opioids in neurons or neural circuits in the spinal dorsal horn, such as increased activity of regulators of G-protein signaling and NMDA receptors (Gutstein and Trujillo, 1993, Zachariou et al., 2003), also could contribute to development of opioid analgesic tolerance. Consistent with this notion, there was a gradual and significant reduction in the baseline mechanical withdrawal threshold (hyperalgesia) in vehicle-treated rats when the effect of morphine was diminished. Some have proposed that PKC γ in the spinal cord is involved in such compensatory changes following chronic morphine treatment (Zeitz et al., 2001). Further studies are warranted to determine how removal of sensory input from TRPV1-expressing sensory neurons blunts the compensatory response to chronic μ opioid agonists in the spinal cord.

A potential confounding factor for this study is that in addition to removal of TRPV1-expressing primary afferent neurons, RTX may induce the loss of second-order neurons in the spinal dorsal horn through transsynaptic degeneration. Transsynaptic degeneration of spinal dorsal horn neurons may be induced by 1) excitotoxic cell death induced by excessive glutamate release from TRPV1-expressing primary afferents stimulated by RTX; and 2) neuronal atrophies due to loss of neurotrophic factors and afferent input by RTX. However, we have shown previously that dorsal horn neurons that express TRPV1 and μ opioid receptors are preserved in RTX-treated animals (Chen and Pan, 2006b). Similarly, it has been shown that neonatal treatment of capsaicin deletes TRPV1-positive DRG neurons and their central terminals but not those CNS neurons that express TRPV1 (Mezey et al., 2000). Although it is uncertain why CNS neurons expressing TRPV1 are preserved in RTX-treated rats, it is possible that RTX may not pass through the blood-brain-barrier or the survival of CNS neurons is independent of neurotrophic factors removed by RTX. Persistent release of glutamate from TRPV1-expressing DRG neurons and their terminals is unlikely since these neurons can die within 20–30 s due to excessive intracellular calcium influx by TRPV1 stimulation (Olah et al., 2001, Wu et al., 2005). Even the neurons are not immediately deleted by RTX, these neurons and their central terminals can rapidly lose the ability to release more glutamate due to rapid down-regulation of voltage-gated calcium channels (Wu et al., 2005). Furthermore, the spinal second-order neurons have synaptic connections with non-TRPV1 presynaptic terminals, and loss of TRPV1-expressing afferent terminals may not remove all the neurotrophic factors important for the survival of spinal second-order neurons. Thus, it seems that loss of second-order neurons due to transsynaptic degeneration in the spinal dorsal horn in RTX-treated rats is less likely. It should be noted that RTX may have some effect on the spinal glial cells since it has been shown that TRPV1 is co-localized with some astrocytes in the spinal cord (Doly et al., 2004). At the present time, the potential effect of RTX on the glial cells in the spinal cord are not known and should be taken into consideration when interpreting the data from this study.

In summary, the present study demonstrates that chronic treatment with morphine primarily down-regulates presynaptic μ opioid receptors present on TRPV1-expressing afferent terminals in the spinal cord. Removal of TRPV1-expressing sensory neurons attenuates the development of morphine analgesic tolerance, possibly as a result of reduced desensitization of μ opioid receptors through PKC γ in the spinal cord. This new information is important to increasing our understanding of the heterogeneous cellular and molecular mechanisms underlying opioid analgesic tolerance and μ opioid receptor desensitization.

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List of abbreviations

DAMGO

(D-Ala², N-Me-Phe⁴, Gly-ol⁵)-enkephalin

CTAP

D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂

DRG

dorsal root ganglion

PKC

protein kinase C

IB₄

Griffonia simplicifolia isolectin B₄

RTX

resiniferatoxin

TRPV1

transient receptor potential vanilloid type 1

Fig. 1. Effect of RTX on TRPV1- and μ opioid receptor-immunoreactivity in the spinal cord. Panel A, confocal images showing TRPV1-immunoreactivity and IB₄-positive afferent terminals in the spinal dorsal horn of vehicle- and RTX-treated rats (scale bar, 100 μ m). Inset: high-magnification images showing co-localization of TRPV1-immunoreactivity and IB₄ in the lamina I and II (scale bar = 5 μ m). Panel B, confocal images showing μ opioid receptor- and TRPV1-immunoreactivity in the spinal dorsal horn of vehicle- and RTX-treated rats (scale bar, 100 μ m). Inset: high-magnification images showing co-localization of μ opioid receptor- and TRPV1-immunoreactivity in the lamina I and II (scale bar = 10 μ m). Co-localization of TRPV1-immunoreactivity and IB₄ labeling/ μ opioid receptor-immunoreactivity is indicated in yellow when the two images are digitally merged. All of the images are single confocal optical sections.

Fig. 2.

Time course of analgesic tolerance following daily intrathecal injection of morphine in vehicle- and RTX-treated rats. Panel A, time course of tolerance development following daily intrathecal injection of 10 μ g of morphine. The effect on the withdrawal threshold was measured 30 min after intrathecal injection of 5 μ g of morphine in 8 control and 9 RTX-treated rats. Panel B, time course of the effect of intrathecal 5 μ g of morphine on the withdrawal threshold in 8 control and 9 RTX-treated rats on day 1 and day 14. Data presented as means $\pm$ SEM. *, $P < 0.05$ compared with the respective control value in each group.

Fig. 3.

Development of the analgesic tolerance following daily intraperitoneal injection of 10 mg/kg of morphine. The effect on the withdrawal threshold was measured 30 min after intraperitoneal injection of 5 mg/kg of morphine in 7 vehicle- and 8 RTX-treated rats. Data presented as means $\pm$ SEM. *, $P < 0.05$ compared with the respective control value in each group. #, $P < 0.05$ compared with the corresponding value in the vehicle group.

Fig. 4. Concentration-effect curve for DAMGO-stimulated [35 S]GTPS γ binding in the dorsal spinal cord membrane obtained on day 14 following daily intrathecal administration of 10 μ g of morphine in vehicle- and RTX treated rats ($n = 6$ experiments in each group). #, $P < 0.05$ compared with the corresponding value in the vehicle group.

Fig. 5.

Confocal images showing μ opioid receptor- and PKC γ -immunoreactivity in the spinal cord dorsal horn of vehicle- and RTX-treated rats. Inset: high-magnification images (scale bar = 10 μ m) showing co-localization of μ opioid receptor- and PKC γ -immunoreactivity in the lamina II. Co-localization of μ opioid receptor- and PKC γ -immunoreactivity is indicated in yellow when the two images are digitally merged. All of the images are single confocal optical sections. Scale bar, 100 μ m.