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ACTIVATION OF PERIPHERAL DELTA-OPIOID RECEPTORS LEADS TO ANTI-HYPERALGESIC RESPONSES IN THE MASSETER MUSCLE OF MALE AND FEMALE RATS

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Abstract—In this project, we examined peripheral δ-opioid receptor (DOR)-mediated anti-hyperalgesic responses in the context of an acute orofacial muscle pain condition in both male and female rats. We also investigated whether the ATP-sensitive K^+ channel (KATP), a downstream target of OR signaling, contributes to DOR-mediated anti-hyperalgesic responses. Local pretreatment of the masseter with a DOR agonist, [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), dose-dependently attenuated capsaicin-induced mechanical hypersensitivity in both male and female rats. However, there were sex differences in the potency of local DPDPE in that a 10-fold higher dose of DPDPE was required in female rats to produce the level of anti-hyperalgesia achieved in male rats. The sex differences in the DPDPE effect may not be fully explained by DOR expression level since there was no significant sex difference in DOR mRNA levels in trigeminal ganglia (TG). Finally, pretreatment of the masseter with the KATP antagonist, glibenclamide, significantly blocked the effects of DPDPE in male rats suggesting that the peripheral DOR effect is mediated by the KATP. These studies revealed novel information about sex differences with regards to peripherally localized DOR-mediated anti-hyperalgesia under an orofacial muscle pain condition.

Key words: trigeminal, muscle pain, sex differences, potassium channels, Sprague–Dawley rats.

The functional role of peripheral opioid receptors (ORs) in attenuating pain and hyperalgesia has been demonstrated for decades (Ferreira and Nakamura, 1979a,b; Sachs et al., 2004; Stein et al., 2003), and an overwhelming amount of animal data supporting the role of peripheral ORs under various pain conditions is continuously being accumulated (Garlicki et al., 2006; Guan et al., 2008; Núñez et al., 2007; Obara et al., 2009). Consistent with the animal data, pain relief from local application of opioids has been reported in patients with chronic rheumatoid and osteoarthritis, ischemic pain, dental pain, pancreatitis, and postoperative visceral pain (Dionne et al., 2001; Duckett et al., 1997; Eisenach et al., 2003; Keskinbora and Aydinli, 2009; Likar et al., 2001; Modi et al., 2009; Rorarius et al., 1999).

While all three major subtypes of opioid receptors, namely, μ-, δ-, and κ-opioid receptors (MORs, DORs, KORs, respectively) have been implicated in peripheral analgesia and/or anti-hyperalgesia, each subtype of OR may be associated with distinct regulatory mechanisms. Therefore, they may provide distinct therapeutic advantages in different pain conditions. There is evidence that direct activation of peripheral DORs leads to potent anti-hyperalgesic effects under inflammatory and neuropathic pain conditions (Kabli and Cahill, 2007; Pacheco et al., 2005; Shinoda et al., 2007; Stein et al., 1989). However, in comparison to MORs and KORs, the role of peripheral DORs is relatively under studied, and the role of peripheral DORs in a muscle pain condition has never been demonstrated.

While sex differences in spinally- and supraspinally-mediated opioid analgesia have been documented, few studies have examined sex differences in peripheral OR-mediated analgesia (Bodnar and Kest, 2010; Craft, 2003; Flores et al., 2003). In a visceral pain model, activation of peripheral MORs produces more potent analgesia in male rats than in females (Ji et al., 2006). Similarly, local morphine in the temporomandibular joint (TMJ) of male rats, but not females, significantly reduces glutamate-evoked jaw muscle activity (Cai et al., 2001). However, a specific KOR agonist administered in the TMJ produces a greater reduction of formalin-induced nociceptive responses in female rats (Clemente et al., 2004). Sex differences in peripheral DOR-mediated analgesia have not been described.

Specific agonists for ORs open inwardly rectifying K^+ channels through the activation of $G_{i/o}$ proteins in neurons (North et al., 1987); one of which is the ATP-sensitive K^+ channel (KATP). Activation or blockade of the KATP in sensory neurons modulates the anti-hyperalgesic responses induced by all three subtypes of ORs in the spinal system (Amarante et al., 2004; Pacheco and Duarte, 2005). While both pore-forming and regulatory subunits of KATP are expressed in trigeminal sensory neurons (Niu et al., 2011), the functional interaction between KATP and ORs in the orofacial model has not been demonstrated.

These observations have led us to investigate (1) whether activation of peripheral DORs effectively attenuates capsaicin-induced mechanical hypersensitivity in the masseter muscle, (2) whether there are sex differences in peripheral DOR responses, and (3) whether the anti-hy-

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Abbreviations: AUC, area under curve; DOR, delta (δ) opioid receptor; DRG, dorsal root ganglia; KATP, ATP-sensitive potassium channels; KOR, kappa (κ) opioid receptor; MOR, mu (μ) opioid receptor; PBS, phosphate buffer solution; TG, trigeminal ganglia; TMJ, temporomandibular joint; VF, von Frey.

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peralgesic responses of peripheral DORs involves the KATP.

EXPERIMENTAL PROCEDURES

Animals

Age matched adult male and female Sprague–Dawley rats (8 weeks old; 250–300 g for males and 225–260 g for females; Harlan, Indianapolis, IN, USA) were used in this experiment. All animals were housed in a temperature-controlled room under a 12:12 light–dark cycle with access to food and water *ad libitum*. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and under a University of Maryland approved Institutional Animal Care and Use Committee protocol. Estrus cycle in female rats was not determined in this study.

Real-time RT-PCR

To quantitatively compare DOR mRNA between male and female trigeminal ganglia (TG), real-time RT-PCR was performed. Total RNA was extracted from TG with Trizol (Invitrogen, Carlsbad, CA, USA) and purified according to the RNeasy kit (Qiagen, MD, USA) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the SuperScript First strand synthesis kit (Invitrogen). SuperScript II (Invitrogen) was used to generate cDNA from 1 μg of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 25 ng of RNA was then performed using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Forest City, CA, USA) in an Eppendorf Mastercycler ep realplex 2.0. The following primers for DOR were used: sense 5′-TGGGTCTTGGCTTCAGGTGT-3′, antisense 5′-CGTGCATACCACTGCTCCAT-5′.

Drug preparation and administration

Capsaicin (Sigma, Saint Louis, MO, USA) was dissolved in ethanol (23%), Tween 80 (7%), and phosphate buffer solution (PBS) (70%). DPDPE (Tocris Cookson, Ellisville, MO, USA) was dissolved in PBS. PBS was 0.01 M phosphate, 0.14 M NaCl, and 3 mM KCl, pH 7.4. Naltrindole and Glibenclamide (Tocris Cookson) was dissolved in DMSO. All drugs were administered intramuscularly into the masseter muscle. In order to make sure that the drugs and their corresponding vehicles were administered in the same target region of the muscle, the injection site was determined by palpating the masseter muscle between the zygomatic bone and the angle of the mandible. Injections were made with a 27-gauge needle. Upon contacting the mandible, the needle was slowly withdrawn into the mid-region of the masseter and injections were made for 5–10 s.

Behavioral studies

It is well established that noxious chemical or mechanical stimulation of the masseter muscle evokes characteristic shaking of the ipsilateral hindpaw in lightly anesthetized rats (Han et al., 2008; Ro et al., 2003; Sánchez et al., 2010). We have previously described the use of this behavior for testing mechanical sensitivity of the masseter muscle in rats (Ro et al., 2007, 2009).

Since pentobarbital metabolism is different between male and female rats, we measured the heart rate of both under different anesthetic regimens in order to identify conditions that elicit comparable physiological responses. It was determined that male rats would receive an initial intraperitoneal (i.p.) injection of 40 mg/kg and female rats 35 mg/kg of sodium pentobarbital for the behavioral studies. A tail vein was connected to an infusion pump (Harvard Apparatus, Pump11) for continuous infusion of pentobarbital.

A level of "light" anesthesia was determined by providing a noxious pinch to the tail or the hindpaw with a serrated forceps. Male rats typically respond to the noxious pinch on the tail with an abdominal contraction and with a withdrawal reflex to the noxious pinch of a hindpaw about 15 min after the initial anesthesia. It typically took about 30–45 min for female rats to show similar responses. Once the animal reached this level, a metal clip calibrated to produce 600 g of force was applied five consecutive times. Experiments were initiated only after the animals showed reliable reflex responses to every clip application regardless of the sex of the animal. During the course of behavioral experiment male rats required additional anesthetic, which was provided via the tail vein. The rate of infusion was adjusted to maintain a relatively light level of anesthesia throughout the duration of the experiment (3 mg/h). Female rats did not require additional pentobarbital.

During the behavioral observation a baseline mechanical threshold for evoking the hindpaw responses was determined 15 min prior to drug injection using the electronic von Frey (VF) anesthesiometer (IITC Life Science, Inc., Woodland Hills, CA, USA). A rigid tip (diameter, 2 mm) attached to the VF meter was applied to the masseter muscle until the animals responded with hindpaw shaking. The animal's head was rested flat against the surface of the table when pressing the anesthesiometer on the masseter in order to provide stability. The threshold was defined as the lowest force necessary to evoke the hindpaw response. Changes in masseter sensitivity were then assessed at 15, 30, 45, 60, and 90 min following drug treatments. We calculated percent changes in VF thresholds following drug treatment with respect to the baseline threshold and plotted against time. In order to assess the overall magnitude of drug-induced changes in masseter sensitivity over time, the area under the curve (AUC) was calculated for the normalized data for each rat using the trapezoid rule. All behavioral observations were made by one experimenter who was blinded to the experimental conditions in order to maintain the consistency of assessing behavioral responses. All animals were kept warm throughout the experiments with thermal blankets.

Experimental and control groups for behavioral studies

To examine whether activation of peripheral DORs blocks capsaicin-induced mechanical hypersensitivity, the masseter muscle was pretreated with a specific agonist for the DOR, [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) (1, 10, 100, and 300 μg/50 μl) or the vehicle, PBS, 10 min prior to capsaicin (0.1%, 100 μl) injection in both male and female rats. Another group of rats was treated with a selective DOR antagonist, naltrindole (100 μg/20 μl) prior to the injection of DPDPE (100 μg) in order to test the receptor specific action of DPDPE. The doses of DPDPE were adapted from a published study (Stein et al., 1989). Since it is possible that high doses of naltrindole can block other opioid receptors, we chose a dose of 100 μg ($\approx$0.33 mg/kg) which is 20 times lower than the dose (20 mg/kg, s.c.) shown to successfully antagonize the effects of the selective DOR agonist [D-Ser^2, Leu^5, Thr^6]enkephalin without blocking the antinociceptive effects of the KOR or MOR agonists morphine and U50488H, respectively (Portoghese et al., 1988).

There is a possibility that DPDPE injected into the masseter can mediate its effects by activating central DORs. To evaluate possible systemic effects, the highest dose of DPDPE (300 μg) was administered into the masseter muscle contralateral to the capsaicin injection in a separate group of animals. In order to investigate whether the peripheral DOR-mediated anti-hyperalgesia involves the KATP, a specific KATP antagonist, glibenclamide (100 μg/20 μl), was administered prior to DPDPE and capsaicin treatments in the masseter muscle of male rats. The highest dose of each drug used in this study was administered in the masseter contralateral to the capsaicin treatment to rule out the possibility of