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Peripheral Neurobiologic Mechanisms of Antiallodynic Effect of Warm Water Immersion Therapy on Persistent Inflammatory Pain

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Water immersion is widely used in physiotherapy and might relieve pain, probably by activating several distinct somatosensory modalities, including tactile, pressure, and thermal sensations. However, the endogenous mechanisms behind this effect remain poorly understood. This study examined whether warm water immersion therapy (WWIT) produces an antiallodynic effect in a model of localized inflammation and whether peripheral opioid, cannabinoid, and adenosine receptors are involved in this effect. Mice were injected with complete Freund's adjuvant (CFA; intraplantar; i.pl.). The withdrawal frequency to mechanical stimuli (von Frey test) was used to determine 1) the effect of WWIT against CFA-induced allodynia and 2) the effect of i.pl. preadministration of naloxone (a nonselective opioid receptor antagonist; 5 μg/paw), caffeine (a nonselective adenosine receptor antagonist; 150 nmol/paw), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; a selective adenosine A_1 receptor antagonist; 10 nmol/paw), and AM630 (a selective cannabinoid receptor type 2 antagonist; 4 μg/paw) on the antiallodynic effect of WWIT against CFA-induced allodynia. Moreover, the influence of WWIT on paw inflammatory edema was measured with a digital micrometer. WWIT produced a significant time-dependent reduction of paw inflammatory allodynia but did not influence paw edema induced by CFA. Naloxone, caffeine, DPCPX, and AM630 injected in the right, but not in the left, hind paw significantly reversed the antiallodynic effect of WWIT. This is the first study to demonstrate the involvement of peripheral receptors in the antiallodynic effect of WWIT in a murine model of persistent inflammatory pain.

Key words: adenosine; analgesia; cannabinoid; chronic pain; opioid; water therapy

Persistent inflammatory pain occurs in response to tissue injury and the subsequent inflammatory response. After inflammation, dramatic alterations in the somatosensory system occur, amplifying responses and increasing sensitivity to peripheral stimuli, such that pain is now activated by normally innocuous or low-intensity stimuli (Woolf and Costigan, 1999). Generally, inflammatory pain disappears after resolution of the initial tissue injury. However, in chronic disorders such as rheumatoid arthritis, pain persists for as long as inflammation is active (Michaud et al., 2007).

Inflammatory pain depends, to some degree, on the peripheral activation of primary sensory afferent neurons.

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In contrast, a series of endogenous ligands related to inhibition of sensory transduction of noxious stimuli at the peripheral level has been described, including opioid peptides, endocannabinoids, and purines (adenosine), which act on different receptors (Carins, 2009). Endogenous opioids have also been implicated in pain modulation (Rogers and Peterson, 2003; Brack et al., 2004). Opioids act via peripheral and central opioid receptors to produce analgesic effects (Iwaszkiewicz et al., 2013; Zeppetella and Davies, 2013). In addition, endogenous opioids regulate inflammation through opioid receptors found on immune cells at the site of inflammation (Rogers and Peterson, 2003; Brack et al., 2004). The endocannabinoid system consists of cannabinoid receptor type 1 (CB_1) and cannabinoid receptor type 2 (CB_2), endogenous ligands, and their synthesizing/metabolizing enzymes (Agarwale et al., 2007; Jhaveri et al., 2007). Ibrahim and coworkers have demonstrated that CB_2 receptor activation in keratinocytes, one type of cell that has been reported to express CB_2 receptors (Ibrahim et al., 2005) and to contain endogenous opioid peptides (Cabot et al., 1997), releases beta-endorphin, which in turn can produce peripheral antinociception by acting upon μ-opioid receptors on primary afferent neurons.

Recently, adenosine receptors have emerged as attractive potential alternatives for the treatment of chronic pain. It has been well documented that adenosine regulates pain transmission in the periphery, and several agents can alter the extracellular availability of adenosine and subsequently modulate pain transmission, particularly by activation of adenosine A_1 receptors (Sawynok and Liu, 2003; Sawynok, 2013).

The use of water for medical treatment is probably as old as mankind. Water immersion (WI) is an approach to the treatment of chronic disorders, such as rheumatoid arthritis (O'Hare et al., 1984). The mechanisms by which warm water immersion therapy (WWIT) reduces inflammatory pain are not fully understood. The net benefit is probably the result of a combination of factors, with mechanical and thermal effects among the most prominent (Sukenik et al., 1999). The thermal effect, which is the result of heat, acts in four ways, vasodilatation, gate control mechanism, elevation of beta-endorphin levels, and muscle relaxation. The mechanical effect can be described as hydromechanical stimuli of the water adapted to the body parts and hydrostatic pressure of water on the skin (Melzack and Wal, 1965; Perl, 2007).

It is clear that, in immersion with or without exercise, temperature and immersion time are key variables to obtain the desired effect in the treatment of painful conditions (Bender et al., 2005). Although these treatments are commonly and ubiquitously used to treat pain, no standard guidelines have been established, and a target temperature for optimal therapeutic effects has yet to be identified. This is largely the result of a lack of understanding regarding the mechanisms through which WWIT affects pain symptoms (Bender et al., 2005; Fioravanti et al., 2011).

The clinical use of WI, as part of the physical therapy for chronic pain treatment, requires detailed knowledge of the peripheral endogenous mechanisms behind of antiallodynic effects induced by WWIT. The present study examines 1) the antiallodynic and antiedematogenic effects of WWIT on lower limbs via local injection in a mouse model of localized inflammation and 2) the potential role of opioid, cannabinoid, and adenosine receptors in the antiallodynic effect WWIT.

MATERIALS AND METHODS

Animals

The experiments were performed after approval of the protocol (13.006.4.08.IV) by the Institutional Ethics Committee (blinded) and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals. Experiments were conducted with male Swiss mice (25–35 g body weight) housed at 22°C ± 2°C under a 12-hr light–dark cycle (lights on at 6:00 AM), with access to food and water ad libitum. Animals were acclimatized to the laboratory for at least 1 hr before testing and were used only once throughout the experiments. The experiments were carried out between 8:00 and 11:00 AM.

Drugs

The following substances were used: dimethyl sulfoxide (DMSO), ethanol, morphine hydrochloride, and N^6-cyclohexyladenosine from Merck (Darmstadt, Germany); caffeine and complete Freund's adjuvant (CFA) from Sigma (St. Louis, MO); adenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), and naloxone hydrochloride from Tocris Bioscience (Ellisville, MO); and AM630 and WIN 55,212-2 from Cayman Chemical (Ann Arbor, MI). DPCPX was dissolved in saline with DMSO that did not exceed 5% and did not cause any effect per se. AM630 and WIN 55,212-2 were dissolved, immediately before use, in DMSO and ethanol, in an amount that did not exceed final concentrations of 1% and 2.5%, respectively. Other substances were dissolved in saline. For drugs delivered by subcutaneous routes, a constant volume of 20 μl/paw was injected. Appropriate vehicle-treated groups were also assessed simultaneously.

Warm Water (35°C) Immersion Therapy

Animals were placed in a plastic box divided into eight compartments (170 × 100 mm), filled with 5.5 liters of shallow (3 cm depth) water at 25°C and 35°C. WWIT consisted of different immersion times: 3, 10, and 30 min at 35°C (acute or chronic exposition). Appropriate control groups were also assessed simultaneously. After each immersion session, animals were gently dried with a cloth towel. Mice in the WWIT and control groups were exposed to shallow water (~25°C) for 3 min once per day on the first, second, and third days. Mice were thus acclimated to the new environment.

CFA-Induced Inflammation and Mechanical Allodynia

Mice were injected (intraplantar; i.pl.) with 20 μl 70% CFA (*Mycobacterium tuberculosis*) as described by Meotti et al. (2006), with minor modifications. The sham group received 20

μl phosphate-buffered saline in the right paw. CFA produced significant hind paw swelling and hyperalgesia. To assess the effects of WWIT on CFA-induced chronic inflammatory pain, animals received WWIT of different durations (3, 10, or 30 min of immersion) 24 hr after i.pl. injection of CFA. Development of mechanical allodynia was evaluated at 0, 15, 30, 60, and 120 min after therapy to verify the time course of WWIT in reducing mechanical allodynia (Meotti et al., 2006; Martins et al., 2013a). To investigate the effects of the long-term treatment on mechanical allodynia, WWIT was performed once per day. Mechanical allodynia was evaluated 30 min after therapy (time with maximal inhibition observed in the acute treatment) for 5 consecutive days. Mechanical allodynia was measured in mice acclimatized to individual clear boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws, as previously described (Mazzardo-Martins et al., 2012; Martins et al., 2013a). The withdrawal response frequency to 10 applications of 0.4 g of von Frey filaments (Stoelting, Wood Dale, IL) is presented as a percentage response, with 100% being 10/10 and 0% being 0/10 responses. An increased number of responses was interpreted as mechanical allodynia. The von Frey filaments of 0.4 g produced a mean withdrawal frequency of about 10–20%, which is considered an adequate value for the measurement of mechanical allodynia (Dutra et al., 2012). All withdrawal latencies were measured manually.

CFA-Induced Hind Paw Edema in Mice

This experiment was performed as previously described by Levy (1969). On the fifth day after CFA i.pl. injection, and after mechanical allodynia evaluation, paw edema was measured as the difference between paw thickness (in micrometers) in the different groups, with a digital micrometer. The difference between the groups indicated the degree of inflammation; this period for observation of paw edema was chosen to verify the effect of daily WWIT treatment on edema formation.

Analysis of Possible Mechanism of Action Peripheral of WWIT (35°C)

To evaluate some of the peripheral mechanisms by which WWIT causes antiallodynia against CFA-induced chronic inflammatory pain, the animals were treated with some classic drugs. The doses of the drugs used were selected based on previous studies (Martins et al., 2012, 2013b; Cidral-Filho et al., 2014) and also based on previous results from our laboratory.

Experiment 1: Involvement of Peripheral Opioid Receptors

To assess the involvement of the opioid system in the antiallodynic effect of 10 min of WWIT, the animals received an i.pl. injection of naloxone (a nonselective opioid receptor antagonist; 5 μg/paw) or saline solution (20 μl/paw) in the right and left hind paws (Martins et al., 2012). After 15 min, the animals were subjected to WWIT for 10 min. Mechanical allodynia was evaluated with the von Frey filament test 30 min after WWIT. Control animals were subjected to cold water and were assessed over the same time intervals. Furthermore, mice were pretreated with an i.pl. injection of saline or naloxone, and after 15 min received morphine (5 μg/paw) or saline (20 μl/paw). These groups were assessed 30 min after morphine or saline treatment.

Experiment 2: Involvement of Peripheral Adenosine A_1 Receptors

Next, we investigated the involvement of peripheral adenosine receptors in the antiallodynic effect produced by 10 min of WWIT. The animals received an i.pl. injection with 20 μl caffeine (a nonselective adenosine receptor antagonist; 150 nmol/paw) or saline solution (20 μl/paw) in the right and left hind paws. After 15 min, the animals were subjected to WWIT for 10 min. Mechanical allodynia was evaluated with the von Frey filament test 30 min after WWIT. Control animals were subjected to cold water and were assessed over the same time intervals. Furthermore, mice were pretreated with an i.pl. injection of saline or caffeine and, after 15 min, received N^6-cyclohexyladenosine (CHA, a selective adenosine A_1 receptor agonist; 10 μg/paw) or saline (20 μl/paw). These groups were assessed 30 min after CHA or saline treatment.

To evaluate the involvement of peripheral A_1Rs in the antiallodynic effect of 10 min of WWIT, in another set of experiments the animals received an i.pl. injection of DPCPX (a selective adenosine A_1 receptor antagonist; 10 nmol/paw) or saline solution (20 μl/paw) in the right hind paw. After 15 min, the animals were subjected to WWIT for 10 min. Mechanical allodynia was evaluated with the von Frey filament test 30 min after WWIT. Control animals were subjected to cold water and were assessed over the same time intervals. Furthermore, mice were pretreated with an i.pl. injection of saline or caffeine and, after 15 min, received CHA (10 μg/paw) or saline (20 μl/paw). These groups were assessed 30 min after CHA or saline treatment.

Experiment 3: Involvement of Peripheral CB_2

To determine the involvement of peripheral CB_2, the animals received an i.pl. injection of AM630 (a selective CB_2 antagonist; 4 μg/paw) or saline solution (20 μl/paw) in the right and left hind paws. After 15 min, the animals were subjected to WWIT for 10 min. Mechanical allodynia was evaluated with the von Frey filament test 30 min after WWIT. Control animals were subjected to cold water and were assessed over the same time intervals. Furthermore, mice were pretreated with an i.pl. injection of saline or AM630 and, after 15 min, received WIN 55,212-2 (a mixed CB_1R/CB_2R agonist; 5 μg/paw) or saline (20 μl/paw). These groups were assessed 30 min after WIN 55,212-2 or saline treatment.

Statistical Analysis

Behavioral testing was analyzed by two-way analysis of variance for repeated measures, with Bonferroni multiple-comparisons posttest or one-way analysis of variance following Student-Newman-Keuls test. Results are presented as mean ± SEM for each group. $P < 0.05$ was considered significant.

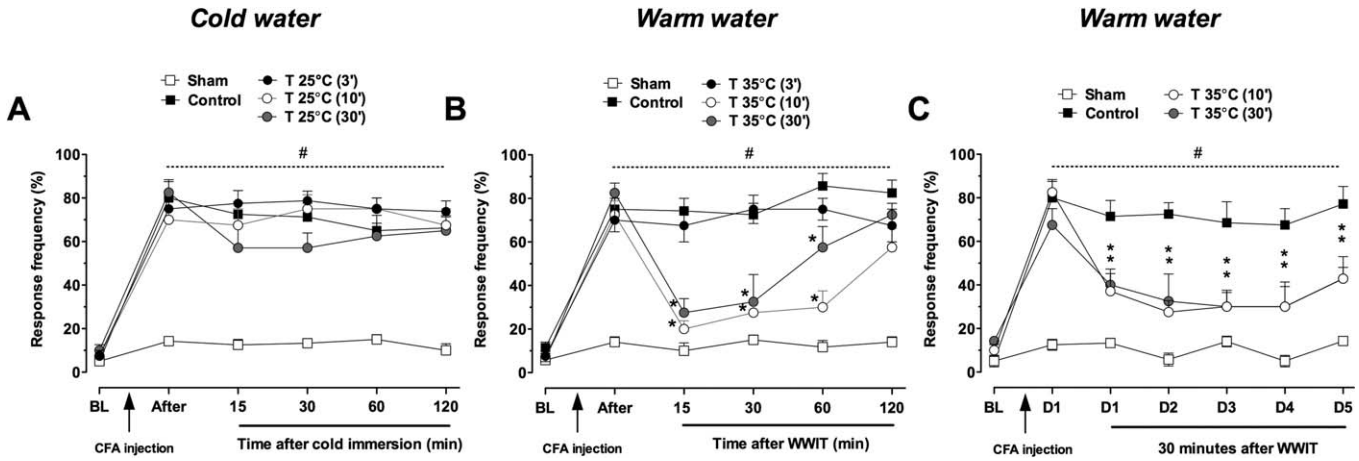

Fig. 1. Effect of WWIT on mechanical allodynia after CFA injection into the paws of mice. Acute treatment with 3-, 10-, and 30-min WI at 25°C (**A**) and with 3-, 10-, and 30-min WWIT at 35°C (**B**) 24 hr after CFA injection. Daily treatment of animals with 10- and 30-min WWIT at 35°C (**C**). Each point represents the mean of eight animals; vertical lines show SEM. BL, baseline; WWIT, warm water immersion therapy; T, temperature. $^{\star}P < 0.05$ compared with control-only group; $^{\#}P < 0.05$ compared with sham group.

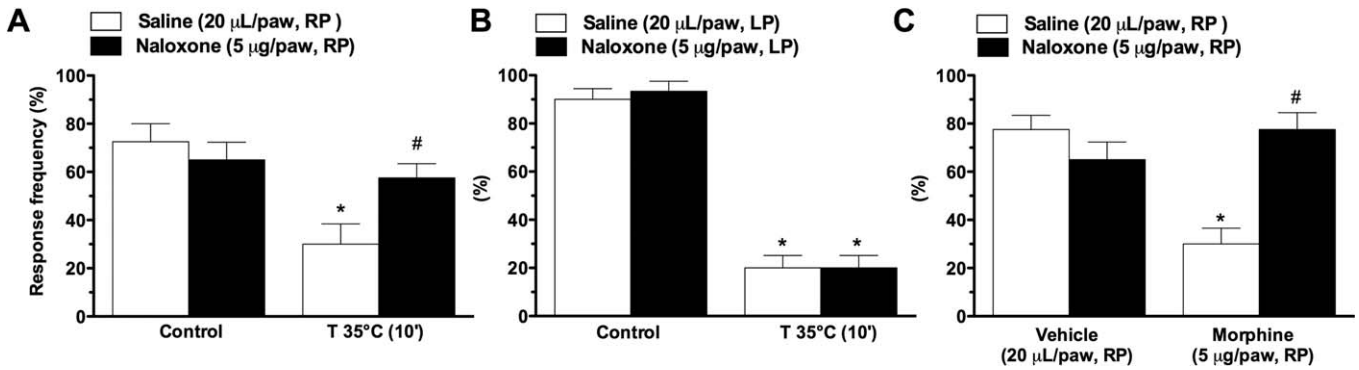

Fig. 2. Involvement of peripheral opioid receptors in antiallodynic effect caused by WWIT. Intraplantar pretreatment with naloxone in the right paw (5 μg/paw; **A**) and naloxone in the left paw (5 μg/paw; **B**) and morphine in the right paw (5 μg/paw; **C**). Each point represents the mean of eight animals; vertical lines show SEM. WWIT, warm water immersion therapy; T, temperature; RP, right paw; LP, left paw. $^{\star}P < 0.05$ compared with saline (control) group; $^{\#}P < 0.05$ compared with saline + T 35°C (10 min)-only group.

RESULTS

Antiallodynic Effect of WWIT on Persistent Inflammatory Pain Model

To evaluate the effects of WWIT on inflammatory pain, we injected CFA into the paws of mice. The results depicted in Figure 1A,B show that acute treatment with 10 or 30 min of WWIT at 35°C reduced the mechanical allodynia induced by the CFA injection. Significant differences between groups were observed 15 ($P < 0.05$), 30 ($P < 0.05$), and 60 min ($P < 0.05$) after WWIT compared with the control group. However, 3, 10, or 30 min of cold (normal) immersion at 25°C had no effect on the response frequency and was not significantly different from the control group (Fig. 1B). In addition, the daily treatment of animals with 10 or 30 min of WWIT at 35°C decreased the mechanical allodynia induced by CFA when evaluated 30 min after treatment. This effect was evident until the fifth day of treatment (Fig. 1C). Nevertheless, 10 or 30 min of WWIT at 35°C had no effect on CFA-induced edema (data not shown).

Peripheral Opioid Receptor Mediates Antiallodynic Effect of WWIT

Results presented in Figure 2A,B indicate that the i.pl. preadministration of naloxone (5 μg/paw) in the right, but not in the left, hind paw significantly ($P < 0.05$) prevented the acute effect of 10 min of WWIT at 35°C against mechanical allodynia induced by CFA in mice. Furthermore, when preadministered in the same dose as previously described for the right hind paw, naloxone

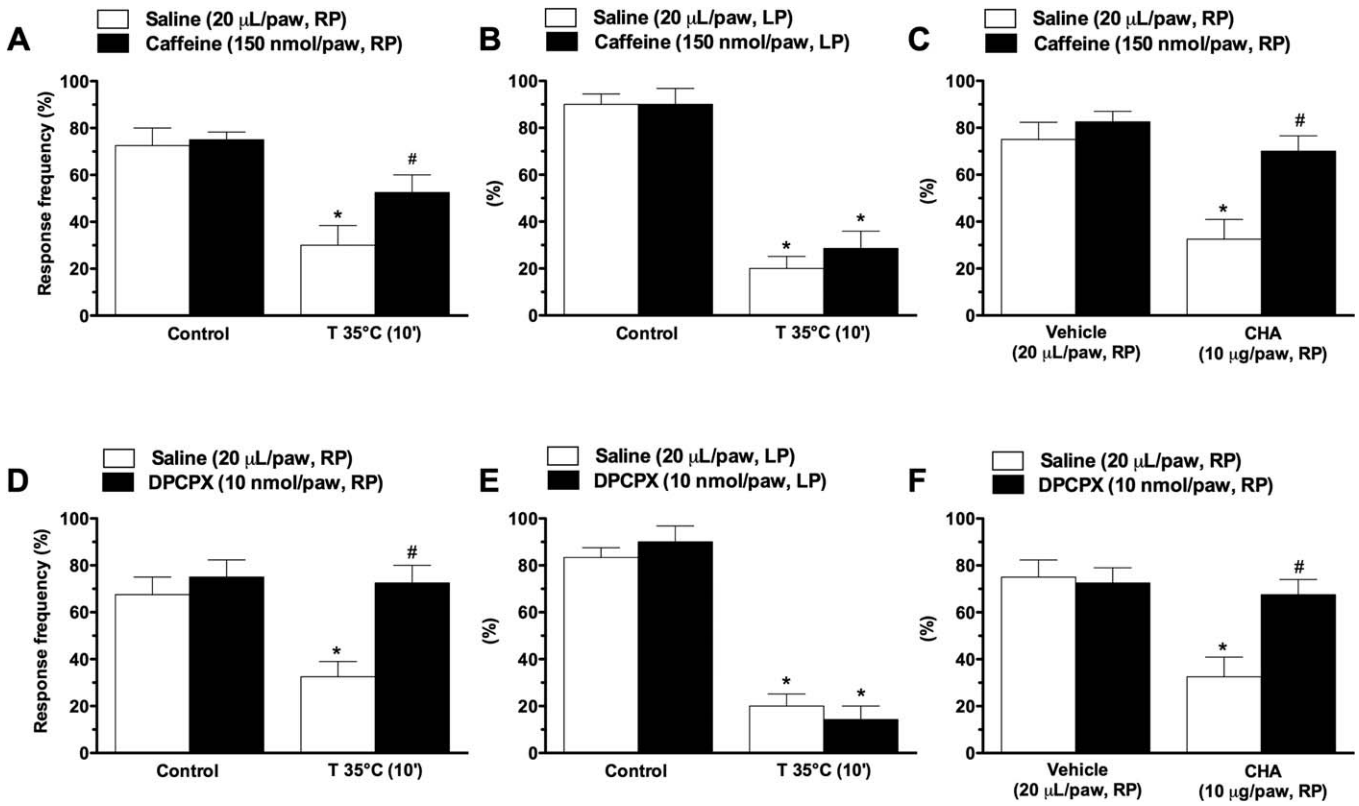

Fig. 3. Involvement of the peripheral adenosine receptors in antiallodynic effect caused by WWIT. Intraplantar pretreatment with caffeine in the right paw (150 nmol/paw; **A**) and caffeine in the left paw (150 nmol/paw; **B**) and the antiallodynic effect of WWIT (T 35°C [10'] and CHA (10 μg/paw; **C**). DPCPX in the right paw (10 nmol/paw; **D**) and DPCPX in the left paw (10 nmol/paw; **E**) and the antiallodynic effect of WWIT (T 35°C [10']) and CHA (10 μg/paw; **F**). Each point represents the mean of eight animals; vertical lines show SEM. WWIT, warm water immersion therapy; T, temperature; RP, right paw; LP, left paw. $^{*}P < 0.05$ compared with saline + control group or saline + vehicle group; $^{\#}P < 0.05$ compared with saline + T 35°C (10 min)-only group.

significantly ($P < 0.05$; Fig. 2C) prevented the antiallodynic effect of morphine (5 μg/paw). The administration of naloxone per se did not affect the animals' response frequency.

Peripheral Adenosine Receptors Are Necessary for the Antiallodynic Effect of WWIT

Results presented in Figure 3A indicate that the i.pl. preadministration of caffeine or DPCPX in the right, but not in the left, hind paw significantly ($P < 0.05$) prevented the acute effect of 10 min of WWIT at 35°C on mechanical allodynia induced by CFA in mice (Fig. 3A,B,D,E). Furthermore, the preadministration of caffeine or DPCPX in the right hind paw also significantly ($P < 0.05$; Fig. 3C–F) prevented the analgesic effect of CHA (10 μg/paw). The administration of caffeine or DPCPX per se did not affect the animals' response frequency.

Peripheral Cannabinoid Receptor Was Involved in the Antiallodynic Effect of WWIT

Results presented in Figure 4A,B indicate that the i.pl. preadministration of AM630 in the right, but not in the left, hind paw significantly ($P < 0.05$) prevented the acute effect of 10 min of WWIT at 35°C on mechanical allodynia induced by CFA in mice. Furthermore, the preadministration of AM630 in the right hind paw also significantly ($P < 0.05$; Fig. 4C) prevented the analgesic effect of WIN 55,212-2 (5 μg/paw) injected in the right hind paw. The administration of AM630 per se did not affect the animals' response frequency.

DISCUSSION

Evidence from clinical studies emphasizes the importance of peripheral drive in maintaining chronic pain. In this regard, for the majority of chronic pain conditions, there is now a large body of evidence strongly suggesting that activity from the periphery is essential not only to initiate but also to maintain painful symptoms (Richards and McMahon, 2013). Inflammatory pain depends, to some degree, on the peripheral activation of primary sensory afferent neurons. Peripheral nerve endings express a variety of inhibitory receptors, such as opioid, cannabinoid, and adenosine receptors, and these receptors are potential targets for therapy (Richards and McMahon, 2013).

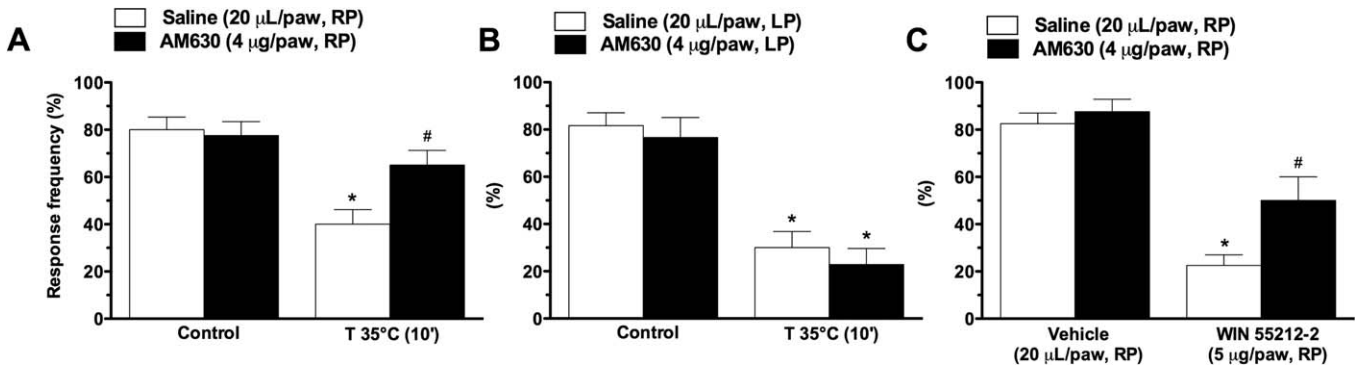

Fig. 4. Involvement of the peripheral cannabinoid receptors in antiallodynic effect caused by WWIT. Intraplantar pretreatment with AM630 in the right paw (4 μg/paw; **A**) and with AM630 in the left paw (4 μg/paw; **B**) and the antiallodynic effect of WWIT; pretreatment with AM630 in the right paw (4 μg/paw; **C**) and the antiallodynic effect of WIN 55,212-2 (5 μg/paw; **C**). Each point represents the mean of eight animals; vertical lines show SEM. WWIT, warm water immersion therapy; T, temperature; RP, right paw; LP, left paw. $^{\star}P < 0.05$ compared with saline + control group or saline + vehicle group; $^{\#}P < 0.05$ compared with saline + T 35°C (10')-only group.

Given that peripheral antinociception can involve activation of multiple receptors on sensory neurons, the ability of WWIT to target several receptors might be of particular interest. The current data show, for the first time, that WWIT on lower limbs reduces persistent inflammatory pain. The data further show the role of the different peripheral endogenous mechanisms in supporting the antiallodynic effect of WWIT.

Clinical Significance of the Persistent Inflammatory Pain Model

Clinically, chronic inflammatory pain, particularly that resulting from osteoarthritis or rheumatoid arthritis, accounts for the largest single population of patients seeking analgesic therapies. There are several rodent models of chronic inflammatory pain that are commonly used to support the development of new therapeutics. Common rodent experimentally induced arthritis models include CFA-induced arthritis of the paw (Kruger and Light, 2010) and monoiodoacetate-induced arthritis of the knee (Fernihough et al., 2004). In these models, pain responses are typically measured by assessing mechanical and/or thermal hypersensitivity of a hind paw (Kruger and Light, 2010).

The current study shows evidence that supports the use of WWIT for the treatment of persistent inflammatory pain and also contributes to the general knowledge of the endogenous mechanisms underlying this effect, insofar as it demonstrates that WWIT presents a time-response analgesic effect in the model of persistent inflammatory pain in mice but had no effect on the edema. It is important to point out that, in the current study, WWIT presented a time-response analgesic effect that is in agreement with the clinical view, in which the analgesic effect occurs between 10 and 30 min in warm temperatures (Wright and Sluka, 2001; Robinson et al., 2002; French et al., 2006). This observation is of utmost relevance because the effectiveness of the treatment can be highly influenced by the correct selection of the duration and temperature for therapy, as was demonstrated here.

WI is widely used in physiotherapy and might even induce a variety of physiological responses, depending on physical parameters such as temperature, immersion time, and hydrostatic pressure (Weston et al., 1987). These physiological changes have demonstrated therapeutic benefits in individuals with rheumatoid arthritis (Melton-Rogers et al., 1996). In addition, WI is used as a part of rehabilitation regimes for respiratory, cardiovascular, and orthopedic disorders (Sato et al., 2007, 2012a,b; Marabotti et al., 2009).

Heat can be applied superficially by application of moist hot packs, use of infrared light, paraffin wax application, or immersion in hot water baths (Robinson et al., 2002; French et al., 2006). WI might have beneficial effects on muscle tone, joint mobility, and pain intensity, supposedly by activating several distinct somatosensory modalities, including tactile, pressure, and thermal sensations (Sato et al., 2012a,b).

Some hypotheses have been suggested to explain the analgesic effect of WWIT; however, none of them has been entirely elucidated. One of the discussion points suggests that hot stimuli might influence muscle tone and pain intensity, helping to reduce muscle spasm and to increase pain threshold in nerve endings (Wright and Sluka, 2001). Another point of discussion is the "gate theory," in which pain relief might be due simply to the temperature and hydrostatic pressure of water on the skin (Melzack and Wal, 1965; Perl, 2007). It has also been suggested that increasing skin temperature or deep tissue temperature would cause vasodilation in the vessels of damaged tissue and increase metabolism and blood flow, which in turn would result in increased removal of inflammatory compounds known to activate and sensitize primary afferent fibers. This would result in less input being transmitted to the spinal cord and higher brain centers and, thus, decreased perception of pain (Wright and Sluka, 2001).

Another point of discussion is the activation, by warm water, of the thermotransient receptor potentials (TRPs), a recently discovered family of ion channels activated by temperature that are expressed in primary sensory nerve terminals, where they provide information about thermal changes in the environment (Vay et al., 2012). The TRPV3 was initially shown to be expressed only in keratinocytes, but later studies have shown its expression in sensory neurons (Facer et al., 2007; Frederick et al., 2007). As a thermoreceptor, TRPV3 is activated by temperatures in the warm range of 33–39°C, with an activation threshold of 33–34°C (Peier et al., 2002; Xu et al., 2002). Similarly to TRPV3, a strong expression for TRPV4 has been found in keratinocytes (Chung et al., 2003), providing further evidence that these cells might be involved in sensing warmth. TRPV4 is activated by temperatures in the warm range of 27–34°C (Chung et al., 2003). TRPV3 is activated by nonnoxious warm temperatures and seems to have an important analgesic function that is exploited by traditional anti-inflammatory preparations. However, there have been some preliminary indications that TRPV3 might also contribute to inflammatory heat pain; if this is confirmed, then antagonists might be of value (Vay et al., 2012). These data from the literature allow us to formulate the hypothesis that thermal stimuli (35°C) could profoundly desensitize the receptor and that this inactivation reduces the sensitivity and other ligands and can be used to reduce pain.

Given these results showing that WI in the conditions evaluated in the present study did not affect CFA-induced edema, it seems more reasonable to reinforce the first theory that relates the benefits of WI to the muscles or sensory nerves. It is worth noting that ourt data demonstrate that WWIT is able to reduce mechanical hyperalgesia in a persistent inflammatory pain model and that the activation of opioid, cannabinoid CB_2, and adenosine A_1 peripheral (paw) receptors seems to contribute to the antiallodynic effect of WWIT.

Involvement of the Opioid Receptors in Antiallodynia by WWIT

Several peripheral endogenous antinociceptive mechanisms are involved in counteracting inflammatory hyperalgesia. Most of these involve the release of opioid peptides (Iwaszkiewicz et al., 2013), endocannabinoids (Agarwal et al., 2007; Jhaveri et al., 2007), or purines (Sawynok and Liu, 2003; Sawynok, 2013). Opioid receptors are widely expressed in the central and peripheral nervous systems and in numerous nonneuronal tissues. Both animal models and human clinical data support the involvement of peripheral opioid receptors in analgesia, particularly in inflammation, in which both opioid receptor expression and efficacy are increased (Iwaszkiewicz et al., 2013). Prior studies show that all three major classes of opioid receptors (μ, δ, and κ) are present on peripheral sensory nerve terminals, both in animals and in humans (Stein et al., 1989; Stein and Lang, 2009). In a single study that used CFA-induced inflammation of the rat paw, locally acting μ, δ, and κ opioid receptor-selective agonists delivered antinociception that was dose dependent, stereospecific, and reversible by receptor-specific antagonists (Stein et al., 1989). Furthermore, κ receptor-selective opioid agonists, when administered subcutaneously to the paw, evoked potent dose-dependent increases in pain thresholds after CFA-induced chronic inflammation, an effect antagonized by naloxone methiodide (Binder et al., 2001).

Of note is the fact that preadministration of naloxone in the right, but not in the left, hind paw significantly prevented the effect of 10 min of WWIT against mechanical hyperalgesia induced by CFA in mice when administered via i.pl. This result suggests, for the first time, that the effects of WWIT are mediated, at least in part, through activation of peripheral opioid receptors. These previous findings together with the current data lead to the hypothesis that WWIT produces an opioid form of analgesia mediated by local peripheral opioid receptors. Furthermore, our findings are in agreement with studies examining opioid peptide production in keratinocyte cells. Recent data have demonstrated the possibility that normal keratinocytes can produce and secrete a precursor pro-opiomelanocortin after various stimuli (e.g., ultraviolet rays, thermal stimuli), which is the common precursor of various endorphins (Ibrahim et al., 2005; Fioravanti et al., 2011). This finding allows us to formulate the fascinating hypothesis that thermal stimuli could be used to condition the skin's production of opioid peptides, thus altering pain threshold.

Involvement of the Cannabinoid Receptors in Antiallodynia by WWIT

Increased sensory sensitivity produced by peripheral inflammatory processes is an important component of many pain states. Cannabinoid receptor agonists inhibit inflammatory hyperalgesia in animal models. Significantly, peripheral cannabinoid receptors might be capable of inhibiting inflammatory hyperalgesia, as shown by the observation that the endogenous cannabinoid receptor agonist anandamide exhibits antihyperalgesic activity when injected locally into the inflamed hind paw of the rat (Richardson, 2000). Two cannabinoid receptor subtypes, CB_1 and CB_2, have been identified and cloned (Matsuda et al., 1990; Munro et al., 1993). The cannabinoid CB_2 receptor expression seems to be found predominantly, but not exclusively, in peripheral tissues with immune functions (Matsuda et al., 1990; Munro et al., 1993; Galiègue et al., 1995). It has also recently been found in the brain, on dorsal root ganglion, in the lumbar spinal cord, on sensory neurons, on microglia, and in peripheral tissues (Sawynok, 2013). Also of interest are our current results showing that local (i.pl.) pretreatment of animals with AM630 (a selective CB_2 receptor antagonist), at a dose that produced no significant effect on mechanical hyperalgesia in the right, but not in the left, hind paw, significantly reversed the antihyperalgesic effect

caused by WWIT. Given the present data, we speculate that the antiallodynic effect of WWIT is probably linked to an activation of peripheral CB_2 receptors.

Involvement of the Adenosine Receptors in Antiallodynia by WWIT

Adenosine is directly involved in the modulation of nociceptive activity. A_1 receptors in the periphery recently have been implicated in antinociception produced by ankle joint mobilization (Martins et al., 2013a), acupuncture (Goldman et al., 2010), and systemic administration of acetaminophen (Liu et al., 2013a) and amitriptyline (Liu et al., 2013b). It has been demonstrated that peripheral administration of A_1 receptor agonists in the rat paw blocks mechanical hyperalgesia induced by prostaglandin E2 (PGE2; Valério et al., 2009).

This study demonstrates that the inhibition of local peripheral antiallodynic effect of WWIT by caffeine, a nonselective adenosine receptor antagonist, does not determine which subtypes of adenosine receptors are involved in this effect. However, because DPCPX, a selective A_1 receptor antagonist, also suppressed the antiallodynic effect of WWIT when administered in the right, but not in the left, hind paw, our results suggest the participation of A_1 receptors in the antiallodynic effect of WWIT. Thus, the antiallodynic effect of WWIT seems to be mediated, at least in part, by peripheral A_1 receptors, inasmuch as it was blocked by local i.pl. injection of the antagonist.

Interactions Among Opioid, Cannabinoid, and Adenosine Receptors in Antiallodynia by WWIT

A recent study by Ibrahim and coworkers (2005) investigated the mechanism through which CB_2 cannabinoid receptor-selective agonists are able to inhibit inflammatory pain responses. The authors demonstrated that CB_2 receptor activation in keratinocytes, one type of cell that has been reported to express CB_2 receptors (Casanova et al., 2003) and to contain endogenous opioid peptides (Cabot et al., 1997), releases beta-endorphin, which in turn can produce peripheral antinociception by acting upon μ opioid receptors on primary afferent neurons. This mechanism allows for the local release of endogenous opioids limited to sites where CB_2 receptors are present (Ibrahim et al., 2005).

Furthermore, it has been shown that cannabinoids, such as Δ9-tetrahydrocannabinol (THC) and morphine, produce a synergistic interaction in arthritic rats (Cox et al., 2007). Early studies in rats by Ghosh and Bhattacharya (1979) demonstrated the enhancement of morphine by an extract of *Cannabis indica*, and the potency of codeine and morphine given orally were shown to be enhanced by orally administered Δ9-THC and Δ6-THC in mice. Although the antinociceptive interactions of opioid receptor/agonists with CB receptor/agonists peripheral have been widely investigated (Ibrahim et al., 2005; Cox et al., 2007; Welch, 2009), the interactions of adenosine receptor/agonists with CB receptor/agonists are not well established.

An interesting study examined the interactions among μ opioid, α2-adrenergic, and adenosine A_1 agonists peripherally administered. Antinociception was determined by assessing the degree of inhibition of PGE2-induced mechanical hyperalgesia by using the Randall-Selitto paw-withdrawal test. These findings suggest that all three receptors are located on the same primary afferent nociceptors. Although any of the agonists administered alone produce antinociception, the authors found that μ opioid, adenosine A_1, and α2 receptors might not act independently to produce antinociception but rather might require the physical presence of the other receptors to produce antinociception by any one agonist (Aley and Levine, 1997). Therefore, we speculate that any one endogenous molecule could mediate the antiallodynic the effect of WWIT and that this effect could require the physical presence of more than one receptor.

CONCLUSIONS

In summary, our data show that WWIT produces local peripheral antihyperalgesic effects in an experimental model of persistent inflammatory pain. Its antihyperalgesic effect is mediated, at least in part, through peripheral opioid, adenosine (A_1), and cannabinoid (CB_2) receptors. These findings could contribute to a better understanding of the mechanism of the peripheral antiallodynic effect of WWIT. WWIT is certainly not a cure for arthritis, but it should be considered as an additional nonpharmacological treatment option in an existing treatment plan.

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