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# Antihyperalgesic effect of $CB_1$ receptor activation involves the modulation of $P2X_3$ receptor in the primary afferent neuron

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ABSTRACT

Cannabinoid system is a potential target for pain control. Cannabinoid receptor 1 ($CB_1$) activation play a role in the analgesic effect of cannabinoids once it is expressed in primary afferent neurons. This study investigates whether the anti-hyperalgesic effect of $CB_1$ receptor activation involves $P2X_3$ receptor in primary afferent neurons. Mechanical hyperalgesia was evaluated by electronic von Frey test. Cannabinoid effect was evaluated using anandamide or ACEA, a non-selective or a selective $CB_1$ receptor agonists, respectively; AM251, a $CB_1$ receptor antagonist, and antisense ODN for $CB_1$ receptor. Calcium imaging assay was performed to evaluated α,β-meATP-responsive cultured DRG neurons pretreated with ACEA. Anandamide or ACEA administered in peripheral tissue reduced the carrageenan-induced mechanical hyperalgesia. The reduction in the carrageenan-induced hyperalgesia induced by ACEA was completely reversed by administration of AM251 as well as by the intrathecal treatment with antisense ODN for $CB_1$ receptor. Also, ACEA reduced the mechanical hyperalgesia induced by bradykinin and by α,β-meATP, a $P2X_3$ receptor non-selective agonist, but not by tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and chemokine-induced chemoattractant-1 (CINC-1). Finally, $CB_1$ receptors are co-localized with $P2X_3$ receptors in DRG small-diameter neurons and the treatment with ACEA reduced the number of α,β-meATP-responsive cultured DRG neurons. Our data suggest that the analgesic effect of $CB_1$ receptor activation is mediated by a negative modulation of the $P2X_3$ receptor in the primary afferent neurons.

## 1. Introduction

The importance of cannabinoid system and its potential therapeutic effect on pain management increased considerably in the last few years. Cannabinoid action is mediated by two receptors, $CB_1$ and $CB_2$, both coupled to inhibitory Gi/o proteins (Ahluwalia et al., 2000; Freund and Hajos, 2003; Hohmann and Herkenham, 1999; Piomelli, 2005). Cannabinoid $CB_1$ receptors are expressed in central nervous system (CNS) (Matsuda et al., 1990; Munro et al., 1993; Zimmer et al., 1999), and in peripheral nervous system specifically in the nociceptive afferent fibers of the dorsal root ganglion (DRG) (Ahluwalia et al., 2000; Hohmann and Herkenham, 1999; Veress et al., 2013), cutaneous tissue (Agarwal et al., 2007; Amaya et al., 2006; Stander et al., 2005), keratinocytes and immune cells (Gaffal et al., 2013; Jean-Gilles et al., 2015; Mai et al., 2015). Cannabinoid $CB_2$ receptors, in turn, occur in CNS and non-neuronal cells of the peripheral tissue, particularly immune cells (Agarwal et al., 2007; Amaya et al., 2006; Pertwee, 2006).

Cannabinoid receptors activation in the peripheral tissue prevents signs of tonic pain induced by administration of inflammatory agent in animals (Gutierrez et al., 2007; Pertwee, 2006). Thus, activation of cannabinoid receptors by anandamide, a non-selective cannabinoid receptor agonist, or ACEA, a selective $CB_1$ receptor agonist, reduces carrageenan-induced inflammatory hyperalgesia in the rat hind paw (Clayton et al., 2002; Gutierrez et al., 2007; Nackley et al., 2003). Mechanical hyperalgesia induced by carrageenan is a model of inflammatory pain widely used since it is sensitive to non-steroidal anti-inflammatory drugs similarly to many inflammatory conditions in

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humans (Ferreira et al., 1993a). Administration of carrageenan in the subcutaneous tissue of rat hind paw leads to the release of bradykinin and subsequent release of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Ferreira et al., 1993b, 1974). These cytokines induce the release of prostaglandins and sympathetic amines, which are final inflammatory mediators that ultimately will sensitize the nociceptors (Gold et al., 1996; Rush and Waxman, 2004).

Moreover, a previous study showed that during carrageenan-induced inflammation the bradykinin triggers ATP release and consequent neuronal $P2X_3$ receptor activation (Oliveira et al., 2009). This activation contributes to the susceptibility of the nociceptor to the action of inflammatory mediators facilitating the development of inflammatory hyperalgesia (Prado et al., 2013). It has been suggested that activation of cannabinoid $CB_1$ receptor negatively modulates P2X receptor activity in vitro (Krishtal et al., 2006). Considering this interaction among $CB_1$ and P2X receptors, it is plausible to hypothesize that the anti-hyperalgesic effect of $CB_1$ receptor activation may be mediated by the decreasing of $P2X_3$ receptor activity. Thus, this study aimed to investigate whether the mechanism by which the activation of peripheral cannabinoid $CB_1$ receptor reduces the carrageenan-induced mechanical hyperalgesia involves the modulation of $P2X_3$ receptor.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed using 233 adult male Wistar rats (150–200 g) in agreement with the guidelines of the IASP on laboratory animal care (Zimmermann, 1983). All animal protocols and experimental procedures followed the guidelines of the Ethics Committee for Animal Research, State University of Campinas, and were approved by the Committee for Multidisciplinary Research in Biological Science Area of Laboratory Animals (CEMIB, protocol number: 2799-1).

The animals (specific pathogen free - SPF) were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle, with food (commercial chow for rodents) and filtered water available ad libitum, in a temperature-controlled room ( ± 23 °C). Testing sessions took place during the light phase (09:00 AM–5:00 PM) in a quiet room maintained at 23 °C. During the tests, the animals had no access to water or food. All efforts were made to minimize both the stress of rats and the number of animals per group. The group size (n) for each experimental group is showed in “Results sections”. Animals were divided randomly into the groups. The experimenter blinded to the experimental groups made all analyses.

### 2.2. Drugs and doses

λ-carrageenan (100 μg/paw (Araldi et al., 2013), Sigma-Aldrich, MO, USA); bradykinin (500 ng/paw (Cunha et al., 2000), Sigma-Aldrich, MO, USA); α,β-methyleneATP lithium salt (α,β-meATP), a non-selective $P2X_3$ receptor agonist (50 μg/paw (Prado et al., 2013), Sigma-Aldrich, MO, USA), were all dissolved in saline (0.9% NaCl). ACEA, a selective cannabinoid $CB_1$ receptor agonist (N-2-Chloroethil)–5Z,8Z,11Z,14Z-eicosatetraenamida] (0.1, 1.0 and 10 μg/paw (Sagar et al., 2005), Tocris Bioscience, Bristol, UK) was diluted in ethanol and saline. Anandamide, a non-selective cannabinoid receptor agonist, was dissolved in Tocrisolve TM 100 [N-2-Hydroxyethil)–5Z,8Z,11Z,14Z-eicosatetraenamide] (0.03, 0.1, 0.3 and 0.9 ng/paw (Schreiber et al., 2012), Tocris Bioscience, Bristol, UK). AM251, a selective cannabinoid $CB_1$ receptor antagonist (80 μg/paw (Richardson et al., 1998), Tocris Bioscience, Bristol, UK) was dissolved in 10% propyleneglycol. Tumor necrosis factor alpha (TNF-α, 2.5 pg/paw; Cunha et al., 2008), Interleukin 1 beta (IL-1β, 0.5 pg/paw; Araldi et al., 2013; Cunha et al., 2008), chemokine-induced chemoattractant-1 (CINC-1, 100 pg/paw, Cunha et al., 2008) were bought from National Institute of Biological Standards and Control, South Mimms, Hertfordshire, UK. α,β-meATP was dissolved in Hanks buffer for primary DRG cultures (30 μM) (Grubb and Evans, 1999).

### 2.3. Subcutaneous injection

Animals were quickly restrained and the drugs or vehicle (50 μl) was subcutaneously injected in the rat intraplantar tissue of the hind paw (Vivancos et al., 2003). A BD Ultra-Fine® needle (30 gauge) with a insulin syringe (30 units) was used to the intraplantar injection.

### 2.4. Intrathecal injection

Based on Papir-Kricheli (Papir-Kricheli et al., 1987), antisense ODN was administered into the subarachnoid space. Rats were briefly anesthetized with 5% halothane (Le Bars et al., 1979), a needle (29 gauge) was inserted in the midline between the lumbar vertebrae (L4 and L5), and antisense ODN was injected at 1 μl/s. Antisense ODN against $CB_1$ receptor (30 μg/10 μl) was intrathecally administered once a day over 4 days (Barclay et al., 2002). Behavioral test was performed following the last day of antisense ODN injection.

### 2.5. Evaluation of the hyperalgesia

Hyperalgesia was measured by electronic pressure-meter paw test for rats. Before testing, rats were placed in acrylic cages (12×20×17 cm) with a wire grid floor during 15–30 min and the paws were tested 2–3 times for adaptation. For the test, a hand-held force transducer with a 0.5 $mm^2$ polypropylene tip (electronic von Frey, TC Inc. Life Science Instruments, USA) was used to induce a paw flexion reflex (Vivancos et al., 2004). A clear view of the rat hind paw was provided by a tilted mirror under the grid floor. The investigator was instructed to handle the tip applying a progressive increasing force, not exceeding 80 g, in the rat hind paw. The tip was applied between the five distal footpads. The mechanical stimulus was repeated from three to six times up to the animal presented three similar measurements. A typical flinch response following the hind paw withdrawal determined the end point. The animals that did not respond consistently were removed from the experimental group. The pressure applied to the hind paw was measured (in grams) and automatically recorded when the paw was withdrawn. The intensity of hyperalgesia was presented as Δ withdraw threshold, obtained by subtracting values measured before the treatment from those obtained after the treatment.

### 2.6. Antisense Oligodeoxynucleotide (AS-ODN)

The intrathecal treatment with antisense ODN was performed to block the $CB_1$ receptor function only in the nociceptor (Prado et al., 2013; Yu et al., 2009). The antisense ODN sequence against $CB_1$ receptor transcript was: 5′- TGAATCATGCGGACCGCGT-3′. The mismatch ODN sequence with six bases changed was: 5′- TTACTCAG GCTGGCCGAGT-3′. Agreeing to NCBI database to Rattus norvegicus there are no other homologous sequences. The ODNs were purchased lyophilized from Exxtend (Campinas, SP, Brazil), reconstituted in saline, aliquoted and stored at −20 °C for using during the treatment.

### 2.7. Analysis of $CB_1$ expression by western blotting

The analysis of cannabinoid $CB_1$ receptor expression was performed to verify the efficacy of intrathecal antisense ODN treatment against this protein. L4 and L5 DGR were harvested, frozen in liquid nitrogen and stored at −80 °C for using in the immunoblotting. To the immunoblotting, DRG samples were homogenized with an ultrasonic homogenizer (Sonic Corporation, USA) in a buffer solution with 1% Triton X-100, 50 mM phosphate buffer pH 7.4, 8 M urea, 2 M thiourea,