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Article

Muscle pain induced by static contraction is modulated by transient receptor potential vanilloid 1 and ankyrin 1 receptors

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Molecular mechanisms involved in the development of muscle pain induced by static contraction are not completely elucidated. This study aimed to evaluate the involvement of the transient receptor potential vanilloid 1 (TRPV1) and the transient receptor potential ankyrin 1 (TRPA1) receptors expressed in peripheral and central terminals of primary afferents projected to gastrocnemius muscle and spinal cord in muscle pain induced by static contraction. An electrical stimulator provided the contraction of rat gastrocnemius muscle and mechanical muscle hyperalgesia was quantified through the pressure analgesimeter Randall-Selitto. AMG9810 and HC030031 were used. When administered in ipsilateral, but not contralateral gastrocnemius muscle, drugs prevented mechanical muscle hyperalgesia induced by static contraction. Similar results were obtained by intrathecal administrations. We propose that, in an inflammatory muscle pain, peripheral and central TRPV1 and TRPA1 work together to sensitize nociceptive afferent fibers, and that TRPV1 and TRPA1 receptors are potential target to control inflammatory muscle pain.

Keywords: Muscle pain. Static contraction. TRPA1 and TRPV1 receptors.

INTRODUCTION

Pain is one of the major health problems of the world. It was estimated that 1 in 5 adults suffer from pain and that another 1 in 10 adults are diagnosed with chronic pain each year, experiencing potentially debilitating conditions (Goldberg, McGee, 2011). For example, low back pain has the highest global burden of disease related to years lived with disabilities around the world (French, Downie, Walker, 2018). Consequently, pain is also a critical socioeconomic problem, reducing quality of life and costing billions annually to world societies (Loeser, 2012). Muscle pain is one of the most important reasons of disability in the world (Collaborators, 2015). The static contraction-induced muscle pain is highly associated to occupational and daily life activities (Luttmann *et al*., 2003), such as work-related neck and shoulder pain, which is associated to static contraction of the trapezius muscle (Pollak *et al*., 2014); low back pain in dentists are usually related to long periods in a sitting posture with static contraction of the low back muscle (Valachi, Valachi, 2003); and the primary cause of back pain during bedrest is a low-intensity static contraction of low back muscle (Baum, Essfeld, 1999). In addition, in patients with chronic muscle pain conditions, static contraction of a muscle induces a potential increase in pain (Umeda, Corbin, Maluf, 2015). In spite of its clinical significance, the mechanism underlying static contraction-induced muscle pain is not completely elucidated.

We have demonstrated that static contraction-induced muscle pain in rats is modulated by peripheral inflammatory mechanisms, which are dependent, at least

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in part, by neutrophil migration, bradykinin, sympathetic amines and prostanoids (Santos *et al.,* 2017). It is well known that an essential mechanism of inflammation is modifications in intracellular Ca^{2+} concentrations (Nuka *et al*., 2018) and, as transient receptor potential (TRP) channels favor Ca^{2+} permeability (Khalil *et al.,* 2018), it is conceivable that, in association with other molecular pathways, TRP channels could contribute to inflammatory processes.

The members of the Transient receptor potential family (TRP family), receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1), has been shown as interesting targets for the control of several pain conditions. TRPV1 is involved with a mouse model of fibromyalgia (Yüksel *et al.,* 2017), inflammatory muscle pain and delayed onset muscle soreness (Fujii *et al.,* 2008), craniofacial muscle nociception and mechanical hyperalgesia (Leo *et al.,* 2017) and visceral hyperalgesia (Shen *et al*., 2017). In addition, the administration of TRPV1 agonist into masseter muscle increases muscle pain (Ro, Lee, Zhang, 2009). TRPV1 receptors are expressed primarily on dorsal root ganglia (DRG) neurons and both on peripheral and central terminals of small and medium size (C and Aδ) nociceptive afferent fibers (c). It seems that TRPV1 are also in microglial cells of brain areas and peripheral inflammatory cells (Marrone *et al*., 2017).

Some unmyelinated, peptidergic nociceptors that express TRPV1, co-express TRPA1 (formerly ANKTM1) (Fernandes, Fernandes, Keeble, 2012; Nielsen *et* al., 2018), furthermore, TRPA1 receptors are also expressed on non-neuronal cells (Fernandes, Fernandes, Keeble, 2012). Similar to TRPV1, TRPA1 is also involved in the development of pain, including mechanical hyperalgesia induced by chemical inflammatory agents into subcutaneous (Martínez-Rojas *et al.,* 2018) and muscle tissue (Ro, Lee, Zhang, 2009; Wang *et al*., 2018) and in neuropathic pain (Wang *et al*., 2019).

Considering the clinical relevance of muscle pain, especially that induced by static contraction, the aim of this study was to evaluate the involvement of TRPV1 and TRPA1 receptors expressed on gastrocnemius muscle and spinal cord dorsal horn in muscle pain induced by static contraction.

MATERIAL AND METHODS

Animals

Male Wistar rats (200–250 g) were used in all experiments. Animals were housed in plastic cages with soft bedding (five/cage) on a 12-12 hour light/dark cycle (lights on at 06:00 a.m.) with water and food available *ad libitum*. All experiments were approved by the Committee on Animal Research of the State of University of Campinas – UNICAMP, protocol number 3277-1 and were in accordance with *Ethical Guidelines for Investigators of Experimental Pain in Conscious Animals* (Zimmermann 1983). One hour previously to the test, animals were maintained in a temperature-controlled room test (± 23◦C) for habituation. Animal suffering and number per group were kept at a minimum.

Static contraction model of muscle pain

To induce static contraction, rats were anesthetized with isoflurane (5 % for induction and 1.5 % for maintenance) and an electrical stimulator (Grass S88X – Grass Technologies, West Warwick, RI, USA) provided pulses via two sterile needle electrodes (27 gauge) directly inserted into the belly of gastrocnemius muscle (10 mm apart). A monophasic current was modulated with 1.6 volts, repeat pulses, 19 ms pulse duration, frequency of 50 Hz for 1 hour (Santos *et al.,* 2017). In the control group (sham), electrodes were inserted but no stimulation was generated. The electrodes in both groups were removed from the gastrocnemius muscle after 1 hour, and the mechanical nociceptive threshold test was performed immediately after the animals woke up from anesthesia (around three minutes).

Drug injections

Intramuscular injections

To injections into gastrocnemius muscle, rats were anesthetized with isoflurane (5 % for induction). They were performed with a 30-gauge needle in a total volume of 50μL (Schiavuzzo *et al.,* 2015).

Intrathecal injections

To intrathecal injections, rats were anesthetized with isoflurane (5 % for induction and 1.5 % for maintenance). A 31-gauge needle was inserted in the subarachnoid space on the midline between L4 and L5 vertebrae. Drugs were injected at 1 µL/s in a volume of 10µL. The animals regained consciousness approximately 1 min after discontinuing the anesthesia.

Drugs and Doses

The selective TRPV1 receptor antagonist, AMG9810, (2E-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-Propenamide: 35, 70 and 105 µg/muscle or 0.03, 0.3 and 3.0 µg, i.t.) and the selective TRPA1 receptor antagonist, HC030031, (1,2,3,6-Tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7-acetamide, 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide: 30, 100 and 300 µg/muscle or 1.0, 4.0 and 16 µg, i.t.) were used. All drugs were provided by Sigma-Aldrich (Brazil). The stock solution of these drugs was diluted in DMSO and after, to work concentration, they were diluted in 0.9 % NaCl.

Mechanical nociceptive threshold test

Mechanical withdrawal thresholds were measured by the Randall-Selitto analgesimeter (Randall, Selitto, 1957) (Insight, Ribeirão Preto, SP, Brazil), which applied a linear mechanical force to the belly of the gastrocnemius muscle of rats (Santos *et al.,* 2017). All testing were performed in the light phase (9:00 a.m. until 5:00 p.m.), in a quiet room with temperature-controlled at 23ºC (Rosland 1991). To reflect the pain threshold of deep tissues, a rounded tip with 2.0mm of diameter was used (Takahashi *et al*., 2006). The baseline muscle-withdrawal threshold was defined as the mean of three tests performed at five-minute intervals before static contraction. Mechanical muscle hyperalgesia was quantified as the change in mechanical nociceptive threshold calculated by subtracting the mean of three measurements taken one hour after the end of static contraction (Santos *et al*., 2017) from the baseline muscle-withdrawal threshold. Therefore, an increase in y-axis represents an increased behavioral response. All experiments were performed with the tester blinded to treatment.

Statistical Analysis

Data were analyzed by one-way ANOVA or Student's t-test. If there was a significant between-subject main effect of treatment group, post hoc contrasts, using the Tukey test, were performed to determine the basis of the significant difference. Data are expressed by the decrease in paw-withdrawal threshold in grams and are presented as means ± SEM. Statistical analysis was run on GraphPad Prism 7.0 software. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Results

TRPV1 receptors are involved in mechanical muscle hyperalgesia induced by static contraction

Five minutes before the beginning of the static contraction, the selective TRPV1 receptor antagonist, AMG9810, was injected into the belly of gastrocnemius muscle. AMG9810 (70 and 105 µg/muscle, but not 35 µg) reduced static contraction-induced mechanical muscle hyperalgesia when compared to control group (p=0,0246, p<0,0001, p=0,7795, respectively; One-way ANOVA, Tukey test, Figure 1A). Contralateral administration of AMG9810 (105 µg/muscle) did not affect the mechanical muscle hyperalgesia (p= 0,2647, Student's t-test, Figure 1A), suggesting its local peripheral action. In the sham group, AMG9810 (105 µg/muscle) did not affect the mechanical muscle withdrawal threshold (p= 0,1230, One-way ANOVA, Tukey test, Figure 1A).

Intrathecal administration of AMG9810 (3.0, but not 0.3 µg and 0.03 µg) previously to static contraction also reduced mechanical muscle hyperalgesia when compared to control group (p<0.0001, p=0,2309, p=0,6443, respectively; One-way ANOVA, Tukey test, Figure 1B). The anti-hyperalgesic effect of intramuscular

and intrathecal administration of AMG9810 did not occur in a dose-dependent manner.

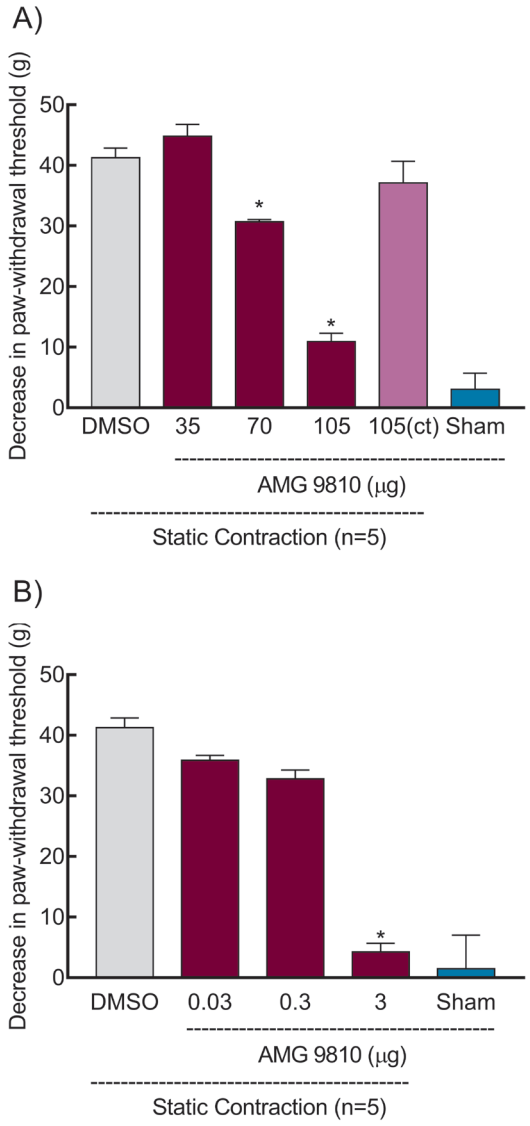

FIGURE 1 - Involvement of TRPV1 receptors in mechanical muscle hyperalgesia induced by static contraction.

A) Intramuscular administration of AMG9810 reduced the mechanical muscle hyperalgesia induced by static contraction when administered in the ipsilateral but not in the contralateral gastrocnemius muscle. The administration of AMG9810 in sham group did not affect the mechanical muscle withdrawal threshold. B) Intrathecal administration of AMG9810 reduced the mechanical muscle hyperalgesia induced by static contraction. The symbol "*" indicates responses significantly lower than that induced by static contraction ($p < 0.05$, ANOVA, Tukey test). Abbreviations: ct – contralateral.

TRPA1 receptors are involved in mechanical muscle hyperalgesia induced by static contraction

Five minutes before the beginning of the static contraction, the selective TRPA1 receptor antagonist, HC030031, was injected into the belly of gastrocnemius muscle. HC030031 (100 and 300 μg/muscle, but not 30 μg) reduced static contraction-induced mechanical muscle hyperalgesia when compared to control group ($p<0,0001$, $p<0,0001$, $p>0,9999$, respectively; ANOVA, Tukey test, Figure 2A). Contralateral administration of HC030031 (300 μg/muscle) did not affect the mechanical muscle hyperalgesia ($p=0,7877$, Student's t-test, Figure 2A), suggesting its local peripheral action. In the sham group, HC030031 (300 μg/muscle) did not affect the mechanical muscle withdrawal threshold ($p=0,9997$, ANOVA, Tukey test, Figure 2A).

Intrathecal administration of HC030031 (4 and 16 μg, but not 1 μg) previously to static contraction also reduced mechanical muscle hyperalgesia when compared to the control group ($p=0,0003$, $p<0,0001$, $p=0,6541$, respectively; ANOVA, Tukey test, Figure 2C). The anti-hyperalgesic effect of intramuscular and intrathecal administration of HC030031 occurred in a dose-dependent manner with an ED_{50} of 141.8 μg and 3.2 μg, respectively (Figures 2B and D).

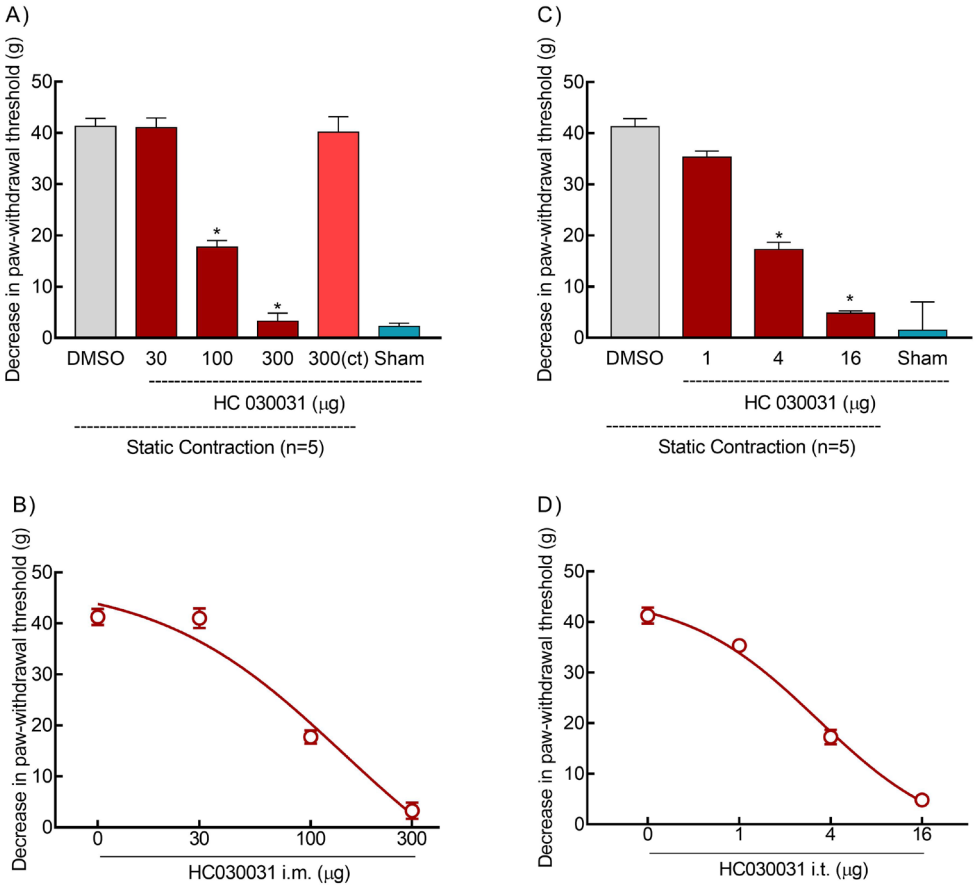

FIGURE 2 - Involvement of TRPA1 receptors in mechanical muscle hyperalgesia induced by static contraction

A) Intramuscular administration of HC030031 reduced the mechanical muscle hyperalgesia induced by static contraction when administered in the ipsilateral but not in the contralateral gastrocnemius muscle. The administration of HC030031 in sham group did not affect the mechanical muscle withdrawal threshold. B) Intrathecal administration of HC030031 reduced the mechanical muscle hyperalgesia induced by static contraction. C) Dose–response analysis of the data for intramuscular administration of HC030031. D) Dose–response analysis of the data for intrathecal administration of HC030031. The symbol "*" indicates responses significantly lower than that induced by static contraction ($p < 0.05$, ANOVA, Tukey test). Abbreviations: ct – contralateral.

DISCUSSION

The present study demonstrated, for the first time, that static contraction-induced mechanical muscle hyperalgesia is modulated by activation of TRPV1 and TRPA1 receptors expressed in peripheral and central terminals of primary afferents projected to gastrocnemius muscle and spinal cord.

The mechanical muscle hyperalgesia induced by static contraction is a model of muscle pain developed by our research group (Santos *et al*., 2017). This mechanical muscle hyperalgesia is associated with an inflammatory process, increased levels of creatine kinase and no histological evidences of structural damage in muscle fibers (Santos *et al.,* 2017). Specifically, the involvement of an inflammatory process was demonstrated by the findings that the static contraction-induced mechanical muscle hyperalgesia was reduced by pre-treatment with the steroidal anti-inflammatory dexamethasone and by the cyclooxygenase inhibitor indomethacin (Santos *et al.,* 2017). Considering that final inflammatory mediators like prostaglandins sensitize nociceptive afferent fibers to contribute to the development of pain (Rush, Waxman, 2004) and that TRPV1 receptors are primarily expressed on peripheral and central terminals of nociceptive afferent fibers (Fernandes, Fernandes, Keeble, 2012), it

is possible to suggest that activation of TRPV1 expressed in peripheral and central terminals of primary afferents projected to gastrocnemius muscle and spinal cord is essential to sensitization induced by final inflammatory mediators in primary afferent fibers. Several studies support our data that TRPV1 contributes to mechanical muscle hyperalgesia (Zhao, Wu, 2018). The mechanism by which TRPV1 contributed to static contraction-induced muscle pain is unknown. However, there are some evidences that a decrease in tissue pH is related to an inflammatory process, and the TRPV1 receptor can be directly activated by a pH under 6.0 (Breese *et al.,* 2005). In addition, the activity of TRPV1 receptors can be upregulated by lipoxygenase products at moderately acidic pH (Hwang *et al.,* 2000) and inflammatory mediators such as prostaglandins, bradykinin and ATP can activate TRPV1 receptors by different G-coupled protein receptors and downstream activation of protein kinases (Huang, Zhang, McNaughton, 2006). Considering that static contraction-induced mechanical muscle hyperalgesia is modulated by inflammatory mediators (Santos *et al.,* 2017), it is possible to suggest that static contraction induced the release of inflammatory mediators associated with a decrease of pH, which in turn induced activation of TRPV1 receptors of primary afferent nociceptors and subsequent increase in nociceptive input to the spinal cord

The present study also demonstrated that, similar to TRPV1 receptors antagonist, the TRPA1 receptor antagonist was effective in reducing static contraction-induced muscle pain when administered intramuscularly or intrathecally. The mechanism by which TRPA1 contributes to this response is unknown. It has been described that TRPA1-deficient mice have an important deficit in bradykinin-induced activation of nociceptive afferent fibers and pain (Cesare, McNaughton, 1996). In addition, bradykinin and G-protein coupled receptors increase signaling of inflammatory pain by sensitizing TRPA1 through PKA and phospholipase C (Wang *et al*., 2008). Taken together, it is possible to suggest that some of the inflammatory mediators released by the static contraction have activated both TRPV1 and TRPA1 channels by intracellular pathways and sensitization of the primary afferent fibers to contribute to mechanical muscle hyperalgesia (Ro, Lee, Zhang, 2009).

Interestingly, 30 % of the sensory neurons expressing TRPV1 also express TRPA1, while 97 % of the sensory neurons expressing TRPA1 also express TRPV1 (Story *et al.,* 2003). TRPV1 and TRPA1 channels are calcium-permeable and form a complex on nociceptive neurons that enables TRPV1 to have an effect on TRPA1 channel opening (Staruschenko, Jeske, Akopian, 2010). Moreover, both TRPV1 and TRPA1 work together to signal a noxious stimulus by desensitizing each other pathways (Ruparel *et al.,* 2008). Finally, activation of these receptors in the spinal cord dorsal horn increases glutamate release (Jeffry *et al.,* 2009; Spicarova, Palecek, 2009) and, consequently, the excitability of interneurons and projection neurons. Therefore, we can not exclude the hypothesis that TRPV1 and TRPA1 may also have interacted with each other to activate nociceptive afferent pathways during static contraction.

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