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ORIGINAL ARTICLE

P2X3 receptors contribute to transition from acute to chronic muscle pain

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Abstract

This study aimed to evaluate whether the development and/or maintenance of chronic-latent muscle hyperalgesia is modulated by P2X3 receptors. We also evaluate the expression of P2X3 receptors and PKCε of dorsal root ganglions during these processes. A mouse model of chronic-latent muscle hyperalgesia, induced by carrageenan and evidenced by PGE_2, was used. Mechanical muscle hyperalgesia was measured by Randall-Selitto analgesimeter. The involvement of P2X3 receptors was analyzed by using the selective P2X3 receptors antagonist A-317491 by intramuscular or intrathecal injections. Expression of P2X3 and PKCε in dorsal root ganglion (L4-S1) were evaluated by Western blotting. Intrathecal blockade of P2X3 receptors previously to carrageenan prevented the development and maintenance of acute and chronic-latent muscle hyperalgesia, while intramuscular blockade of P2X3 receptors previously to carrageenan only reduced the acute muscle hyperalgesia and had no effect on chronic-latent muscle hyperalgesia. Intrathecal, but not intramuscular, blockade of P2X3 receptors immediately before PGE_2, in animals previously sensitized by carrageenan, reversed the chronic-latent muscle hyperalgesia. There was an increase in total and phosphorylated PKCε 48 h after the beginning of acute muscle hyperalgesia, and in P2X3 receptors at the period of chronic muscle hyperalgesia. P2X3 receptors expressed on spinal cord dorsal horn contribute to transition from acute to chronic muscle pain. We also suggest an interaction of PKCε and P2X3 receptors in this process. Therefore, we point out P2X3 receptors of the spinal cord dorsal horn as a pharmacological target to prevent the development or reverse the chronic muscle pain conditions.

Keywords Muscle · Hyperalgesia · P2X3 receptors · PKC epsilon

Introduction

Even with the advance of health science, chronic pain represents an important health problem around the world [1] and chronic pain is considered on the top ten conditions responsible for the most years lived with disability (YLD) globally [2]. It is already known that in 80% of cases, pain could persist forever [3]. Specifically, disabling musculoskeletal pain is responsible for 28 million of disability-adjusted life years (DALYs) [2, 4] and affects over 40% of the general population [5], 13–47% of whom experience chronic pain [6]. Chronic pain is not a simple acute pain that lasts longer, it is a sum of plastic changes in transduction and transmission systems of the nociceptive signal, including primary afferent neurons, dorsal root ganglions, and structures of the central nervous system [7].

The chronic muscle pain can be triggered by hyperalgesic mediators, which activate transiently the protein kinase C epsilon (PKCε) [8, 9] or the signaling molecules of the PKC activation cascade [10]. The maintenance of chronic muscle pain seems to involve more lasting modifications, such as modulation of nociceptive afferent fibers by kinase activation cascade and/or in the expression of some receptors in the nociceptive fibers [11–13]. It is interesting to point out that P2X3 receptor, one of the most important receptors related to transmission and modulation of nociceptive signals (Oliveira-Fusaro et al. 2017; Teixeira et al. 2016), including the

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nociceptive muscle tissue signaling [14, 15], has an involvement with the PKC signaling pathway [16–19].

We have recently demonstrated that the intramuscular and intrathecal pharmacological blockade of P2X3 receptors previously to static contraction of gastrocnemius muscle prevents the development of acute mechanical muscle hyperalgesia [14]. It is important to point out that the long-term static contraction of the gastrocnemius muscle induced a local inflammation. Considering the clinical relevance of chronic muscle pain, now using a mouse model of chronic-latent muscle hyperalgesia induced by an inflammatory insult [8], we aimed to evaluate whether the development and/or maintenance of chronic-latent mechanical muscle hyperalgesia are modulated by the P2X3 receptors. We also evaluate the expression of P2X3 receptors and PKCε protein of dorsal root ganglions (L4-S1) during the development and maintenance of chronic-latent muscle hyperalgesia.

Material and methods

Animals

Male Swiss mice (6 weeks old) from CEMIB (Multidisciplinary Center for Biological Research, UNICAMP) were used based on IASP guidelines on using laboratory animals [20] and the National Council for the Control of Animal Experimentation (CONCEA, Brazil). The procedures were also approved by the Committee on Animal Research of the State University of Campinas (protocol number 3883-1). The animals were housed in standard plastic cages (five animals per cage) in an animal room with controlled environmental conditions, with free access to food and water, in exception during the experiments. Before starting any experimental protocol, the animals were habituated to the test room for 1 h. The experimental sessions were performed during the light phase, between 9:00 a.m. and 5:00 p.m., in a quiet room with the temperature maintained at 23 °C [21].

Model of chronic-latent muscle hyperalgesia

The protocol of chronic-latent muscle hyperalgesia was adapted in mice in the present study from Dina and collaborators [8]. Briefly, carrageenan (100 μg) was injected into the belly of the gastrocnemius muscle to induce mechanical muscle hyperalgesia and, 10 days later, Prostaglandin E_2 (PGE_2, 1 μg) was injected at the same local to reveal the state of chronic-latent muscle hyperalgesia. Hyperalgesic thresholds were measured immediately before the carrageenan administration (baseline) and after 1, 3, 6, 24, 48, 72, 96, and 144 h. Over again, the hyperalgesic threshold was measured immediately before PGE_2 injection and after 1, 4, 24, 48, and 168 h.

Mechanical muscle nociceptive threshold test

Randall-Selitto analgesimeter (Insight, Ribeirão Preto, SP, Brazil) was used to quantify the mechanical muscle hyperalgesia of the gastrocnemius muscle [22]. This equipment applies gradual mechanical linear strength in the gastrocnemius muscle of mice paw. Initially, the baseline muscle-withdrawal threshold was evaluated by three measures at intervals of 5 min before the injection of carrageenan or PGE_2. The mechanical muscle hyperalgesia was quantified as the change in mechanical nociceptive threshold calculated by subtracting the mean of measurements taken in each time point after the injections of carrageenan and PGE_2. All the in vivo experiments were performed by a blinded tester. Before the animal's habituation in the experimental room, another researcher, who was not involved in the behavior tests, blinded the animal groups by naming them in alphabetic letters. The blinded tester performed the Randall-Selitto tests and collected all tissues for molecular analysis. Finally, the real names of the groups were revealed to allow the interpretation of the behavior results and sample separation.

Administration of drugs

For drug injections, animals were briefly anesthetized by inhalation of isoflurane (3–4% to induction and 1.5% to maintenance/100% of oxygen). The animals recovered conscience 1 min after discontinuing the anesthesia.

Intramuscular administration: Drugs or their vehicles were administrated directly in the belly of the gastrocnemius muscle of mice [15]. The 30-gauge needle was connected with polyethylene catheter and to a Hamilton syringe (50 μL). The final injection volume was 20 μL.

Intrathecal administration: The ultra-fine syringe (1 mL) with a 31-gauge needle was inserted in the subarachnoid space on the midline between L4 and L5 vertebrae [23]. Drugs were injected at 1 μL/s in a volume of 5 μL.

Drugs and doses

We used the following drugs: carrageenan (100 μg) [24]; PGE_2 (1 μg) [8]; and the selective P2X3 receptor antagonist, 5-([(3-phenoxybenzyl) [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] [amino]carbonyl)-1,2,4-benzene-tricarboxylic acid (A-317491), 60 μg/20 μL via intramuscular [24] and 20 μg/5 μL via intrathecal. The stock solution of PGE_2 (1 μg/μL) was prepared in 10% ethanol and additional dilutions made with isotonic saline (0.9% NaCl) to a final ethanol concentration lower than 1%. All other drugs were dissolved in saline (0.9% NaCl) and were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting

A pull of tree dorsal root ganglions (L4-S1), collected from different time points of acute and chronic-latent muscle hyperalgesia, was lysed in a lysis buffer (Tris/HCl, pH 7.4; Na_2 ethylenediaminetetraacetic acid [EDTA], pH 8.0; DTT; Triton X-100; and protease inhibitor cocktail [Roche Diagnostics GmbH, Mannheim, Germany]). In the case of phosphorylated PKCε analysis, the phosphatase activity inhibitors (PhosStop, Roche Diagnostics GmbH, Mannheim, Germany) were added to the lysis buffer. The lysates were centrifuged at 12,000×*g* for 20 min at 4 °C, and the supernatants were collected and transferred to an − 80 °C freezer until being used for the Western blotting analysis. An aliquot from the supernatant was used to determine the total protein content by the Bradford method [25]. About 30 μg of total protein extract was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes and blocked with Tris-buffered saline with Tween 20 (TBS-T) containing 5% non-fat milk. Next, the membranes were incubated overnight at 4–8 °C temperature with the desired primary antibodies diluted in TBS-T containing 5% non-fat milk. Membranes were washed with TBS-T and incubated for 1 h at room temperature with the appropriate specific horseradish peroxidase-conjugated anti-immunoglobulin (Ig)G (Jackson ImmunoResearch Inc., USA) diluted in TBS-T containing 5% non-fat milk. Finally, membranes were washed with TBS-T and immunoreactive proteins were detected by enhanced chemiluminescence using an enhanced chemiluminescence (ECL) reagent (Cell Signaling, USA). Primary antibodies are as follows: anti-P2X3 (1:200, rabbit polyclonal, Alomone, Jerusalem, Israel); anti-PKCε (1:1000, rabbit polyclonal, Cell Signaling Technologies, Denvers, MA, USA); Rabbit anti-PKCε phospho S729 (1:500, Abcam, Cambridge, MA, USA); Rabbit anti-β-tubulin (1:1000, Cell Signaling Technologies, Denvers, MA, USA). Secondary antibody is as follows: polyclonal anti-IgG anti-rabbit (1:5000, Jackson ImmunoResearch Inc., West Grove. PA, USA). G:BOX Chemi XRQ gel doc system was used to capture membrane images after 1 min of exposition. All images were analyzed by Image J program. All blotting experiments were done three times to confirm data.

Statistical analysis

Behavior tests Kolmogorov-Smirnov test were done to define if data assumed a normal Gaussian distribution. Results related to mechanical muscle hyperalgesia were analyzed by one-way ANOVA, two-way ANOVA, or Student *t* test. To identify the significance between subjects of the treated group, post hoc contrast was performed, using Tukey test or Bonferroni test. To evaluate the main effect of treatments on mechanical muscle hyperalgesia, area under the curve (AUC) were performed to acute (from 1 to 72 h) and persistent (1–168 h) periods and analyzed by Student's *t* test or one-way ANOVA followed by Tukey post hoc. Line graphs data are expressed by means ± SD, and AUC graphs are expressed by scatter dot plot—means ± SD.

Western blotting analysis Grubbs' test was done to recognize outliers' samples. After, the Shapiro-Wilk normality test was used to identify if the data assumed Gaussian distribution. For data with Gaussian distribution, we applied a non-pared and parametric Student's *t* test, and for data without Gaussian distribution, we choose a non-paired and non-parametric Student *t* test with Mann-Whitney comparison. Western Blotting data are expressed by box and whiskers—SD.

All data were analyzed by GraphPad Prism 7.0 software. For all tests, significance was set at $p < 0.05$.

Results

Model of chronic-latent muscle hyperalgesia in mice

Carrageenan administration (Cg, 100 μg) into the belly of the gastrocnemius muscle of mice induced an acute mechanical muscle hyperalgesia up to 72 h when compared with the saline control group ($p < 0.05$, two-way ANOVA, Tukey's test, Fig. 1a). Three days after the nociceptive threshold has returned to baseline levels (144 h after Cg administration; Fig. 1a), the inflammatory mediator prostaglandin E_2 (PGE_2, 1 μg) was administered in the same place of carrageenan or saline. A chronic-latent mechanical muscle hyperalgesia state was established in animals previously sensitized by carrageenan (1, 4, 24, 48, and 168 h after PGE_2 injection) when compared with the saline control group ($p < 0.05$, two-way ANOVA, Tukey's test, Fig. 1a). The AUC of the acute (1–72 h) and chronic (1–168 h) period confirmed the results of the temporal analyses ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 1b and c). Also, the comparison between scales in grams of the AUC graphs evidenced that the intensity of persistent muscle hyperalgesia was greater than the intensity of acute muscle hyperalgesia (Fig. 1b and c).

Involvement of peripheral P2X3 receptors in the development and maintenance of chronic-latent muscle hyperalgesia

Intramuscular pretreatment with the selective P2X3 receptor antagonist A-317491 (60 μg/20 μL) before Cg administration reduced, but not prevented, the development of acute mechanical muscle hyperalgesia between 3 and 48 h ($p < 0.05$, two-way ANOVA, Tukey's test, Fig. 2a) and was inefficient to prevent the development and maintenance of chronic-latent muscle hyperalgesia ($p > 0.05$, two-way ANOVA, Tukey's test, Fig. 2a). To isolate the acute from chronic phase of muscle hyperalgesia, A-317491 was administered into the gastrocnemius muscle immediately before PGE_2 in animals

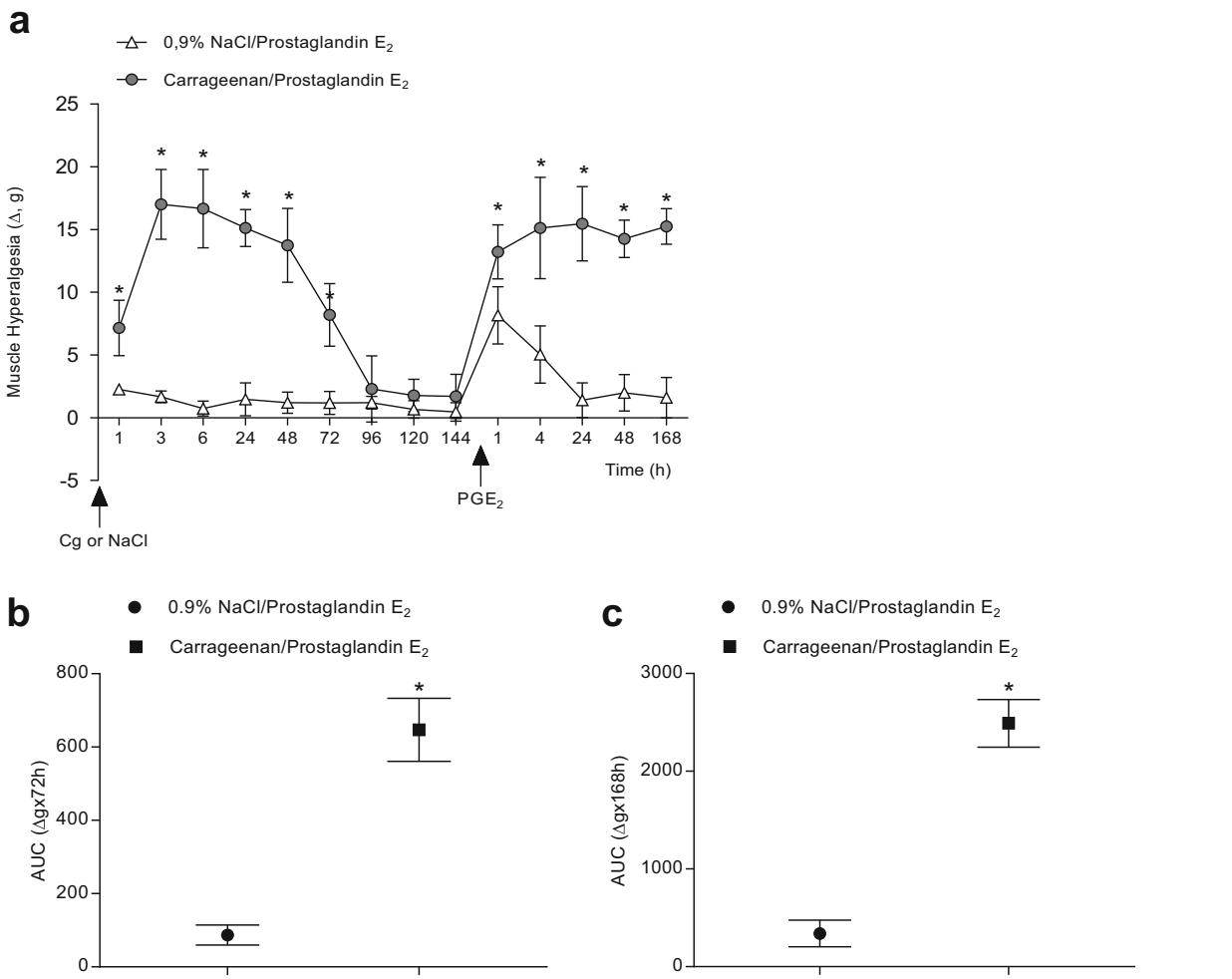

Fig. 1 Model of chronic-latent muscle hyperalgesia in mice. **a** Administration of carrageenan (Cg, 100 μg) in the gastrocnemius muscle induced acute mechanical muscle hyperalgesia between 1 and 72 h when compared with 0.9% NaCl ($n = 5$). Administration of PGE_2 (1 μg) in the gastrocnemius muscle in animals previously sensitized by Cg revealed a chronic state of muscle hyperalgesia when compared with gastrocnemius previously challenged by 0.9% NaCl ($n = 5$). **b** AUC characterization of acute muscle hyperalgesia period induced by Cg between 1 and 72 h. **c** AUC characterization of chronic-latent muscle hyperalgesia period induced by PGE_2 between 1 and 168 h. The asterisk "*" symbol indicates difference to 0.9% NaCl/PGE_2 ($p < .05$, for **a**—two-way ANOVA and Tukey's test, for **b** and **c**—Student's *t* test)

previously sensitized by Cg. The results showed that A-317491 previously to PGE_2 did not prevent or reduce the development and maintenance of chronic-latent muscle hyperalgesia ($p > 0.05$, two-way ANOVA, Tukey's test, Fig. 2c). The AUC of the acute (1–72 h) and chronic (1–168 h) period confirmed the results of the temporal analyses ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 2b and c).

Involvement of spinal P2X3 receptors in the development and maintenance of chronic-latent muscle hyperalgesia

Intrathecal (L4-L5) pretreatment with the selective P2X3 receptor antagonist A-317491 (20 μg/5 μL) before Cg administration prevented the development of acute mechanical muscle hyperalgesia between 3 and 48 h ($p < 0.05$, two-way ANOVA, Tukey's test, Fig. 3a) and also the development and maintenance of chronic-latent muscle hyperalgesia ($p < 0.05$, two-way ANOVA, Tukey's test, Fig. 3a). To isolate the acute from chronic phase of muscle hyperalgesia, A-317491 was administered intrathecally immediately before PGE_2 in animals previously sensitized by Cg. The results showed that A-317491 previously to PGE_2 reversed the chronic-latent muscle hyperalgesia ($p < 0.05$, two-way ANOVA, Tukey's test, Fig. 3a). The AUC of the acute (1–72 h) and chronic-latent period confirmed the results of the temporal analyses ($p < 0.05$, one-way ANOVA, Tukey's test, Fig. 3b and c).

P2X3 receptor expression in dorsal root ganglion (L4-S1) during acute and chronic-latent muscle hyperalgesia

There was an increase in P2X3 protein expression in dorsal root ganglion at 24 h after PGE_2 administration in animals

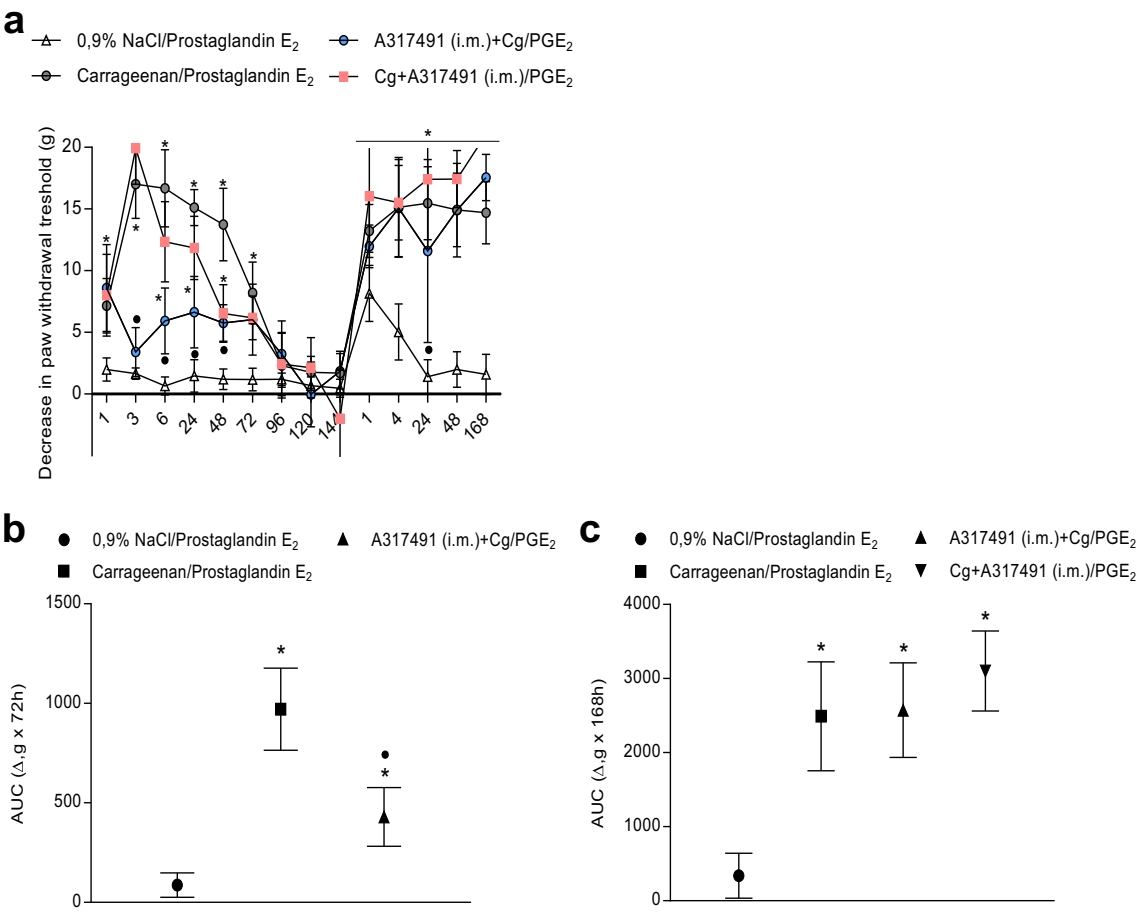

Fig. 2 Involvement of peripheral P2X3 receptors in the development and maintenance of chronic-latent muscle hyperalgesia. **a** Intramuscular A-317491 before Cg reduced the acute but not the chronic muscle hyperalgesia ($n = 5$), and A-317491 immediately before PGE_2 did not reduced the chronic muscle hyperalgesia ($n = 5$). **b** AUC from acute period demonstrated reduction of acute muscle hyperalgesia by the intramuscular pretreatment with A-317491. **c** AUC from chronic period demonstrated that intramuscular pretreatment with A-317491 (before Cg or PGE_2) did not reduce chronic muscle hyperalgesia. The asterisk "*" symbol indicates difference to 0.9% NaCl/PGE_2 ($p < 0.05$, for **a**—two-way ANOVA and Tukey's test, for **b** and **c**—one-way ANOVA and Tukey's test). The bullet "•" symbol indicates difference to Cg/PGE_2 ($p < 0.05$, for **a**—two-way ANOVA and Tukey's test, for **b** and **c**—one-way ANOVA and Tukey's test)

previously sensitized by Cg ($p < 0.05$, Student's t test, Fig. 4e). No changes were found at 6, 24, and 48 h after Cg administration ($p > 0.05$, Student's t test, Fig. 4a–c, respectively) and at 4 after PGE_2 administration in animals previously sensitized by Cg ($p > 0.05$, Student's t test, Fig. 4d).

Total PKCε protein expression in dorsal root ganglion (L4-S1) during acute and chronic-latent muscle hyperalgesia

There was an increase in total PKCε protein in dorsal root ganglion at 48 h after Cg administration ($p < 0.05$, Student's t test, Fig. 5c). No changes were found at 6 and 24 h after Cg administration ($p > 0.05$, Student's t test, Fig. 5a and b, respectively) and 4 h after PGE_2 administration in animals previously sensitized by Cg ($p > 0.05$, Student's t test, Fig. 5d).

Phosphorylated PKCε (S729) protein expression in dorsal root ganglion (L4-S1) during acute and chronic-latent muscle hyperalgesia

There was an increase in phosphorylated PKCε protein expression in dorsal root ganglion at 48 h after Cg administration ($p < 0.05$, Student's t test, Fig. 6c). No changes were found at 6 and 24 h after Cg administration ($p > 0.05$, Student's t test, Fig. 6a and b, respectively) and 4 and 24 h after PGE_2 administration in animals previously sensitized by Cg ($p > 0.05$, Student's t test, Fig. 5d and e, respectively).

Discussion

In the present study, we demonstrated that the development and maintenance of chronic-latent mechanical muscle hyperalgesia are modulated by the P2X3 receptors expressed

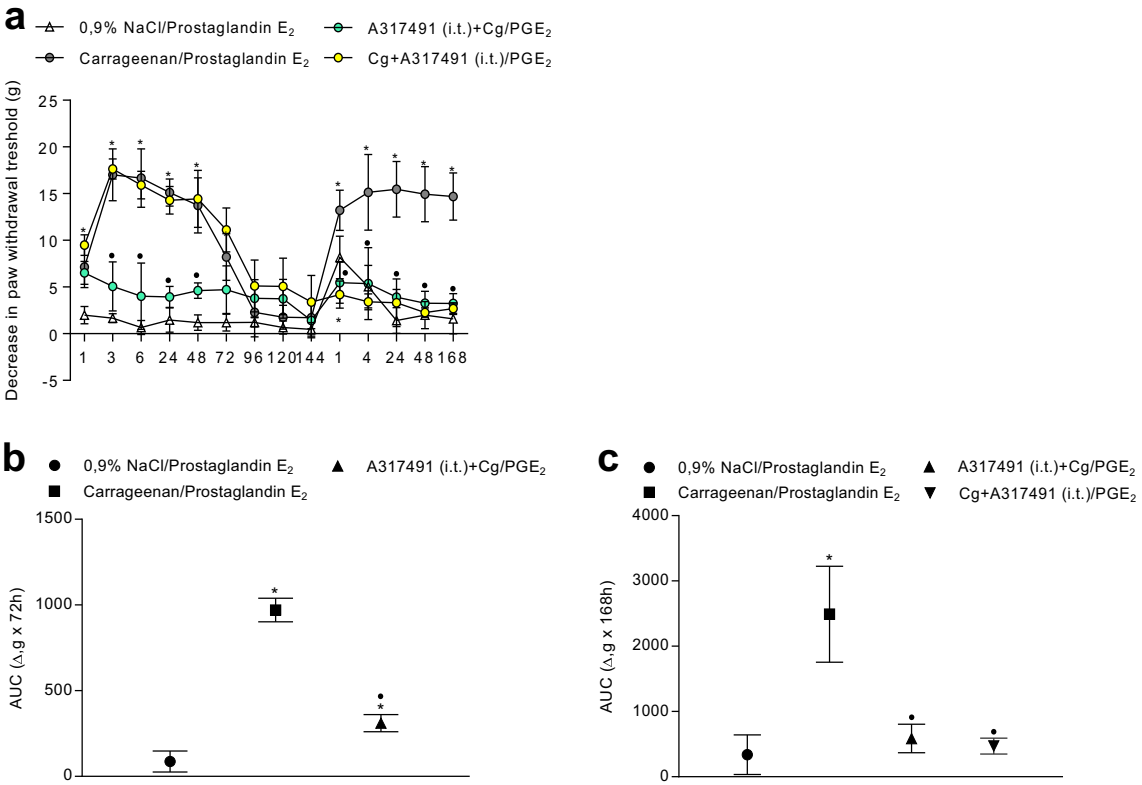

Fig. 3 Involvement of spinal P2X3 receptors in the development and maintenance of chronic-latent muscle hyperalgesia. **a** Intrathecal A-317491 before Cg prevented the acute and chronic muscle hyperalgesia ($n = 5$), and intrathecal A-317491 immediately before PGE_2 also prevented the chronic muscle hyperalgesia ($n = 5$). **b** AUC from acute period demonstrated prevention of acute muscle hyperalgesia by the intrathecal pretreatment with A-317491. **c** AUC from chronic period demonstrated prevention of chronic muscle hyperalgesia by the intrathecal pretreatment with A-317491. The asterisk "*" symbol indicates difference to 0.9% NaCl/PGE_2 ($p < .05$, for **a**—two-way ANOVA and Tukey's test, for **b** and **c**—one-way ANOVA and Tukey's test). The bullet "•" symbol indicates difference to Cg/PGE_2 ($p < 0.05$, for **a**—two-way ANOVA and Tukey's test, for **b** and **c**—one-way ANOVA and Tukey's test)

on the spinal cord dorsal horn, but not on the gastrocnemius muscle. We also suggest an interaction of PKCε and P2X3 in this process.

The acute muscle pain does not raise major concerns, as its symptoms usually resolve quickly without therapeutic interventions or even with simple interventions, such as analgesic and anti-inflammatory drugs. On the other hand, the long-term persistence or recurrence of muscle pain after resolution of acute symptoms makes the chronic muscle pain a major health problem. Despite the little attention given to acute muscle pain, the inflammation that induces acute muscle pain may also induce plastic latent changes on muscle tissue and/or nociceptive system, which in turn enhances the susceptibility to the development of chronic muscle pain. The model of chronic-latent muscle hyperalgesia developed by Dr. Levine's group [8] and standardized for mice in the present study demonstrates that after the resolution of carrageenan-induced acute muscle hyperalgesia, the injection of PGE_2 at the same intramuscular site induces a markedly prolonged hyperalgesic response, confirming the relevance of an acute inflammatory environment to a priming state of the primary afferent fibers that enhances the susceptibility to chronic muscle pain.

We have recently demonstrated that P2X3 receptors expressed on gastrocnemius muscle and spinal cord dorsal horn seem to be essential to the development of the acute mechanical muscle hyperalgesia induced by static contraction of the gastrocnemius muscle [26]. It is important to point out that the long-term static contraction produces musculoskeletal pain by similar mechanisms to carrageenan, such as final inflammatory mediators and neutrophil migration [14, 27, 28]. In another study, we demonstrated that, in subcutaneous tissue, the development of carrageenan-induced acute mechanical hyperalgesia is also dependent on peripheral and spinal P2X3 receptors [24]. Now, we confirm that both peripheral and spinal P2X3 receptors contribute to acute mechanical muscle hyperalgesia induced by an inflammatory insult and demonstrated that only the spinal P2X3 receptors are involved in the development and maintenance of chronic-latent mechanical muscle hyperalgesia. This is because intrathecal blockade of P2X3 receptors previously to the inflammatory insult (carrageenan) prevented the development and maintenance of acute and chronic-latent mechanical muscle hyperalgesia, while blockade of the P2X3 receptors on gastrocnemius muscle previously to the inflammatory insult only

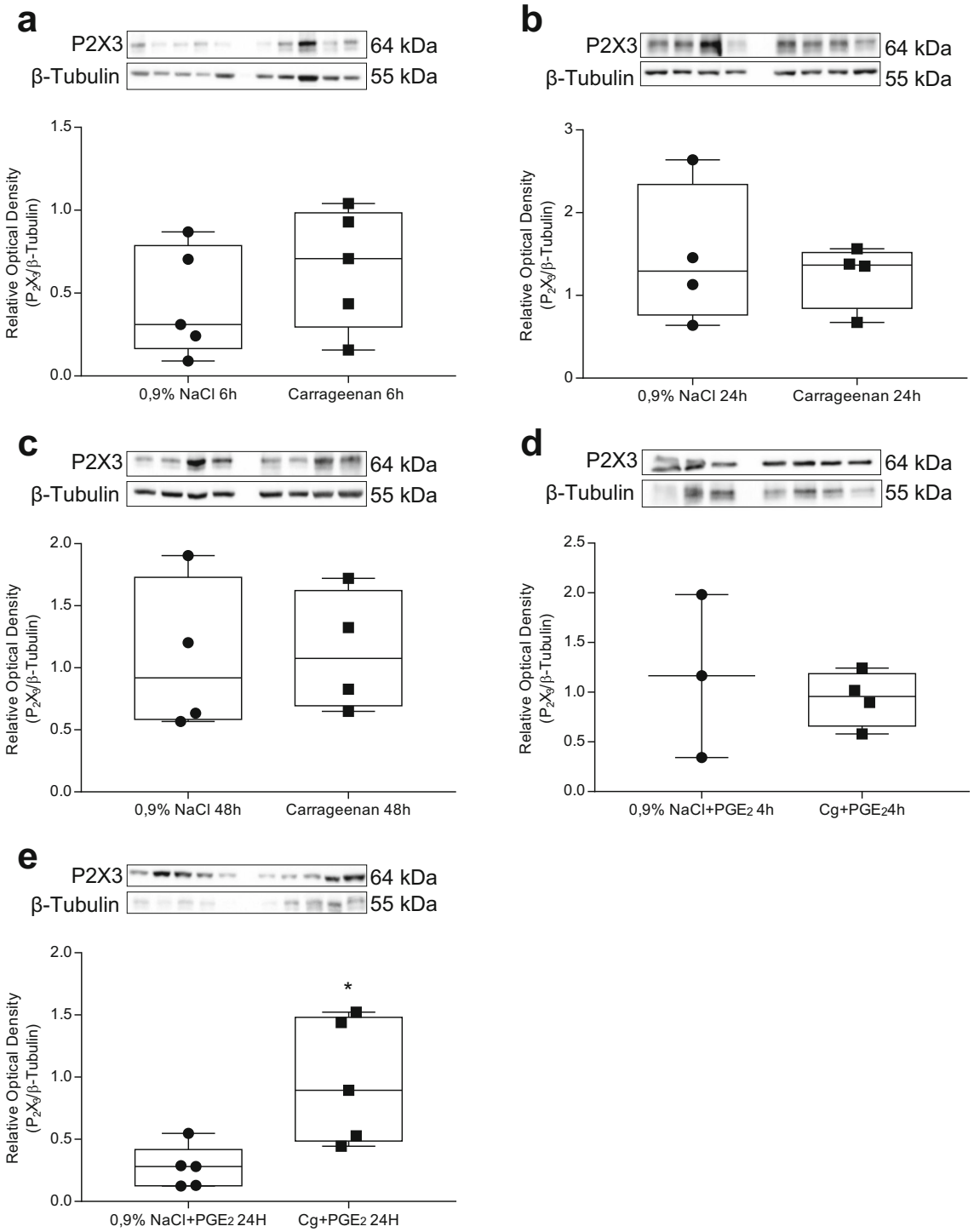

Fig. 4 P2X3 receptor expression in dorsal root ganglion (L4-S1) during acute and chronic-latent muscle hyperalgesia. No chances in P2X3 were observed 6 h (**a**, $n = 5$), 24 h (**b**, $n = 4$), and 48 h (**c**, $n = 4$) after Cg administration ($p > 0.05$, Student's t test) and after 4 h of PGE_2 administration in animals previously sensitized by Cg ($p > 0.05$, Student's t test, $n = 3$ for NaCl and $n = 4$ for Cg, **d**). An increase in P2X3 protein expression in dorsal root ganglion was found at 24 h after PGE_2 administration in animals previously sensitized by Cg ($p < 0.05$, Student's t test, $n = 5$, **e**)

reduced the acute mechanical muscle hyperalgesia and had no effect on chronic-latent mechanical muscle hyperalgesia. It is interesting to point out that the blockade of spinal, but not peripheral, P2X3 receptors immediately before PGE_2 in animals previously sensitized by carrageenan reversed the chronic-latent mechanical muscle hyperalgesia, suggesting that not only the spinal P2X3 receptors involved in acute muscle pain modulate the chronic-latent muscle pain but that

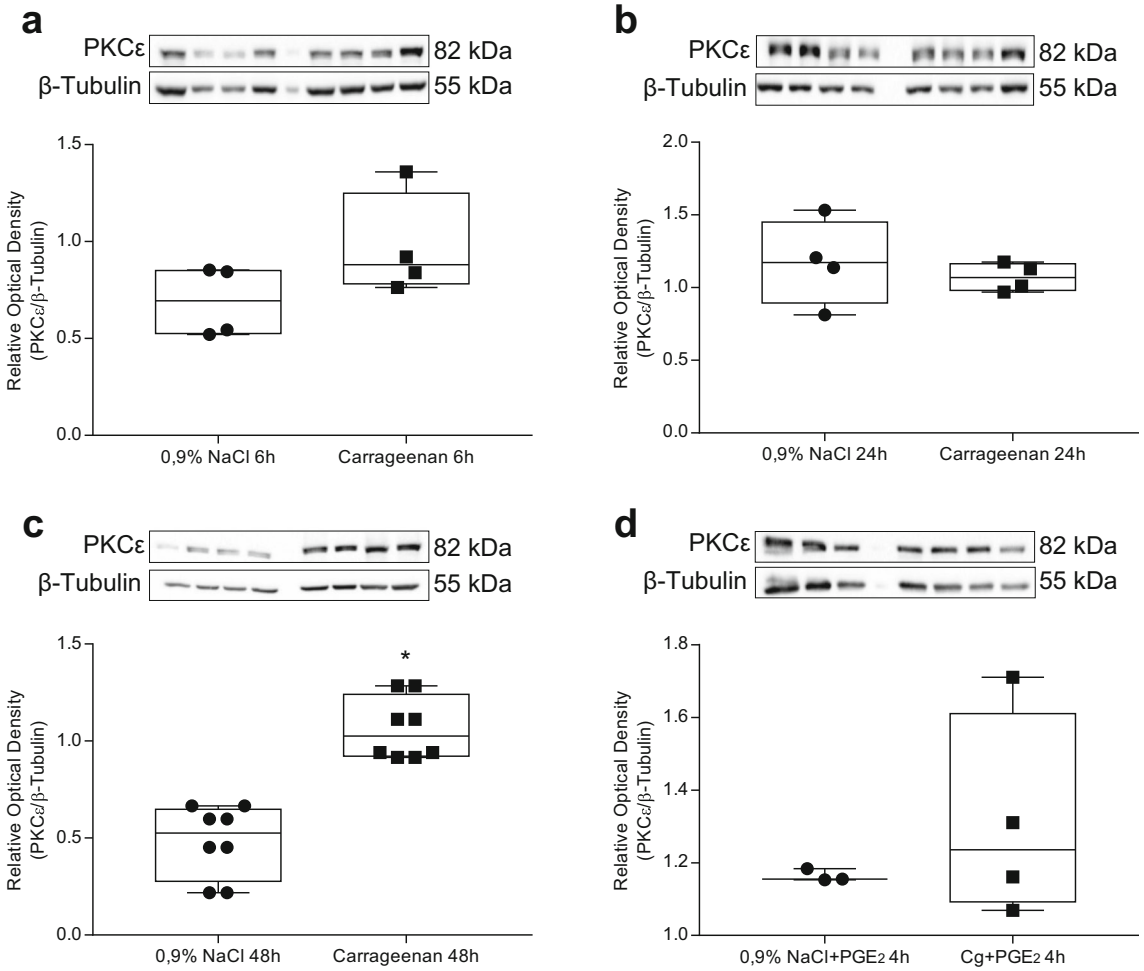

Fig. 5 Total PKCε protein expression in dorsal root ganglion (L4-S1) during acute and chronic-latent muscle hyperalgesia. No changes in total PKCε protein expression were found 6 h (**a**, $n = 4$) and 24 h (**b**, $n = 4$) after Cg administration and 4 h after PGE_2 administration (**d**, $n = 3$ for NaCl and $n = 4$ for Cg). There was an increase in total PKCε protein expression 48 h after Cg administration (**c**, $n = 4$). The asterisk "*" symbol indicates difference to 0.9% NaCl/PGE_2 ($p < 0.05$, Student's t test)

the second insult also activates spinal P2X3 receptors, which in turn contribute to the modulation of chronic muscle pain.

The mechanism underlying the transition from acute to chronic muscle pain by spinal P2X3 receptors is unknown; however, considering P2X3 are ionotropic receptors associated with Ca^{2+} and Na^{+} channels [29–33], it is possible to hypothesize that the inflammatory process triggered by carrageenan and by the second painful insult have induced a release of ATP in the spinal cord dorsal horn [34], which in turn activated spinal P2X3 receptors and sensitized central nociceptive pathways. Also, the P2X3 receptors expressed on central terminals of dorsal root ganglions terminating in the inner lamina II of the dorsal horn of the spinal cord can be particularly important to chronic muscle pain since their activation facilitates the release of excitatory neurotransmitters like glutamate and, consequently, the sensitization of central nociceptive pathways [31, 35].

It is widely known that the P2X3 receptors are predominantly expressed in key structures of the nociceptive pathway, such as C- and Aδ-primary afferent neurons and on the central projections of these primary sensory neurons within the dorsal horn of the spinal cord [36–38]. It was recently demonstrated that during an inflammation, PGE_2 increases the P2X3 receptor–mediated responses by activating the cAMP-Epac-PKC signaling pathway [16, 39]. It is important to note that chronic-latent muscle pain is modulated by PKCε [8–10] and by neuroplastic changes of the nociceptive signaling structures [11–13], including expression of CREB (cyclic adenosine monophosphate response element-binding proteins)-dependent genes for hyperalgesic priming [12]. Also, the increase in expression of P2X3 receptors seems to depend on the activation of PKCε [40] and the phosphorylation of CREB [41]. Therefore, our data that there was an increase in total and phosphorylated PKCε 48 h after the beginning of acute muscle hyperalgesia and in P2X3 receptors at the period of chronic muscle hyperalgesia, both in dorsal root ganglions, confirm the interaction of PKCε and P2X3 receptors in the transition

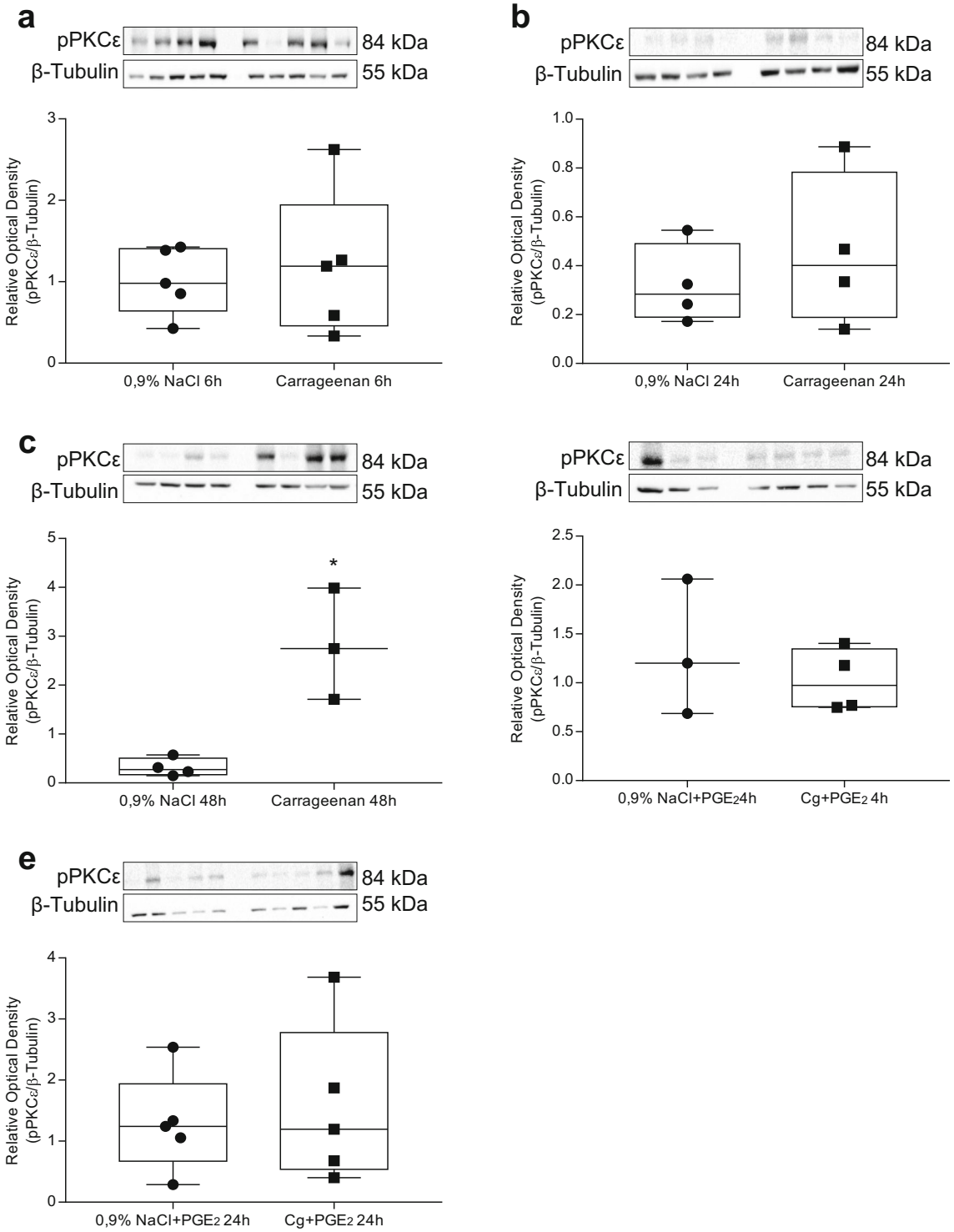

Fig. 6 Phosphorylated PKCε (S729) protein expression in dorsal root ganglion (L4-S1) during acute and chronic-latent muscle hyperalgesia. No changes in phosphorylated PKCε protein expression were found 6 h (**a**, $n = 5$) and 24 h (**b**, $n = 4$) after Cg administration and 4 h (**d**, $n = 3$ for NaCl and $n = 4$ for Cg) and 24 h (**e**, $n = 4$) after PGE_2 administration in animals previously sensitized by Cg ($p > 0.05$, Student's t test). There was an increase in phosphorylated PKCε protein expression 48 h after Cg administration (**c**, $n = 4$). The asterisk "*" symbol indicates difference to 0.9% NaCl/PGE_2 ($p < 0.05$, Student's t test). No changes in phosphorylated PKCε expression were found after 24 h of PGE_2 administration

from acute to chronic muscle pain and in the maintenance of the chronic muscle pain.

Although our data present evidences of the essential involvement of P2X3 receptors expressed on spinal cord dorsal

horn in the transition from acute to chronic muscle pain, we cannot exclude the involvement of other mechanisms, such as the downstream calcium signaling molecule α calmodulin–dependent protein kinase II [10] and the lower levels of G protein–coupled receptor kinase 2 (GRK2) in neurons during priming state [42].

Conclusion

It is well-known that P2X3 receptors play a crucial role in several types of pain in different tissues, like diabetic neuropathy [17], cancer pain [43], articular hyperalgesia [44, 45], visceral [46, 47], neuropathic, and inflammatory pain [24, 48, 49]. Clinically, one of the most relevant aspects of inflammatory pain is the development of chronic pain following acute inflammation, e.g., as produced in repetitive strain disorders. In the present study, we used a clinically relevant model of chronic-latent muscle pain and showed that P2X3 receptors expressed on spinal cord dorsal horn contribute to the transition from acute to chronic muscle pain. We also suggest an interaction of PKCε and P2X3 receptors in this process. Therefore, we point out the P2X3 receptors of the spinal cord dorsal horn as a pharmacological target to prevent the development and maintenance or to reverse the chronic muscle pain conditions.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval All the procedures followed the guidelines on using laboratory animals from IASP [1] and approved by the Committee on Animal Research of the State University of Campinas (license number 3883-1).

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