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Papel do receptor purinérgico P2X7 na gênese da dor inflamatória e os mecanismos periféricos envolvidos

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Mestre em Odontologia, Área de concentração em Fisiologia Oral

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"Tudo tem seu tempo e até certas manifestações mais vigorosas e originais entram em voga ou saem de moda. Mas a sabedoria tem uma vantagem: é eterna."

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RESUMO

Dados obtidos recentemente em nosso laboratório demonstram que a ativação dos receptores P2X3 e P2X2/3 pelo ATP endógeno é essencial para o desenvolvimento da hiperalgesia mecânica induzida pela carragenina no tecido subcutâneo da pata de ratos. Além dos receptores P2X3, dos sete subtipos de receptores P2X, os receptores P2X7 também possuem um papel importante nos processos de dor e inflamação. No entanto, o papel desses receptores na hiperalgesia mecânica induzida pela carragenina no tecido subcutâneo da pata de ratos e o mecanismo pelo qual a ativação dos receptores P2X7 contribui para essas respostas hiperalgésicas ainda não eram conhecidos. Portanto, os objetivos desse trabalho foram (1) Estudar se o ATP endógeno, via ativação de receptores P2X7, contribui para a hiperalgesia mecânica induzida pela carragenina e caracterizar em que período de tempo o ATP endógeno, via ativação de receptores P2X7, contribui com o desenvolvimento dessa resposta hiperalgésica e (2) Testar a hipótese de que o mecanismo pelo qual a ativação dos receptores P2X7 contribui para a hiperalgesia mecânica induzida pela carragenina é através da sensibilização indireta dos nociceptores aferentes primários. De acordo com o objetivo (1): A co-administração da carragenina com os antagonistas de receptor P2X7, oATP e A-438079, reduziu significativamente a hiperalgesia mecânica induzida pela carragenina e essa resposta hiperalgésica foi reduzida 1, 2, 3 e 6 horas após as administrações. De acordo com o objetivo (2): A co-administração dos antagonistas de receptor P2X7, oATP e A-438079, com a carragenina reduziu significativamente o aumento na concentração tecidual das citocinas pró-inflamatórias TNF-α, IL-6 e CINC-1 mas não de IL-1β induzida pela carragenina. Os resultados do primeiro objetivo demonstram que a ativação dos receptores P2X7 pelo ATP endógeno contribui para a hiperalgesia induzida pela carragenina e os resultados do segundo objetivo mostram que o mecanismo pelo qual os receptores P2X7 contribuem para a hiperalgesia mecânica induzida pela carragenina é através da sensibilização indireta dos nociceptores aferentes primários mediada pela liberação prévia de citocinas inflamatórias como TNF-α, IL-6 e CINC-1. Esses resultados sugerem que, como a ativação dos receptores P2X7 pelo ATP endógeno é fundamental para o desenvolvimento da hiperalgesia inflamatória, os receptores P2X7

podem ser alvos farmacológicos interessantes para o desenvolvimento de medicamentos usados no controle da dor inflamatória.

Palavras-chave: ATP, receptores purinérgicos P2X7, hiperalgesia mecânica, citocinas inflamatórias.

ABSTRACT

We have recently demonstrated that activation of P2X3 and P2X2/3 receptors by endogenous ATP is essential for the development of carrageenan-induced mechanical hyperalgesia in the subcutaneous tissue of the rat's hind paw. In addition to P2X3 receptors, among the seven subtypes of P2X receptors, P2X7 receptors also have an important role in pain and inflammation. However, the role of these receptors in carrageenan-induced mechanical hyperalgesia in the subcutaneous tissue of the rat's hind paw and the mechanism by which the activation of P2X7 receptors contributes to these hyperalgesic responses were not yet known. Therefore, the aim of this study were (1) To study whether endogenous ATP via activation of P2X7 receptors contributes to carrageenan-induced mechanical hyperalgesia and characterize the period of time in which endogenous ATP via activation of P2X7 receptors, contributes to the development of carrageenan-induced mechanical hyperalgesia and (2) To test the hypothesis that the mechanism by which activation of P2X7 receptors contributes to carrageenan-induced mechanical hyperalgesia is through an indirect sensitization of the primary afferent nociceptors. According to the first aim: Co-administration of carrageenan with the P2X7 receptor antagonists, oATP or A-438079, significantly reduced carrageenan-induced mechanical hyperalgesia. This hyperalgesic response was reduced 1, 2, 3 and 6 hours after administrations. According to the second aim: Co-administration of the P2X7 receptor antagonists, oATP or A-438079 with carrageenan significantly reduced the increase in tissue concentration of proinflammatory cytokines TNF-α, IL-6 and CINC-1, but not IL-1β induced by carrageenan. The results of the first aim demonstrate that activation of P2X7 receptors by endogenous ATP contributes to the hyperalgesia induced by carrageenan and the results of the second aim show that the mechanism by which P2X7 receptors contribute to carrageenan-induced mechanical hyperalgesia is through indirect sensitization of the primary afferent nociceptors mediated by previous release of inflammatory cytokines such as TNF-α, IL-6 and CINC-1. Because the activation of P2X7 receptors by endogenous ATP is essential for the development of inflammatory hyperalgesia, P2X7 receptors may be potential pharmacological targets for developing drugs to control inflammatory pain.

Keywords: ATP, P2X7 purinergic receptors, mechanical hyperalgesia, inflammatory cytokines.

SUMÁRIO

I – INTRODUÇÃO

A dor é um dos problemas mais sérios da nossa sociedade e a principal causa da procura pela assistência à saúde. Dentre os vários tipos de dor, a de origem inflamatória é a mais comum, o que tem motivado o desenvolvimento de estudos sobre os mecanismos envolvidos no desencadeamento da dor inflamatória, com a finalidade de descobrir novos alvos farmacológicos para servir de base no desenvolvimento de novos medicamentos. Dor pode ser definida como uma percepção desagradável associada à nocicepção. Essa definição envolve dois componentes: percepção e nocicepção. Percepção dolorosa é uma função integrativa, modulada por condições motivacionais, emocionais, psicológicas e pela história pregressa individual (Mersky, 1986). Nocicepção resulta da ativação de uma população específica de neurônios aferentes primários, que transmitem informação nociceptiva para o sistema nervoso central (Millan, 1999, Julius and Basbaum, 2001).

Após uma lesão tecidual, uma resposta inflamatória é gerada por macrófagos locais e amplificada por células sanguíneas migratórias, como os neutrófilos (van Furth et al., 1985, Laskin and Pendino, 1995). Tem sido demonstrado que, durante esse processo, ocorra a liberação de mediadores inflamatórios (Cunha et al., 1992, Ferreira et al., 1993), que estimulam a síntese das prostaglandinas e a liberação das aminas simpatomiméticas, que por sua vez irão sensibilizar os nociceptores aferentes primários (Gold et al., 1996, Rush and Waxman, 2004). Além desses mediadores inflamatórios, estudos recentes demonstram o importante papel do nucleotídeo adenosina 5'-trifosfato (ATP) como mediador da hiperalgesia inflamatória (Wu et al., 2004, McGaraughty et al., 2005, Oliveira et al., 2005, Wang et al., 2007, Oliveira et al., 2009, Teixeira et al., 2009 *in press*).

O modelo de inflamação induzido pela carragenina tem sido amplamente utilizado no estudo da hiperalgesia inflamatória em animais (Fulgenzi et al., 2005, Oliveira et al., 2005, Otuki et al., 2005, Oliveira et al., 2009, Teixeira et al., 2009 in press) uma vez que, semelhante a muitas condições inflamatórias em humanos, a carragenina induz uma resposta hiperalgésica que é significativamente reduzida por antiinflamatórios não esteroidais (Moncada et al., 1973, Ferreira et al., 1974). A carragenina induz hiperalgesia mediada pela liberação de dois mediadores inflamatórios finais, as prostaglandinas e as

aminas simpatomiméticas, que sensibilizam diretamente as fibras aferentes primárias (Gold et al., 1996, Rush and Waxman, 2004). A produção desses mediadores depende da liberação prévia de uma cascata de citocinas, que envolve inicialmente a formação da bradicinina, que por sua vez, induz à liberação da citocina pró-inflamatória TNFα (Ferreira et al., 1993). Esta citocina desencadeia a liberação de duas vias distintas de citocinas, uma mediada pelas IL-1β e IL-6 que estimulam a síntese da cicloxigenase-2 (COX-2), convertendo o ácido araquidônico em prostaglandinas e outra mediada pela IL-8 (em humanos) ou CINC-1 (em ratos) que estimula a produção das aminas simpatomiméticas (Cunha et al., 1991, Cunha et al., 1992, Lorenzetti et al., 2002).

Dados obtidos em nosso laboratório, demonstram a participação do ATP endógeno no desenvolvimento da hiperalgesia inflamatória induzida pela carragenina na articulação temporomandibular e no tecido subcutâneo da pata de ratos através da ativação de receptores purinérgicos (Oliveira et al., 2005, Oliveira et al., 2009, Teixeira et al., 2009 in press). Durante o processo de inflamação, o ATP é liberado para o meio extracelular e contribui com o desenvolvimento da hiperalgesia inflamatória, via ativação dos receptores purinérgicos P2X (Dell'Antonio et al., 2002a, Honore et al., 2002, Fulgenzi et al., 2005, Oliveira et al., 2005, McGaraughty et al., 2007, Fulgenzi et al., 2008, Oliveira et al., 2009, Teixeira et al., 2009 in press).

Os receptores purinérgicos P2X são canais ionotrópicos ativados pelo ATP, cuja ativação induz despolarização da membrana celular (Dubyak and el-Moatassim, 1993, Valera et al., 1994). Dentre os sete subtipos de receptores purinérgicos P2X (P2X1-P2X7) (Buell et al., 1996), o subtipo P2X7 tem sido muito estudado recentemente pelo seu envolvimento em processos de dor e hiperalgesia na pele (Dell'Antonio et al., 2002a, Dell'Antonio et al., 2002b, Chessell et al., 2005, Fulgenzi et al., 2005, McGaraughty et al., 2007), em vísceras (Fulgenzi et al., 2008) além de tecidos articulares como a ATM de ratos (Teixeira et al., 2009 in press). Os receptores P2X7 são seletivamente expressos em células de origem hematopoiéticas, incluindo mastócitos, linfócitos, eritrócitos, fibroblastos e macrófagos periféricos (Surprenant et al., 1996, Collo et al., 1997, Mancino et al., 2001). No SNC, receptores P2X7 funcionais estão localizados na micróglia, astrócitos e em células de Schwann (Collo et al., 1997, Sim et al., 2004) e nos gânglios das raízes dorsais de ratos,

esses receptores parecem ser seletivamente localizados em células da glia, mas não em neurônios (Zhang et al., 2005).

Estudos demonstraram que em animais "knockout" para receptores P2X7 não há desenvolvimento de resposta hiperalgésica após lesão neuropática (Chessell et al., 2005) ou após o tratamento com CFA (complete Freund's adjuvant) (Chessell et al., 2005, Fulgenzi et al., 2008). Além disso, antagonistas seletivos de receptor P2X7 reduzem a dor neuropática e visceral (Fulgenzi et al., 2008), alodinía mecânica induzida por lesão neuropática (Honore et al., 2006, McGaraughty et al., 2007), hiperalgesia térmica (Honore et al., 2006, Fulgenzi et al., 2008) e mecânica (Dell'Antonio et al., 2002a, Dell'Antonio et al., 2002b) induzidas pelo CFA além de reduzir a hiperalgesia térmica (Fulgenzi et al., 2005, Honore et al., 2006) induzida pela carragenina.

Ademais, tem sido descrito que os receptores P2X7 apresentam um importante papel no desenvolvimento dos processos inflamatórios por modularem a produção de mediadores inflamatórios (Lister et al., 2007). Recentes estudos demonstraram que durante a inflamação induzida por CFA ou LPS (lipopolissacarídeos), os receptores P2X7 contribuem com a liberação de citocinas pró-inflamatórias relacionadas à produção de mediadores inflamatórios finais (Ferrari et al., 1997, Hide et al., 2000, Solle et al., 2001, Colomar et al., 2003, Chessell et al., 2005, Gourine et al., 2005, Mingam et al., 2008) e que, durante a inflamação induzida pela carragenina, os receptores P2X7 contribuem com a liberação de quimiocinas pró-inflamatórias (MCP-1, IP-10 e IL-8) relacionadas à migração e ativação de células inflamatórias (Fulgenzi et al., 2005). Além disso, estudos in vitro têm demonstrado que, após a estimulação por LPS, a maturação e liberação da forma ativa da IL-1β depende da ativação dos receptores P2X7, pois a ativação desses receptores ativa a enzima caspase-1, que converte a pro-IL-1β (forma inativa) em IL-1β (forma ativa) e libera a IL-1β para o meio extracelular (Perregaux and Gabel, 1994, Sanz and Di Virgilio, 2000, Solle et al., 2001, Colomar et al., 2003, Kahlenberg and Dubyak, 2004).

Recentemente, demonstramos que a ativação dos receptores P2X3 e P2X2/3, pelo ATP endógeno, é essencial para o desenvolvimento da hiperalgesia inflamatória induzida pela carragenina no tecido subcutâneo da pata de ratos, através da sensibilização indireta dos nociceptores aferentes primários mediada pela liberação de citocinas e sensibilização

direta dos nociceptores aferentes primários (Oliveira et al., 2009). Porém o papel dos receptores P2X7 nesse processo ainda não havia sido elucidado.

Um recente estudo demonstrou que o antagonista de receptor P2X7, oATP, reduz significativamente a hiperalgesia térmica, a presença das quimiocinas e o infiltrado de macrófagos induzido pela administração de carragenina na pata de ratos (Fulgenzi et al., 2005). Embora esse estudo tenha demonstrado que os receptores P2X7 contribuem para o desenvolvimento da hiperalgesia térmica induzida pela carragenina, muitos estudos envolvendo diferentes agentes inflamatórios e receptores importantes na resposta hiperalgésica demonstraram que os mecanismos de desenvolvimento da hiperalgesia térmica e mecânica são distintos (Spraggins et al., 2001, Zheng and Chen, 2001, Hama et al., 2003, Huang et al., 2004, Kanai et al., 2007). Por exemplo, foi demonstrado que a administração subcutânea do antagonista seletivo de receptor P2X3 e P2X2/3 bloqueia a hiperalgesia mecânica induzida pela carragenina (Oliveira et al., 2009), enquanto que hiperalgesia térmica induzida pela carragenina não é afetada pelo mesmo antagonista (McGaraughty et al., 2003), sendo plausível considerar que os mecanismos envolvidos na contribuição dos receptores P2X7 na hiperalgesia térmica e mecânica induzida pela carragenina também sejam diferentes.

Tendo em vista a importância clínica da hiperalgesia mecânica em estados inflamatórios, não se sabia se a ativação de receptores P2X7 também contribuía para o desenvolvimento da hiperalgesia mecânica neste modelo, e, em caso afirmativo, quais os mecanismos envolvidos na contribuição dos receptores P2X7 para esta resposta hiperalgésica.

Portanto, o objetivo desse trabalho foi estudar o mecanismo pelo qual a ativação dos receptores P2X7, pelo ATP endógeno, contribui para a hiperalgesia mecânica induzida pela carragenina. Para isso, foi investigado se o ATP endógeno, via ativação de receptores P2X7, contribui para a hiperalgesia mecânica induzida pela carragenina e, em caso positivo, caracterizar em que período de tempo o ATP endógeno, via ativação de receptores P2X7, contribui com o desenvolvimento dessa resposta hiperalgésica, avaliando a habilidade dos antagonistas seletivos para receptores P2X7 de reduzirem a hiperalgesia mecânica induzida pela carragenina. Além disso, foi avaliado se a ativação dos receptores

P2X7 pelo ATP endógeno contribui para a hiperalgesia mecânica induzida pela carragenina através da sensibilização indireta dos nociceptores aferentes primários. Para testar essa hipótese, foi avaliado se os antagonistas seletivos de receptores P2X7 reduzem a liberação endógena das citocinas inflamatórias TNF-α, IL-1β, IL-6 e CINC-1 induzida pela carragenina.

II – PROPOSIÇÃO

O objetivo do presente trabalho foi investigar a participação dos receptores purinérgicos P2X7 na hiperalgesia inflamatória do tecido subcutâneo da pata de ratos e os mecanismos periféricos envolvidos.

O presente estudo está apresentado em formato alternativo, conforme deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 001/98

III – CAPÍTULO

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Peripheral mechanisms underlying the essential role of P2X7 receptors in the development of inflammatory hyperalgesia

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Abstract

Activation of P2X7 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia. Given the clinical importance of mechanical hyperalgesia in inflammatory states, we hypothesized that the activation of P2X7 receptors by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia, and that this contribution is mediated by an indirect sensitization of the primary afferent nociceptors. Co-administration of the selective P2X7 receptors antagonist, A-438079, or the P2X7 receptors antagonist, oATP, with carrageenan blocked the mechanical hyperalgesia induced by carrageenan and significantly reduced the increased concentration of TNF-α, IL-6 and CINC-1, but not of IL-1β induced by carrageenan in the subcutaneous tissue of the rat's hind paw. We concluded that activation of P2X7 receptors by endogenous ATP is essential to the development of the mechanical hyperalgesia induced by carrageenan in the subcutaneous tissue. Furthermore, we showed that this essential role of P2X7 receptors in the development of carrageenan-induced mechanical hyperalgesia is mediated by an indirect sensitization of the primary afferent nociceptors dependent on the previous release of TNF-α, IL-6 and CINC-1, but not of IL-1β.

Keywords: mechanical inflammatory hyperalgesia, P2X7 receptors, ATP, carrageenan, cytokines.

Introduction

P2X receptors are ligand-gated ionotropic channels activated by ATP inducing membrane depolarization and consequent cytosolic Ca^{2+} increase (Caporali et al., 2008). Recent reports show that the P2X7 receptors subunit are predominantly expressed in cells of the immune system (Surprenant et al., 1996, Chiozzi et al., 1997, Collo et al., 1997, Gu et al., 2000, Kim et al., 2001, Mancino et al., 2001, Sim et al., 2004, Zhang et al., 2005) that have an important role in the processes of pain and hyperalgesia (Dell'Antonio et al., 2002a, Dell'Antonio et al., 2002b, Fulgenzi et al., 2005, Donnelly-Roberts and Jarvis, 2007, Lister et al., 2007, McGaraughty et al., 2007, Fulgenzi et al., 2008, Honore et al., 2009).

Studies in P2X7 receptors knockout animals showed that there is no development of the hyperalgesic responses after neuropathic injury or treatment with complete Freund's adjuvant (CFA) (Chessell et al., 2005, Fulgenzi et al., 2005). Moreover, P2X7 receptor selective antagonists reduce neuropathic and visceral pain (Fulgenzi et al., 2008), mechanical allodynia induced by neuropathic injury (Honore et al., 2006, McGaraughty et al., 2007), CFA-induced thermal (Honore et al., 2006, Fulgenzi et al., 2008, Honore et al., 2009) and mechanical hyperalgesia (Dell'Antonio et al., 2002a, Dell'Antonio et al., 2002b) and carrageenan-induced thermal hyperalgesia (Fulgenzi et al., 2005, Honore et al., 2006). Furthermore, P2X7 receptors have an important role in the development of inflammatory processes induced by CFA or LPS, by modulating the secretion of cytokines by immune cells, particularly IL-1β, IL-18, TNF-α and IL-6, all of which play an important role in mediating inflammatory responses (Ferrari et al., 1997, Hide et al., 2000, Solle et al., 2001, Colomar et al., 2003, Chessell et al., 2005, Gourine et al., 2005, Mingam et al., 2008).

The subcutaneous administration of carrageenan has been widely used as a model of inflammatory hyperalgesia in animals (Fulgenzi et al., 2005, Oliveira et al., 2005, Otuki et al., 2005, Oliveira et al., 2009) because similarly to many inflammatory conditions in humans, it induces a hyperalgesic response that is reduced by nonsteroidal anti-inflammatory drugs (Moncada et al., 1973, Ferreira et al., 1974). Despite the clinical importance of mechanical hyperalgesia in inflammatory states, it is not known whether the

activation of P2X7 receptors also contributes to the development of mechanical hyperalgesia in this model, and, if so, which mechanisms underlie the contribution of P2X7 receptors to this hyperalgesic response.

Therefore, the aim of this study was to test the hypothesis that the activation of P2X7 receptors by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia, and, if so, whether cytokines are involved in this contribution.

Experimental procedures

Materials and Methods

Animals

Male Wistar rats (200-250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB) - University of Campinas, were used in this study. The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (±23°C). Experimental protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number: 1389-1) and conformed to IASP guidelines for the study of the pain in animals (Zimmermann, 1983). Animal suffering and the number of rats per group were kept at a minimum.

Drugs and doses

The follow drugs were used: λ-carrageenan (Cg; 30, 100, 300 and 600µg/paw, (Oliveira et al., 2009)); Adenosine 5′-triphosphate-2′,3′-dialdehyde: Oxidized ATP (oATP), an irreversible inhibitor of P2X7 receptor (Di Virgilio, 2003) (0.5, 2.0 and 6.0µg/paw, (Dell'Antonio et al., 2002b)); 3-((5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl)methyl pyridine: A-438079, an antagonist that selectively blocked only P2X7 receptors (Honore et al., 2006, McGaraughty et al., 2007) and was essentially devoid of activity at other P2 receptors (Nelson et al., 2006) (100 µg and 300µg/paw, (McGaraughty et al., 2007)). A-438079 was obtained from Tocris Bioscience (Ellisville, MO) and all other drugs were obtained from Sigma-Aldrich (MO, USA). All drugs were dissolved in sterile saline (0.9% NaCl).

Subcutaneous Injections

Drugs or their vehicle were locally administrated in the subcutaneous dorsal tissue of rat's hind paw by tenting the skin and puncturing it with a 30-gauge needle prior to injecting the test agent, as previously described (Oliveira et al., 2007). The needle was

connected to a catheter of polyethylene and also to a Hamilton syringe (50 µl). The animals were briefly restrained and the volume of injection was 50 µl.

Mechanical paw withdrawal nociceptive threshold test

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland, 1991). The Randall-Selitto nociceptive paw-withdrawal flexion reflex test (Randall and Selitto, 1957) was performed using an Ugo-Basile analgesymeter (Stoelting, Chicago, IL, USA), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw (Oliveira et al., 2007). The nociceptive threshold was defined as the force in grams, which the rat withdrew its paw. The baseline paw-withdrawal threshold was defined as the mean of three tests performed at 5-min intervals before test agents were injected. Mechanical hyperalgesia was quantified as the change in mechanical nociceptive threshold calculated by subtracting the mean of three mechanical nociceptive threshold measurements taken after injection of the test agent from the mean of the three baseline measurements.

ELISA procedure

An adaptation of ELISA (Safieh-Garabedian et al., 1995) was used to determine whether oATP or A-438079 was able to reduce carrageenan-induced release of TNF-a, IL-1 b, IL-6 and CINC-1. The subcutaneous tissues of dorsum of the rat's hind paw were collected 3-hours (Oliveira et al., 2009) after the subcutaneous administration of carrageenan or its vehicle (0.9% NaCl). These tissues were weighed and homogenized in the same weigh/volume proportion in a solution of phosphate-buffered saline (PBS) containing 0.4M NaCl, 0.05% Tween 20, 0.5% bovine serum albumine (BSA), 0.1mM phenyl-methylsulfonyl fluoride, 0.1mM benzotonic chloride, 10mM EDTA, and 20Kl/ml aprotinine (Sigma, USA). The samples were centrifuged at 10000rpm for 15min at 4ºC and the supernatants were stored at -70ºC for posterior use to evaluate the protein levels of TNF-α, IL-1β, IL-6 and CINC-1 in the subcutaneous tissue of rat's hind paw. The cytokines were quantified by the follows kits: TNF-α - Rat TNFalpha/ TNFSF1A Quantikine ELISA Kit (R&D Systems, catalog number RTA00); IL-1β - Rat IL-1 beta/IL-1F2 Quantikine

ELISA Kit (R&D Systems, catalog number RLB00), IL-6 - Rat IL-6 Quantikine ELISA Kit, 2nd Generation (R&D Systems, catalog number: R6000B) and CINC-1 - Rat CINC-1 Quantikine ELISA Kit (R&D Systems, catalog number RCN100). All procedures followed the instructions of the manufacturer R&D Systems. All procedures were repeated two times to guarantee the authenticity of the results.

Statistical analysis

To determine if there were significant differences ($p<0.05$) between treatment groups, One-way ANOVA or T-test was performed. If there was a significant between-subjects main effect of treatment group following One-way ANOVA, post hoc contrasts using the Tukey test were performed to determine the basis of the significant difference. For data shown in Fig. 2C a Two-way repeated measures ANOVA with one between subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) were used to determine whether there were significant ($p<0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. Data are expressed in figures by the decrease in paw-withdrawal threshold or the tissue concentration of cytokine and presented as means ± S.E.M.

Results

Carrageenan-induced mechanical hyperalgesia

The subcutaneous administration of carrageenan (100, 300 or 600µg/paw), but not 30µg/paw, induced a dose-related mechanical hyperalgesia measured 3 hours after the carrageenan administration (Fig. 1, $p<0.05$, ANOVA post hoc Tukey test). This response reached its maximum at the dose of 300µg/paw. Therefore, 300µg/paw of carrageenan was used in subsequent experiments. The baselines were not significantly different between groups ($p >0.05$, Tukey test).

Effect of P2X7 receptors antagonists on carrageenan-induced mechanical hiperalgesia

To verify whether endogenous ATP via activation of P2X7 receptors contributes to carrageenan-induced mechanical hiperalgesia, the non-selective P2X7 receptor antagonist oATP or the selective P2X7 receptors antagonist A-438079 was co-administered with carrageenan (300µg/paw) in the subcutaneous tissue of the rat's hind paw and the mechanical hyperalgesia was measured 3 hours after the carrageenan administration. oATP (0.5, 2.0 and 6.0µg/paw, Fig. 2A) and A-438079 (100 and 300µg/paw, Fig. 2B) significantly reduced carrageenan-induced mechanical hyperalgesia ($p<0.05$, one-way ANOVA post hoc Tukey test). The highest doses of these antagonists did not affect carrageenan-induced mechanical hyperalgesia when applied on the contralateral paw (Fig. 2A and 2B, $p>0.05$, ANOVA post hoc Tukey test), confirming their local peripheral action. Co-administration of oATP (6.0µg/paw) or A-438079 (300µg/paw,) with carrageenan (300µg/paw, Fig. 2C) blocked the hyperalgesic response 1, 2, 3 and 6 hours after the carrageenan administration ($p<0.05$, Two-way ANOVA, Bonferroni post test).

Co-administration of oATP (6.0µg/paw, Fig. 3A) or A-438079 (300µg/paw, Fig. 3B) with carrageenan (300µg/paw) ($p<0.05$, ANOVA post hoc Tukey test), but not their administration ½, 1, 2 and 3 hours after the carrageenan administration ($p>0.05$, ANOVA post hoc Tukey test) significantly reduced carrageenan-induced mechanical hyperalgesia.

Effect of P2X7 receptors antagonists on carrageenan-induced local increase in cytokines concentration

To verify whether endogenous ATP via activation of P2X7 receptors contributes to the release of pro-inflammatory cytokines induced by carrageenan, oATP (6.0μg/paw), A-438079 (300μg/paw) or 0.9% NaCl was co-administrated with carrageenan (300μg/paw) in the subcutaneous tissue of the rat's hind paw and the local concentrations of TNF-α, IL-1β, IL-6 and CINC-1 were quantified 3 hours after the administration of carrageenan. oATP and A-438079 significantly reduced the concentration of TNF-α (Fig. 4A), IL-6 (Fig. 4C) and CINC-1 (Fig. 4D) ($p<0.05$, One-way ANOVA post hoc Tukey test), but not the concentration of IL-1β (Fig. 4B) ($p>0.05$, One-way ANOVA post hoc Tukey test). The subcutaneous injection of 0.9% NaCl alone did not affect the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naïve rats ($p>0.05$, One-way ANOVA post hoc Tukey test).

Discussion

Role of P2X7 receptor in carrageenan-induced mechanical hiperalgesia

In this study we demonstrated that the irreversible inhibitor of P2X7 receptor oATP (Di Virgilio, 2003) or the selective P2X7 receptors antagonist A-438079 (Honore et al., 2006, McGaraughty et al., 2007) blocked carrageenan-induced mechanical hyperalgesia in the subcutaneous tissue of rat´s hind paw 1, 2, 3 and 6 hours after its administration and had no effect when applied on the contralateral hind paw. The contralateral administrations of P2X7 antagonists were performed to ensure that only local P2X7 receptors of the peripheral tissue were targeted. These findings strongly suggest that the activation of peripheral P2X7 receptors by endogenous ATP not only contributes but it is essential to the development of the mechanical hyperalgesia induced by carrageenan in the subcutaneous tissue.

Many studies involving different inflammatory agents and receptors important to the hyperalgesic response have shown that the mechanisms involved in the development of thermal and mechanical hyperalgesia differ from each other (Spraggins et al., 2001, Zheng and Chen, 2001, Hama et al., 2003, Huang et al., 2004, Kanai et al., 2007). For example, the subcutaneous administration of selective P2X3 and P2X2/3 receptor antagonist A-317491 block the mechanical (Oliveira et al., 2009) but not the thermal hyperalgesia induced by carrageenan (McGaraughty et al., 2003). However, P2X7 receptors contribute to both the mechanical (current findings) and thermal hyperalgesia (Fulgenzi et al., 2005, Honore et al., 2006).

The data of the present study demonstrated that peripheral P2X7 receptors seem to be essential to the development, but not to the maintenance of the hyperalgesic response. This is because oATP and A-438079 blocked the carrageenan-induced mechanical hyperalgesia when they were co-administered with carrageenan, but not when they were administered ½, 1, 2 or 3 hours after the carrageenan administration.

It is well known that carrageenan induces hyperalgesia by two distinct pathways that ultimately result in the local release of prostaglandins and sympathetic amines (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993). These inflammatory

mediators directly sensitize the primary afferent nociceptor (Gold et al., 1996, Rush and Waxman, 2004). Therefore, the blockade of the carrageenan-induced mechanical hyperalgesia by the P2X7 receptors antagonists suggests that the activation of peripheral P2X7 receptors must be crucial to prostaglandin and sympathetic amines-mediated sensitization of the primary afferent nociceptor.

Release of cytokines

It has been proposed that carrageenan induces the release of pro-inflammatory cytokines in a cascade manner (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993). Bradykinin induces the release of TNF-α, which in turn triggers the release of IL-1β, IL-6 and CINC-1 that ultimately induce the synthesis of the final inflammatory mediators prostaglandins and sympathetic amines, respectively (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993). The data in this study showed that co-administration of the P2X7 receptors antagonist oATP or A-438079 with carrageenan significantly reduced the release of TNF-α, IL-6 and CINC-1 induced by carrageenan in the subcutaneous tissue of the rat's hind paw. The importance of TNF-α on the development of mechanical hyperalgesia was previously demonstrated by the ability of thalidomide or polyclonal rat TNF-α antibody to block carrageenan-induced hyperalgesia (Parada et al., 2003). Taken together, these findings indicate that the essential role of P2X7 receptors in the development of the carrageenan-induced mechanical hyperalgesia is mediated, at least in part, by the release of cytokines, in particular the TNF-α. These results may suggest that the P2X7 receptors have a broad role in the activation and potentiation of the inflammatory processes modulating the release of proinflammatory cytokines into the subcutaneous tissue. This is consistent with the evidence that the P2X7 receptors are predominantly found in macrophages and other cells of immunological origin (Surprenant et al., 1996, Chiozzi et al., 1997, Collo et al., 1997, Gu et al., 2000, Kim et al., 2001, Mancino et al., 2001, Sim et al., 2004, Zhang et al., 2005), where they can trigger a series of cellular responses such as membrane permeabilization, activation of caspases, cytokine release, cell proliferation, and apoptosis, demonstrating a role for this P2X7 receptor in inflammatory diseases (North, 2002, Baraldi et al., 2003).

Although oATP and A-438079 have significantly reduced the TNF-α release, they did not block it. This finding suggests that the release of TNF-α induced by carrageenan may be mediated by distinct pathways and that only one of them depends on P2X7 activation. Recently, our group demonstrated that similarly to P2X7 receptors antagonists, the P2X3 and P2X2/3 receptors antagonist A-317491 also reduces the TNF-α release without block it (Oliveira et al., 2009), which indicates that in addition to P2X7, P2X3 and P2X2/3 receptors activated by endogenous ATP, are also involved in the release of TNF-α.

It has been described that P2X7 receptors mediate the release de IL-1β, TNF-α and IL-6 (Ferrari et al., 1997, Hide et al., 2000, Solle et al., 2001, Gourine et al., 2005), so the effect of oATP and A-438079 in the carrageenan-induced release TNF-α and IL-6 in the subcutaneous tissue of the rat's hind paw is consistent with previous reports that P2X7 receptor plays a pivotal role in cytokine release (Gourine et al., 2005).

In vitro studies have reported that the P2X7 receptor has an important role in the inflammatory response, because the activation of P2X7 receptors triggers the activation of the enzyme caspase-1, conversion of pro- IL-1β in IL-1β and release of IL-1β in response to LPS stimulation models (Ferrari et al., 1996, Ferrari et al., 1997, Sanz and Di Virgilio, 2000, Solle et al., 2001, Colomar et al., 2003, Kahlenberg and Dubyak, 2004). Moreover, a recent study *in vivo* showed that in IL-1αβ knockout mice, the antinociceptive effects of the blockade of the P2X7 receptor in CFA-induced thermal hyperalgesia are mediated by modulation of IL-1β activity (Honore et al., 2009). This is in contrast to our findings that the administration of the P2X7 receptor antagonists did not reduce the release of IL-1β induced by carrageenan in the subcutaneous tissue of the rat's hind paw, which suggest that under this condition, the P2X7 receptors do not contribute to the release of IL-1β. The difference between the previous and the current findings may be due to the different inflammatory agents used.

The findings that the co-administration of oATP or A-438079 with carrageenan did not alter the release of IL-1β, suggest that the release of IL-1β may not depend on the presence of TNF-α, as previously suggested (Cunha et al., 1992, Lorenzetti et al., 2002). Importantly, the suggestion that the release of IL-1β depends on the presence of TNF-α was

not based on the quantification of cytokines but rather on the findings that the hyperalgesia induced by the injection of carrageenan, but not IL-1β, is prevented by the administration of antibody anti-TNF-α (Cunha et al., 1992). Indeed, consistent with the idea that the release of IL-1β does not depend on the presence of TNF-α, it has been demonstrated that the concentration of IL-1β but not of TNF-α is increased after the injection of carrageenan in the gastrocnemius muscle (Loram et al., 2007). Recently our group demonstrated that similarly to P2X7 receptors antagonists, the P2X3 and P2X2/3 receptors antagonist A-317491 do not alter the release of IL-1β (Oliveira et al., 2009), which supports the idea that IL-1β is not entirely dependent on TNF-α to be released. However, we can not exclude the possibility that the residual concentration of TNF-α observed after the co-administration of oATP or A-438079 with carrageenan could be enough to keep the concentration of IL1-β elevated.

Our previous findings (Oliveira et al., 2009), taken together with our current findings suggest that both P2X7 and P2X3 and P2X2/3 are essential and may act together in the development of mechanical hyperalgesia induced by carrageenan in the subcutaneous tissue. This suggestion is further supported by the recent finding that the expression and release of proinflammatory mediators induced by P2X1 and P2X3 receptors activation were lower in mast cells P2X7-deficient as compared with wild-type mast cells (Bulanova et al., 2009).

In summary, we conclude that activation of P2X7 receptors by endogenous ATP is essential to the development of the mechanical hyperalgesia induced by carrageenan. Furthermore, we showed that this essential role of P2X7 receptors in the development of the carrageenan-induced mechanical hyperalgesia is mediated by an indirect sensitization of the primary afferent nociceptors dependent on the previous release of TNF-α, but not IL-1β. Finally, the finding that blockade of P2X7 receptors prevented the development of inflammatory hyperalgesia suggests that P2X7 receptor is clearly involved in preclinical models of pain and inflammation and that selective antagonists for the P2X7 receptors may be potential targets for the development of new analgesic drugs to control inflammatory pain.

Figures and legends

Figure 1

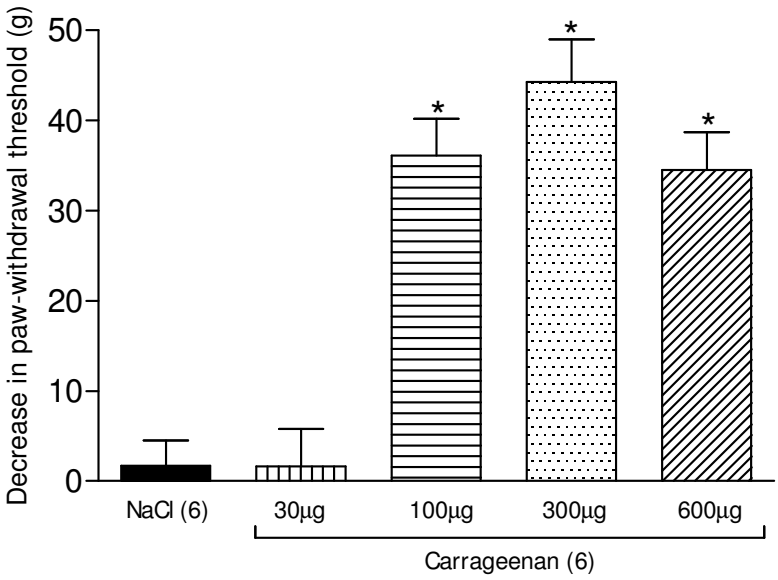
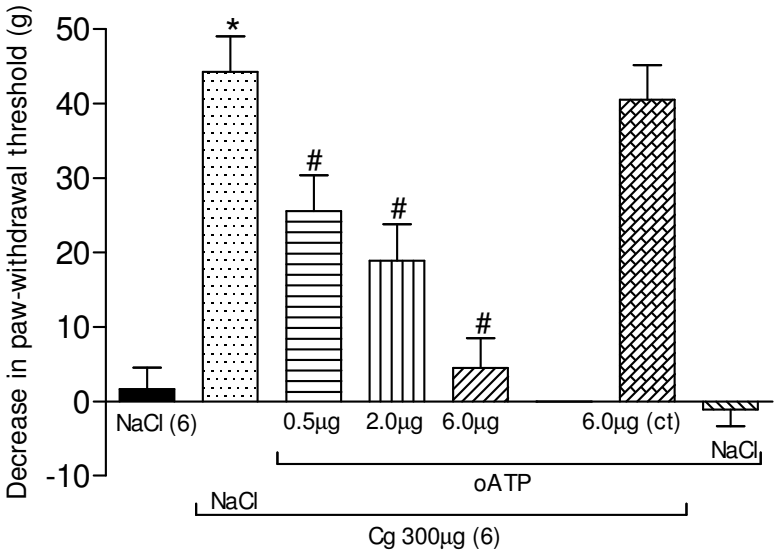


Fig. 1 - Carrageenan induced mechanical hyperalgesia the subcutaneous tissue of the rat's hind paw

Carrageenan (100, 300 or 600µg/paw) induced a dose-related mechanical hyperalgesia. In this and in the subsequent figures the mechanical hyperalgesia was measured 3 hours after the carrageenan administration and the number of rats used is between parentheses. The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test).

Figure 2

A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50
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30
20
10
0
-10
*
#
#
#
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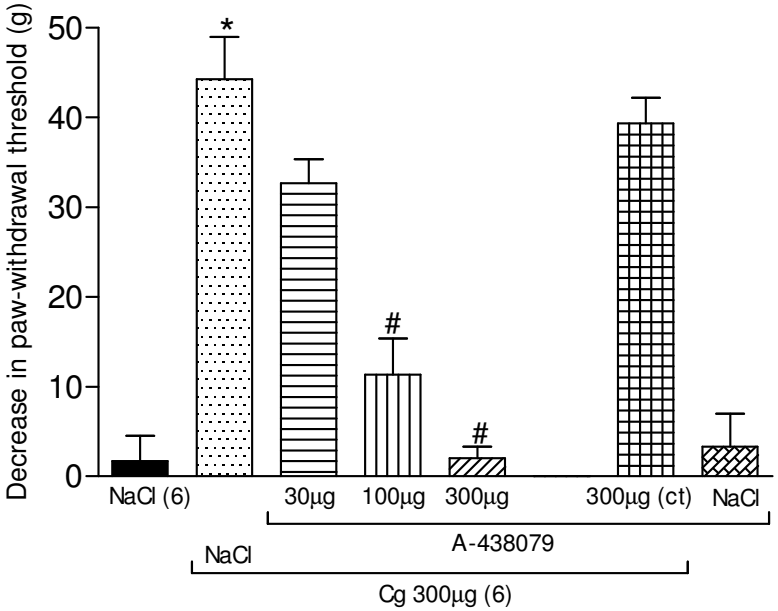
2.0µg
6.0µg
6.0µg (ct)
NaCl
oATP
NaCl
Cg 300µg (6)

B


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
#
#
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30µg
100µg
300µg
300µg (ct)
NaCl
A-438079
NaCl
Cg 300µg (6)

C

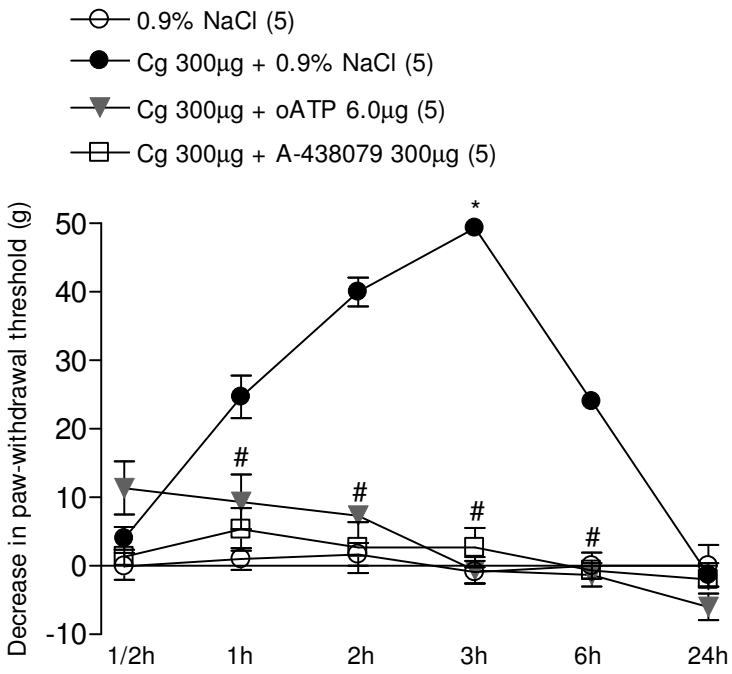


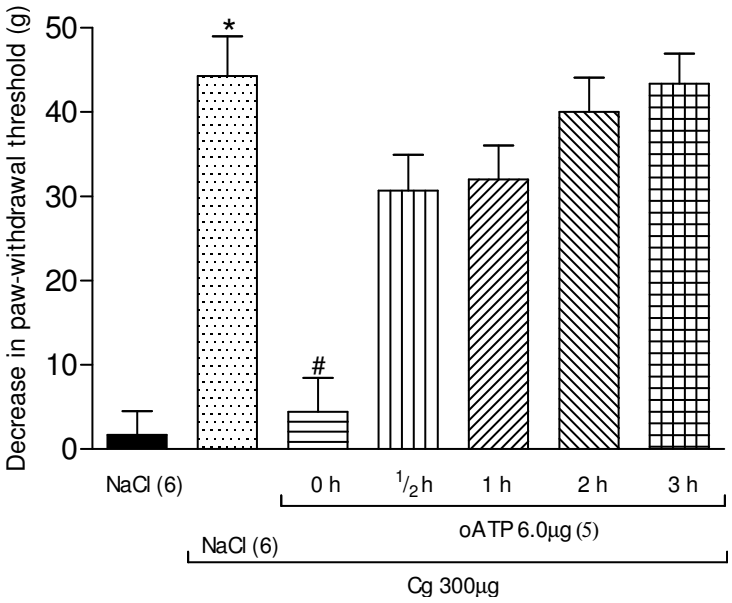
Fig. 2 - Effect of the co-administration of P2X7 receptors antagonists with carrageenan on carrageenan-induced mechanical hyperalgesia

Co-administration of the P2X7 receptors antagonist oATP (0.5, 2.0 and 6.0µg/paw, **A**) or A-438079 (100 and 300µg/paw, **B**) with carrageenan (Cg, 300µg/paw) significantly reduced the carrageenan-induced mechanical hyperalgesia, as indicated by the symbol "#" ($p<0.05$, Tukey test). The highest doses of the antagonists applied on the contralateral paw (ct) did not affect the carrageenan-induced mechanical hyperalgesia ($p>0.05$, Tukey test). oATP (6.0µg/paw, **A**) or A-438079 (300µg/paw, **B**) applied with 0.9% NaCl did not induced mechanical hiperalgesia by itself ($p<0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test).

The temporal analysis of effect of P2X7 receptors antagonists on carrageenan-induced mechanical hyperalgesia showed that they blocked the hyperalgesic response 1, 2, 3 and 6 hours after the carrageenan administration, as indicated by the symbol "#" ($p<0.05$, Bonferroni post test, **C**). The symbol "*" indicates the peak of the hyperalgesic response induced by carrageenan ($p<0.05$, Bonferroni post test).

Figure 3

A


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
#
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1/2 h
1 h
2 h
3 h
oATP 6.0µg (5)
NaCl (6)
Cg 300µg

B

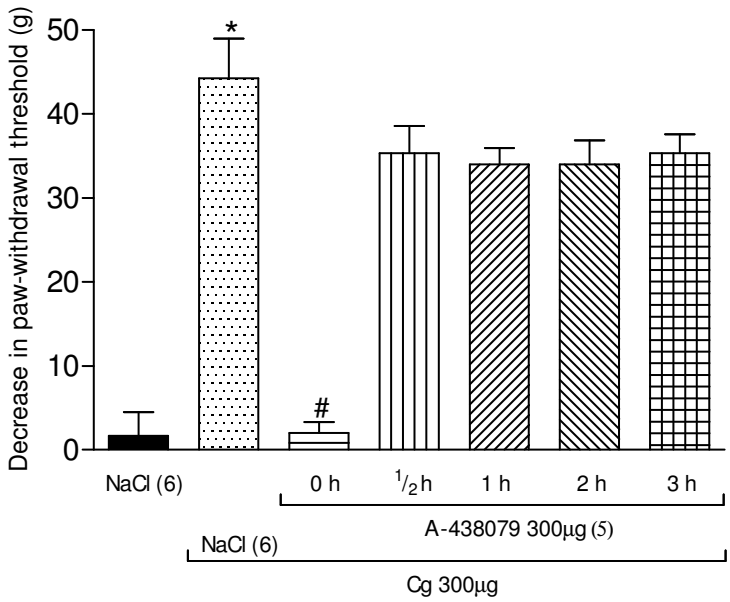
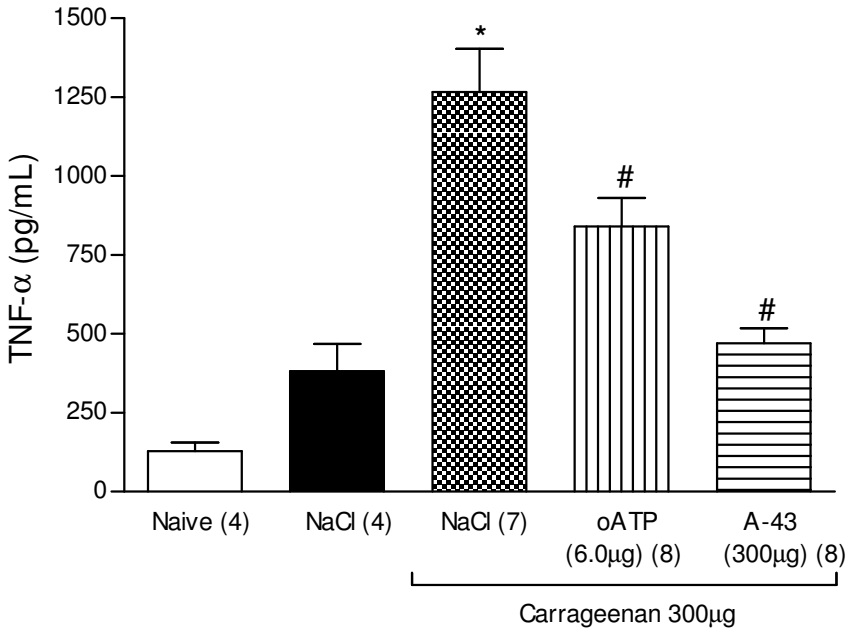

Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
#
NaCl (6)
0 h
1/2 h
1 h
2 h
3 h
A-438079 300µg (5)
NaCl (6)
Cg 300µg

Fig. 3 - Effect of P2X7 receptors antagonists co-administered or administrated ½, 1, 2, and 3 hours after the carrageenan administration on carrageenan-induced mechanical hyperalgesia

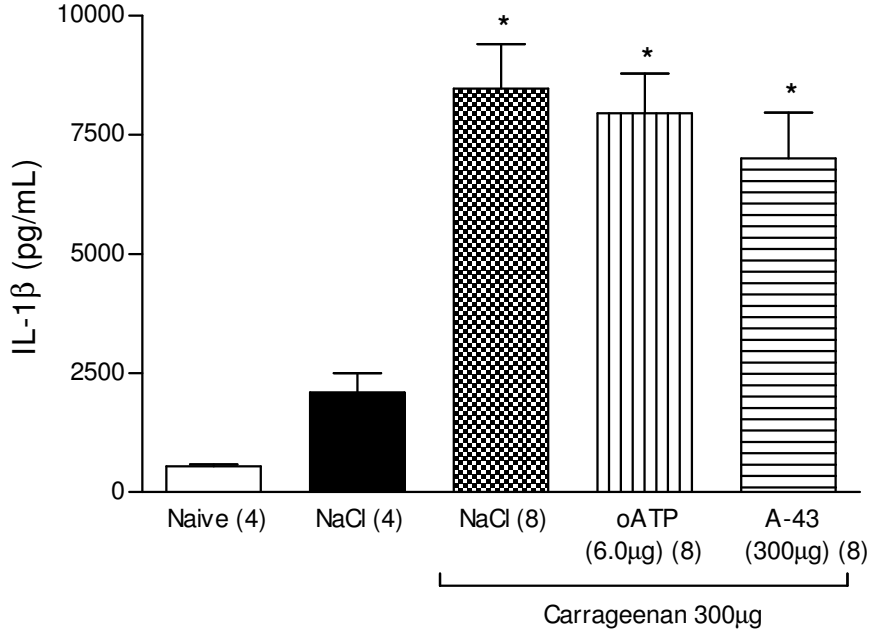
Co-administration (0 h) of oATP (6.0µg/paw, **A**) or A-438079 (300µg/paw, **B**) with carrageenan (Cg, 300µg/paw), but not their administration ½, 1, 2 and 3 hours after the carrageenan administration ($p>0.05$, ANOVA post hoc Tukey test) significantly reduced carrageenan-induced mechanical hyperalgesia, as indicated by the symbol "#" ($p<0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test).

Figure 4

A



B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7500
5000
2500
0
IL-1β (pg/mL)
*
*
*
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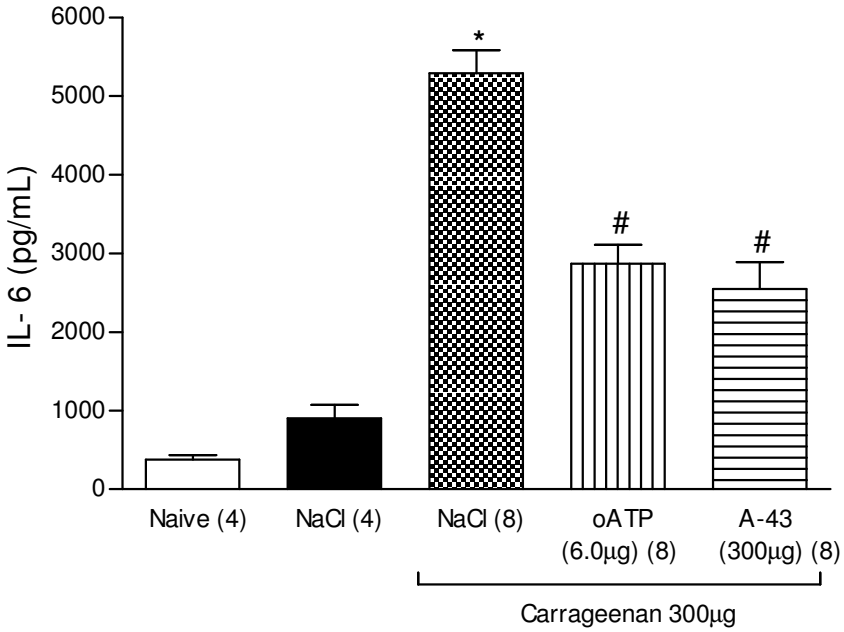
NaCl (4)
NaCl (8)
oATP
(6.0µg) (8)
A-43
(300µg) (8)
Carrageenan 300µg

C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5000
4000
3000
2000
1000
0
IL- 6 (pg/mL)
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Naive (4)
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oATP
(6.0µg) (8)
A-43
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Carrageenan 300µg

D

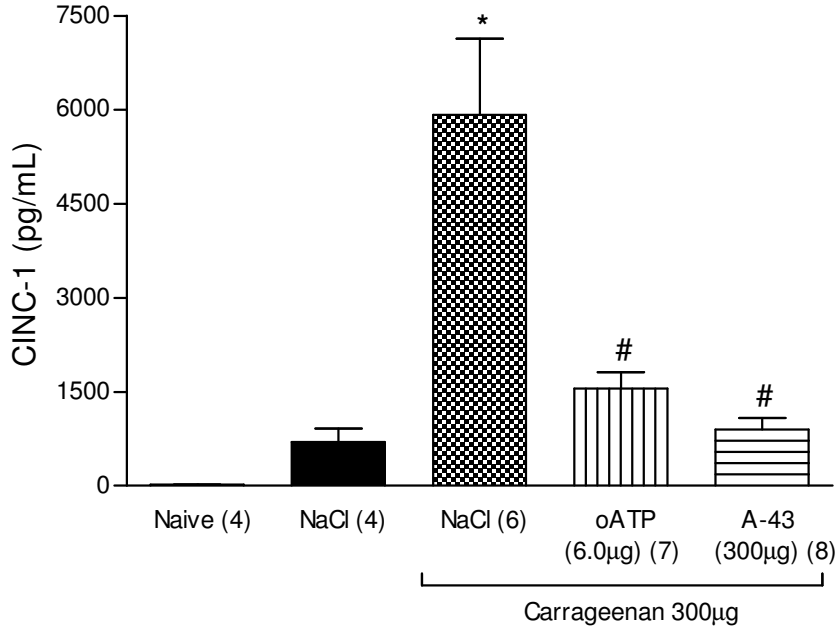

Fig. 4 - Effect of P2X7 receptors antagonists on carrageenan-induced cytokines release

Co-administration of oATP (6.0µg/paw) or A-438079 (300µg/paw) significantly reduced the concentration of TNF-α (Fig. **A**), IL-6 (Fig. **C**) and CINC-1 (Fig. **D**), as indicated by the symbol "#" ($p<0.05$, Tukey test), but did not reduce the concentration of IL-1β (Fig. **B**) ($p>0.05$, Tukey test). The subcutaneous injection of 0.9% NaCl alone did not affect the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive rats ($p>0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl and by naive rats ($p<0.05$, Tukey test).

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IV – CONCLUSÕES

O presente trabalho demonstrou que o ATP endógeno via ativação dos receptores P2X7 contribui para a hiperalgesia mecânica induzida pela carragenina através de uma sensibilização indireta dos nociceptores aferentes primários, mediada pela liberação prévia das citocinas pró-inflamatórias TNF-α, IL-6 e CINC-1. Os resultados obtidos sugerem que, como a ativação dos receptores P2X7 pelo ATP endógeno é fundamental para o desenvolvimento da hiperalgesia inflamatória, os antagonistas seletivos de receptores P2X7 podem ser alvos farmacológicos interessantes para o desenvolvimento de medicamentos usados no controle da dor inflamatória.

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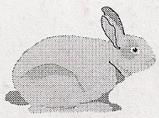

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C E R T I F I C A D O

Certificamos que o Protocolo nº **1389-1**, sobre **"Papel do receptor purinérgico P2X7 na gênese da dor inflamatória"**, sob a responsabilidade de **Profa. Dra. Claudia Herrera Tambeli / Juliana Maia Teixeira**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **12 de dezembro de 2007.**

C E R T I F I C A T E

We certify that the protocol nº **1389-1**, entitled **"Role of purinergic P2X7 receptor in genesis of inflammatory pain"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **december 12, 2007**

Campinas, 12 de dezembro de 2007.

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Piracicaba, 08 de Março de 2010.

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