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MECANISMOS ENVOLVIDOS NA AÇÃO HIPERALGÉSICA DO ATP EM RATOS

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas para a obtenção do título de Doutor em Odontologia – área de concentração em Fisiologia Oral.

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“O fim é belo e incerto... depende de como você vê
o novo, o credo, a fé que você deposita em você e só”.

(Fernando Anitelli)

RESUMO

Recentes estudos demonstram que a ativação de receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia inflamatória. Portanto, os objetivos desse trabalho foram: (1) Estudar o mecanismo pelo qual a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia mecânica induzida no modelo da inflamação causada pela carragenina, (2) Verificar se o ATP endógeno e a ativação dos receptores P2X3,2/3 contribui para a hiperalgesia induzida pelos mediadores inflamatórios Bradicinina, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 e dopamina e, verificar o mecanismo pelo qual essa contribuição ocorre, e (3) Estudar o mecanismo pelo qual a ativação dos receptores P2X1,3,2/3 induz hiperalgesia mecânica. De acordo com o objetivo (1): A co-administração do antagonista seletivo de receptor P2X3,2/3, A-317491, ou do antagonista de receptor P2X1,3,2/3,1/5, TNP-ATP, com carragenina bloqueou a hiperalgesia mecânica, reduziu significativamente o aumento na concentração de TNF-α e CINC-1 mas não de IL-1β, e reduziu levemente a migração de neutrófilos induzida pela carragenina. Administração intratecal de oligonucleotídeos antisense contra receptor P2X3 reduziu significativamente a expressão de receptores P2X3 no nervo safeno e a hiperalgesia mecânica induzida pela carragenina. De acordo com o objetivo (2): A co-administração de A-317491 ou TNP-ATP com bradicinina, mas não com TNF-α, IL-1β, IL-6, CINC-1, PGE_2 ou dopamina, preveniu de forma dose-dependente a hiperalgesia mecânica. TNP-ATP ou A-317491 não afetou a migração de neutrófilos nem a liberação das citocinas TNF-α, IL-1β, IL-6 e CINC-1 induzidas pela bradicinina. De acordo com o objetivo (3): A administração subcutânea do agonista de receptor P2X1,3,2/3, α,β-meATP, induziu hiperalgesia mecânica dose-dependente, que foi significativamente reduzida pelo A-317491, pelo inibidor de cicloxigenase, indometacina, e pelos antagonistas β_1 ou β_2-adrenérgicos, atenolol e ICI 118,551 respectivamente, pelo antagonista seletivo de receptor de bradicinina B1 ou B2, DALBK e Bradyzide respectivamente, e pelo

inibidor não seletivo das selectinas, fucoidan. Além disso, α,β-meATP induziu liberação endógena das citocinas TNF-α, IL-1β, IL-6 e CINC-1 e migração de neutrófilos. Os resultados do primeiro objetivo demonstram que a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia induzida pela carragenina através da sensibilização indireta dos nociceptores aferentes primários mediada pela liberação prévia de TNF-α, e através da sensibilização direta dos nociceptores aferentes primários. Os resultados do segundo objetivo demonstram que a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia induzida pela bradicinina por um mecanismo que independe da liberação de citocinas e migração de neutrófilos. Finalmente, os resultados do terceiro objetivo demonstram que o α,β-meATP induz hiperalgesia mecânica através de uma sensibilização indireta dos nociceptores aferentes primários mediada pela síntese de prostaglandinas, liberação de aminas simpatomiméticas, liberação de citocinas e migração de neutrófilos. Em uma perspectiva clínica-terapêutica, esses resultados sugerem que, como a ativação dos receptores P2X3,2/3 pelo ATP endógeno é fundamental para o desenvolvimento da hiperalgesia inflamatória, os receptores P2X3,2/3 podem ser alvos farmacológicos interessantes para o desenvolvimento de medicamentos usados no controle da dor inflamatória. Ressalta-se ainda que o efeito analgésico dos antagonistas de receptores P2X3,2/3 inibiu a hiperalgesia em uma magnitude comparável à dos antiinflamatórios esteroidais.

Palavras-chave: ATP, receptores purinérgicos, hiperalgesia, neutrófilos, mediadores inflamatórios, antisense.

ABSTRACT

Activation of P2X3,2/3 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia. Therefore, the aims of this study were: (1) To study the mechanism underlying activation of P2X3,2/3 receptors by endogenous ATP contributes to mechanical hyperalgesia induced by the carrageenan model of inflammation, (2) To verify whether endogenous ATP and P2X3,2/3 receptors activation contributes to the hyperalgesia induced by inflammatory mediators bradykinin, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 and dopamine and, to verify the mechanism underlying this contribution occurs, (3) To study the mechanism underlying activation of P2X1,3,2/3 receptors induces mechanical hyperalgesia. According to the first aim: Co-administration of the selective P2X3,2/3 receptors antagonist, A-317491, or the P2X1,3,2/3,1/5 receptors antagonist, TNP-ATP, with carrageenan blocked the mechanical hyperalgesia, significantly reduced the increased concentration of TNF-α and CINC-1 but not of IL-1β, and reduced only slightly neutrophil migration induced by carrageenan. Intrathecal administration of oligonucleotides antisense against P2X3 receptors significantly reduced the expression of P2X3 receptors in the saphenous nerve and significantly reduced the mechanical hyperalgesia induced by carrageenan. According to the second aim: Co-administration of A-317491 or TNP-ATP with bradykinin, but not with TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine, prevented mechanical hyperalgesia in a dose-response manner. TNP-ATP or A-317491 did not affect either neutrophil migration or the release of TNF-α, IL-1β, IL-6 and CINC-1 induced by bradykinin. According to the third aim: Subcutaneous injection of the P2X1,3,2/3 receptors agonist, α,β-meATP, induced a dose-dependent mechanical hyperalgesia, which was significantly reduced by local injection of A-317491, the cyclo-oxygenase inhibitor, indomethacin, the β_1-adrenoceptor antagonist atenolol or the β_2- adrenoceptor antagonist ICI 118,551, the selective B1-receptor antagonist DALBK or B2-receptor antagonist bradyzide, and the nonspecific selectin inhibitor fucoidan. Also, α,β-meATP induced the

release of the cytokines TNF-α, IL-1β, IL-6 and CINC-1 and neutrophils migration. The results of the first aim demonstrate that P2X3,2/3 receptors activation by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia by an indirect sensitization of the primary afferent nociceptors dependent on the previous release of TNF-α and by a direct sensitization of the primary afferent nociceptors. The results of the second aim demonstrate that endogenous ATP via activation of P2X3,2/3 receptors mediated bradykinin-induced mechanical hyperalgesia by a mechanism that was not dependent on neutrophil migration or release of cytokines. Finally, the results of the third aim demonstrate that, α,β-meATP induces mechanical hyperalgesia by an indirect action on the primary afferent nociceptor of the subcutaneous tissue of rat's hind paw mediated by release of bradykinin, prostaglandin, sympathomimetic amines, pro-inflammatory cytokines and by neutrophil migration. In a clinical and therapeutic perspective, these results suggest that, considering that activation of P2X3,2/3 receptors by endogenous ATP is essential to the development of inflammatory hyperalgesia, these P2X3,2/3 receptors may be potential targets for the development of new drugs to control inflammatory pain. Also, it is important to point out that the analgesic effect of P2X3,2/3 receptors antagonists inhibited the hyperalgesia in a magnitude comparable to steroidal anti-inflammatory drugs

Key words: ATP, purinergic receptors, hyperalgesia, neutrophils, inflammatory mediators, antisense.

SUMÁRIO

1. INTRODUÇÃO

A dor é um dos problemas mais sérios da nossa sociedade e a principal causa da procura pela assistência à saúde. Gera altos custos aos cofres públicos, uma vez que milhares de pessoas se afastam do trabalho temporariamente ou permanentemente devido às diversas condições dolorosas (Phillips, 2003; Steenstra et al., 2006). Dentre as várias causas de dor, a de origem inflamatória é a mais comum em nossa sociedade. Embora antiinflamatórios com ação analgésica sejam amplamente utilizados no controle da dor inflamatória, os efeitos colaterais provocados por esses medicamentos têm motivado os estudos sobre os mecanismos envolvidos no desenvolvimento da dor inflamatória, com a finalidade de se descobrir novos alvos farmacológicos que servirão de base para o 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 sugerido que durante esse processo ocorra liberação de mediadores inflamatórios, tais como a bradicinina, TNF-α, IL-1β, IL-6, IL-8 (Cunha et al., 1992, Ferreira et al., 1993a), que estimulam a síntese das prostaglandinas e liberação das aminas simpatomiméticas, as quais sensibilizam diretamente os nociceptores aferentes primários (Gold et al., 1996, Rush and Waxman, 2004). Além desses mediadores inflamatórios, recentes estudos 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).

O ATP está presente em concentrações milimolares em todas as células do corpo (McCleskey and Gold, 1999), uma vez que é uma importante fonte de energia das células. Experimentos realizados em 1959 demonstraram que algumas fibras nervosas sensoriais liberavam ATP (Holton, 1959) e essa descoberta levou em 1972 à proposição do termo neurônios purinérgicos (Burnstock, 1972). Esses achados foram muito significativos, pois evidenciaram o papel extracelular do ATP, que até então era somente conhecido pela sua função intracelular. Atualmente existem inúmeras evidências da ação do ATP extracelular como molécula sinalizadora em diversos processos fisiológicos e patológicos (Khakh and North, 2006). No meio extracelular o ATP exerce suas funções por meio da ativação de receptores conhecidos como purinérgicos. Em 1978, Burnstock propôs a distinção de dois tipos de receptores purinérgicos, nomeados de P1 e P2, os quais medeiam as funções fisiológicas da adenosina e do ATP, respectivamente (Abbracchio and Burnstock, 1998). Entre 1992-1996, vários estudos demonstraram a diversificada distribuição desses receptores nos tecidos de mamíferos. Em 1994, estudos que evidenciaram as diferenças estruturais e propriedades eletrofisiológicas dos receptores P2 levaram Abbracchio e Burnstock a propor um novo sistema de divisão dos receptores P2 em duas grandes famílias: Receptores P2X — (ionotrópicos ligante-dependentes) — e receptores P2Y — acoplados à proteína G (metabotrópicos).

Uma vez que o ATP é normalmente encontrado no citoplasma das células, existem diversas circunstâncias nas quais o ATP pode ser liberado e atuar como um mediador periférico de dor (Hamilton, 2002). Sob situações de inflamação, o ATP pode deixar o meio intracelular e contribuir com o desenvolvimento da hiperalgesia inflamatória via ativação dos receptores P2X. Essa idéia vem se consolidando cientificamente após evidências de que o RNAm dos receptores P2X3, um dos sete subtipos clonados do receptor P2X (P2X1 – P2X7), é

abundantemente expresso nos neurônios sensoriais nociceptivos dos gânglios das raízes dorsais da medula espinhal (Chen et al., 1995, Kennedy and Leff, 1995, Lewis et al., 1995), especialmente nos neurônios sensoriais de pequeno diâmetro, particularmente as fibras C (Chen et al., 1995). Recentes estudos, que utilizaram modelos comportamentais nociceptivos (Bland-Ward and Humphrey, 2000, Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty et al., 2005, Oliveira et al., 2005), animais knockout para receptor P2X3 (Cockayne et al., 2000, Souslova et al., 2000), oligonucleotideos antisense P2X3 (Barclay et al., 2002, Honore et al., 2002a) e antagonistas seletivos de receptores P2X3,2/3 (Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty et al., 2005, Sharp et al., 2006), demonstraram que o ATP endógeno e os receptores P2X3,2/3 estão envolvidos com o desenvolvimento da dor em diferentes condições inflamatórias. Além disso, demonstrou-se que a administração do agonista seletivo de receptores P2X1,3,2/3, α,β-metileno ATP (α,β-meATP), induz hiperalgesia térmica (Hamilton et al., 1999, Waldron and Sawynok, 2004), alodinía mecânica (Tsuda et al., 2000, Wang et al., 2007) e hiperalgesia mecânica (Barclay et al., 2002) na pele da pata de ratos.

Entretanto, o mecanismo pelo qual o ATP endógeno, via ativação dos receptores P2X3,2/3, participa do desenvolvimento da hiperalgesia inflamatória permanece desconhecido. Portanto, os objetivos desse trabalho foram: (1) Estudar o mecanismo pelo qual a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia mecânica induzida no modelo da inflamação causada pela carragenina. Para isso, avaliamos se a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia mecânica induzida pela carragenina mediada pela sensibilização indireta e/ou direta dos nociceptores aferentes primários. Para testar a hipótese da ação indireta, avaliamos se antagonistas seletivos de receptores P2X3,2/3 reduzem a liberação endógena das citocinas inflamatórias TNF-α, IL-1β e CINC-1 e a migração de neutrófilos induzidos pela carragenina. Para testar a hipótese da ação direta, avaliamos se o tratamento com a administração intratecal de oligonucleotídeo antisense P2X3

reduz a hiperalgesia mecânica induzida pela carragenina. (2) Verificar se a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia induzida pelos mediadores inflamatórios bradicinina, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 e dopamina e, verificar o mecanismo pelo qual essa contribuição ocorre. Para testar a hipótese de que o ATP e a ativação dos receptores P2X3,2/3 contribui para a hiperalgesia induzida pelos mediadores inflamatórios, testamos a habilidade dos antagonistas de receptores P2X3,2/3 em reduzir a hiperalgesia mecânica induzida pela Bradicinina, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 e dopamina. Para avaliarmos se o ATP e a ativação dos receptores P2X3,2/3 contribui para a hiperalgesia induzida pelos mediadores inflamatórios através de um mecanismo indireto, testamos a habilidade dos antagonistas de receptores P2X3,2/3 em reduzir a migração de neutrófilos e a liberação de citocinas induzidas pelos mediadores inflamatórios cuja hiperalgesia é mediada pelo ATP endógeno. (3) Estudar o mecanismo pelo qual a ativação dos receptores P2X1,3,2/3 induz hiperalgesia mecânica. Para isso, avaliamos se o agonista de receptor P2X1,3,2/3 α,β-meATP induz hiperalgesia mecânica no tecido subcutâneo da pata de ratos através da sensibilização indireta dos nociceptores aferentes primários. Para testar a hipótese da ação indireta verificamos se os antagonistas seletivos de receptores B1 ou B2, DALBK e bradyzide, respectivamente, o inibidor da cicloxigenase indometacina e os antagonistas seletivos de receptores β_1- ou β_2 atenolol ou ICI 118,551, respectivamente, reduzem a hiperalgesia mecânica induzida pelo α,β-meATP. Testamos também se o α,β-meATP induz liberação das citocinas TNF-α, IL-1β, IL-6 e CINC-1 e migração de neutrófilos, que participam do desenvolvimento da hiperalgesia induzida pela ativação dos receptores P2X1,3,2/3.

CAPÍTULO 1

O presente artigo foi submetido ao periódico “Pain”.

Peripheral mechanisms underlying the essential role of P2X3,2/3 receptors in the development of inflammatory hyperalgesia

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Abstract

Activation of P2X3,2/3 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 P2X3,2/3 receptors by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia, and, that this contribution is mediated by an indirect and/or a direct sensitization of the primary afferent nociceptors. Co-administration of the selective P2X3,2/3 receptors antagonist, A-317491, or the P2X1,3,2/3,1/5 receptors antagonist, TNP-ATP, with carrageenan blocked the mechanical hyperalgesia induced by carrageenan, significantly reduced the increased concentration of TNF-α and CINC-1 but not of IL-1β induced by carrageenan, and slightly reduced the neutrophil migration induced by carrageenan. Given that pro-inflammatory cytokines induce neutrophil migration, this partial reduction of neutrophil migration probably resulted from the inability of the P2X3,2/3 receptors antagonist in inhibiting the production of IL-1β in the site of inflammation. Intrathecal administration of oligonucleotides antisense against P2X3 receptors during seven days significantly reduced the expression of P2X3 receptors in the saphenous nerve and significantly reduced the mechanical hyperalgesia induced by carrageenan. We concluded that activation of P2X3,2/3 receptors by endogenous ATP is essential to the development of the mechanical hyperalgesia induced by carrageenan. Furthermore, we showed that this essential role of P2X3,2/3 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 and by a direct sensitization of the primary afferent nociceptors.

Keywords: mechanical inflammatory hyperalgesia, P2X3,2/3 receptors, ATP, carrageenan, cytokines, antisense.

Introduction

P2X receptors are a family of ligand-gated ion channels activated by extracellular ATP that are involved in pain mechanisms. Recent reports using behavioral nociceptive models with gene knockout methods (Cockayne et al., 2005), antisense oligonucleotide technologies (Barclay et al., 2002, Honore et al., 2002a) and selective P2X3,2/3 receptors antagonist (Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty et al., 2005, Sharp et al., 2006) indicate that the activation of P2X3,2/3 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia.

The subcutaneous administration of carrageenan has been widely used as a model of inflammatory hyperalgesia 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 P2X3,2/3 receptors also contributes to the development of mechanical hyperalgesia in this model, and, if so, which mechanisms underlie the contribution of P2X3,2/3 receptors to this hyperalgesic response.

Therefore, the aims of this study were to test the hypothesis that the activation of P2X3,2/3 receptors by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia, and, that this contribution is mediated by an indirect and/or a direct sensitization of the primary afferent nociceptors. To test the

hypothesis that the activation of P2X3,2/3 receptors by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia, we explored the ability of P2X3,2/3 receptor antagonists to reduce carrageenan-induced mechanical hyperalgesia. To test the hypothesis that this contribution of P2X3,2/3 receptors is mediated by an indirect sensitization of the primary afferent nociceptors, we explored the ability of P2X3,2/3 receptor antagonists to reduce the endogenous release of the inflammatory cytokines TNF-α, IL-1β and CINC-1 and the neutrophil migration induced by carrageenan (Ferreira et al., 1993b, Jain et al., 2001, Loram et al., 2007). Finally, to test the hypothesis that the activation of P2X3,2/3 receptors by endogenous ATP contributes to carrageenan-induced mechanical hyperalgesia through a direct sensitization of the primary afferent nociceptors, we evaluated if treatment with intrathecal administration of oligonucleotides (ODN) antisense against P2X3 receptors reduces carrageenan-induced mechanical hyperalgesia.

Experimental procedures

Drugs and doses

The following drugs were used: carrageenan (Cg; 30, 100, 300 and 600μg/paw); the P2X1,3,2/3,1/5 receptor antagonist, 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP; 80, 160 and 240μg/paw), the selective P2X3/2/3 receptor antagonist, 5-([(3-Phenoxybenzyl) [(1S)-1,2,3,4-tetrahydro-1naphthalenyl] [amino]carbonyl)-1,2,4-benzene- tricarboxylic acid (A-317491; 6.0, 20, 60 and 180μg/paw) and the nonspecific selectin inhibitor fucoidan (25mg/Kg, i.v., (Zhang et al., 2001) were obtained from Sigma Chemicals (St Louis, Missouri, USA). TNF-α (0.8pg/paw), IL-1β (0.15 pg/paw) and CINC-1 (1.0pg/paw) were obtained from R&D Systems (Minneapolis, USA). Those doses of cytokines are the sub-maximal doses obtained from a previous dose-response experiment (data not shown). All drugs were dissolved in saline (0.9% NaCl).

Subjects

Male albino Wistar rats weighing 200 – 350g were used. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of IASP on using laboratory animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas - Unicamp. Animal suffering and the number of animals per group were kept at a minimum. 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 on a temperature-controlled room test (± 23ºC) for a 1-hour habituation period prior to the test.

Subcutaneous Injections

Drugs or their vehicle were subcutaneously injected in the dorsum of the 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., 2007a). 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 50μl.

Intrathecal injections

The method for intrathecal ODN injection was based on the technique of Papir-Kricheli and colleagues (Papir-Kricheli et al., 1987). Briefly, for each injection rats were anesthetized with 1/3 O_2 – 2/3 N_2O and halothane at 5 and 1.5%, respectively (Le Bars et al., 1979). A 26-gauge needle was inserted in the subarachnoid space on the midline between L4 and L5 vertebrae. ODN was injected at 1 μl/ s. The animals regained consciousness approximately 1 min after discontinuing the anesthetic. A dose of 80 μg of P2X3 receptor ODN antisense or mismatch was intrathecally administered in a volume of 10 μl once daily for 7 days (Barclay et al., 2002). The behavioral assessment was conducted on next day of the last injection.

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., 2007a). 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.

Antisense oligodeoxynucleotides (ODNs)

The functional blockade of P2X3 receptors expression on peripheral sensory neurons was realized by the intrathecal injection of ODN antisense. The followed ODN antisense sequence of 19-mer was used: 5'-T A A T C C G A C A C G T C C A T G A -3'. The mismatch-ODN sequence, 5′-T A **T** T C C **C** A C **T** C G **A** C **G** A T **C** A -3′, corresponded to the antisense sequence except that six bases were changed (denoted by bold face). The corresponding GenBank accession number and ODN position within the cDNA sequence are X90651 and 401-420. A search of the NCBI database to *Rattus*

norvegicus identified no other sequences homologous to that used in this experiment. The ODN was purchased from Erviegas (SP, Brazil), lyophilized and reconstituted in 0.9% NaCl. The ODN was aliquoted and stored at –20ºC.

Western blot analysis of P2X3 receptor expression

Eight animals in each group were used for immunoblot study. To assess the efficacy of antisense ODN treatment, immediately after the behavioral test a 1.0 cm section of saphenous nerves of anesthetized rats were removed 1.5 cm proximal to the knee-level bifurcation, in order for detectable levels of protein, homogenized in cold RIPA buffer (1 % Igepal CA-630, 0.5 % sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 10 mg/ml aprotinin, 1 mM sodium orthovanadate in PBS buffer, pH 7.4) and stored at -70ºC (Parada et al., 2003a). The protein concentration was determined by using the Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 40μg total protein were boiled in loading Laemmli buffer (BioRad, USA); thereafter, each aliquot was loaded onto an 8% polyacrylamide gel. After electrophoresis separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membrane was blocked in TBST (20 mM Tris-HCL, 150 mM NaCl, and 0.1 % Tween 20) containing 5 % non-fat dry milk for 2h at room temperature, followed by incubation with P2X3 rabbit polyclonal IgG (1:1000; Neuromics) overnight at 4ºC, rinsed six times with TBST, and then incubated for 1h in goat anti-rabbit IgG peroxidase conjugate (1:3000, Sigma). Membrane was visualized using ECL solution (Pierce), and exposure to x-

ray film (Kodak) in a dark room. Films were scanned into Image Quant 5.2 for analysis. Banding specificity was determined by omission of primary antibody from the Western blot protocol. To compensate for any differences in the amount of loaded protein, the intensity of the P2X3 receptor band was divided by the intensity of α-tubulin (Sigma, USA) band for each sample.

ELISA procedure

An adaptation of ELISA (Safieh-Garabedian et al., 1995) was used to determine if TNP-ATP or A-317491 was able to reduce the carrageenan-induced release of TNF-α, IL-1β and CINC-1. The subcutaneous tissues of dorsum of the rat's hind paw were collected 180min post the subcutaneous injection 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-methyl-sulfonyl fluoride, 0.1mM benzotonic chloride, 10mM EDTA, and 20KI/ml aprotinine (Sigma, USA). The samples were centrifuged at 10,000rpm for 15min at 4ºC and the supernatants were stored at -70ºC for posterior use to evaluate the protein levels of TNF-α, IL-1β and CINC-1 in the subcutaneous tissue of rat's hind paw. The cytokines were quantified by the follows kits: TNF-α - Rat TNF-alpha/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) 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 five times to guarantee the authenticity of the results.

Measurement of myeloperoxidase activity (MPO)

The neutrophil migration to the site of carrageenan administration in the skin of rat's hind paw was evaluated by the myeloperoxidase (MPO) kinetic-colorimetric assay as previously described (Bradley et al., 1982). Approximately 0.5 cm^2 of cutaneous tissue was harvested 180 minutes after the subcutaneous injection of carrageenan. The samples were homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 1.015 M NaEDTA) followed by centrifugation at 3000 rpm for 15 min. The pellet was subjected to hypotonic lyses (1.5 mL of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). After that, the pellet was snap-frozen in liquid nitrogen three times and was centrifuged at 10,000 rpm for 15 min and was re-homogenized. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM). Results were calculated by comparing the optical density of hind paw tissue supernatant with a standard curve of neutrophil (> 95% purity) numbers. The results were presented as number of neutrophils x 10^6/mg tissue. 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. Data are expressed in figures by the decrease with paw-withdrawal threshold and presented as means $\pm$ S.E.M.

Results

Carrageenan-induced mechanical hyperalgesia

Subcutaneous injection of carrageenan (300μg/paw) in the dorsum of the rat's hind paw induced a significant mechanical hyperalgesia 60, 120 or 180 min. after its administration, that peaked at 180 min. (Fig. 1A, $p<0.05$, Tukey test). Therefore, in further experiments, the mechanical hyperalgesia was evaluated only 180 min after the injection of carrageenan.

Subcutaneous injection of carrageenan (100, 300 or 600μg/paw) induced a dose-related mechanical hyperalgesia (Fig.1B, $p<0.05$, Tukey test) that reached its maximum at the dose of 300μg/paw.

Effect of P2X3,2/3 receptors antagonists on carrageenan-induced mechanical hyperalgesia

Co-administration of the P2X1,3,2/3,1/5 receptors antagonist TNP-ATP (Fig. 2A; 160 or 240μg/paw) or the selective P2X3,2/3 receptors antagonist A-317491 (Fig. 2B; 60 or 180μg/paw) with carrageenan (300μg/paw) blocked carrageenan-induced mechanical hyperalgesia. The highest doses of these antagonists did not affect carrageenan-induced mechanical hyperalgesia when applied on the contralateral paw (Figs. 2A and 2B, $p>0.05$, Tukey test) confirming their peripheral action.

Co-administration of TNP-ATP (240μg/paw) with carrageenan (300μg/paw) and the administration of this antagonist 60 min (Fig. 2C, $p<0.05$, Tukey test), but not 120 or 180 min (Fig. 2C, $p>0.05$, Tukey test) after the carrageenan administration significantly reduced carrageenan-induced mechanical hyperalgesia.

Effect of P2X3,2/3 receptors antagonists on carrageenan-induced local increase in cytokines concentration

To verify whether endogenous ATP via activation of P2X3,2/3 receptors contributes to the release of pro-inflammatory cytokines induced by carrageenan, TNP-ATP (240μg/paw), A-317491 (60μg/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 and CINC-1 were quantified 180 min after the administration of carrageenan. TNP-ATP and A-317491 significantly reduced ($p<0.05$, Tukey test) the concentration of TNF-α (Fig. 3A) and CINC-1 (Fig. 3C), but not ($p>0.05$, Tukey test) that of IL-1β (Fig. 3B). The concentration of TNF-α and CINC-1 induced by the co-administration of TNP-ATP or A-317491 with carrageenan was significantly greater than that induced by 0.9% NaCl alone ($p<0.05$, T test). The subcutaneous injection of 0.9% NaCl alone did not affect ($p>0.05$, Tukey test) the endogenous concentration of TNF-α, IL-1β and CINC-1.

Effect of TNP-ATP on neutrophils migration induced by carrageenan

To verify whether endogenous ATP via activation of P2X1,3,2/3,1/5 receptors contributes to the neutrophils migration induced by carrageenan, TNP-ATP (240μg/paw) or 0.9% NaCl was co-administrated with carrageenan (300μg/paw) and the MPO activity in the subcutaneous tissue of rat's hind paw was quantified 180 min after the carrageenan injection. TNP-ATP significantly reduced (Fig. 4A, $p<0.05$, Tukey test) the MPO activity induced by carrageenan when compared to 0.9% NaCl (control group). Also, pre-treatment with fucoidan (25mg/Kg, i.v.) 20 min before the carrageenan injection significantly reduced (Fig. 4A, $p<0.05$, Tukey test) the MPO activity when compared to either rats treated with TNP-ATP or 0.9% NaCl.

To verify whether neutrophils migration contributes to carrageenan-induced mechanical hyperalgesia, rats were treated with fucoidan (25 mg/kg, i.v.) 20 min before the carrageenan injection and 180 min later, the mechanical nociceptive threshold was evaluated. Pre-treatment with fucoidan significantly reduced (Fig. 4B, $p<0.05$, Tukey test) the mechanical hyperalgesia induced by carrageenan, and this reduction was not significantly different from that induced by TNP-ATP (Fig. 4B, $p>0.05$, Tukey test).

Effect of intrathecal treatment with ODN antisense against P2X3 receptors on carrageenan-induced mechanical hyperalgesia

To verify whether neuronal P2X3 receptor contributes to carrageenan-induced mechanical hyperalgesia, rats were pre-treated with ODN (80μg/day, 7

days) antisense or mismatch against P2X3 receptor. ODN antisense significantly reduced the P2X3 receptor expression on saphenous nerve (Fig. 5A) and the carrageenan-induced mechanical hyperalgesia (Fig. 5B) when compared to mismatch ($p<0.05$, paired t-test).

Discussion

Role of P2X3,2/3 receptor in carrageenan-induced mechanical hyperalgesia

Co-administration of the selective P2X3,2/3 receptors antagonist A-317491 (Jarvis et al., 2002) or the P2X1,3,2/3,1/5 receptors antagonist TNP-ATP (Jarvis et al., 2001) with carrageenan blocked carrageenan-induced mechanical hyperalgesia. These findings strongly suggest that the activation of P2X3,2/3 receptors by endogenous ATP not only contributes but is essential to the development of the mechanical hyperalgesia induced by carrageenan. Importantly, the role of P2X3,2/3 receptors in this hyperalgesic response seems to be essential only to its development but not to its maintenance. This is because TNP-ATP blocked carrageenan-induced mechanical hyperalgesia when it was co-administered with carrageenan, but not when it was administered 60, 120 or 180 min 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 sympathomimetic amines (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993a). These inflammatory mediators directly sensitize the primary afferent nociceptor (Gold et al., 1996, Rush and Waxman, 2004). Therefore, the blockade of carrageenan-induced inflammatory hyperalgesia induced by the co-administration of P2X3,2/3 receptor antagonists with carrageenan suggests that the activation of P2X3,2/3 receptors must be crucial to prostaglandin- and sympathomimetic amines-mediated sensitization of the primary afferent nociceptor.

Mechanisms underlying the essential role of P2X3,2/3 receptors in the development of carrageenan-induced mechanical hyperalgesia

Release of cytokines

Co-administration of A-317491 or TNP-ATP with carrageenan significantly reduced the increased concentration of TNF-α and CINC-1 induced by carrageenan. 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., 2003a). Taken together, these findings indicate that the essential role of P2X3,2/3 receptors in the development of carrageenan-induced mechanical hyperalgesia is mediated, at least in part, by the release of cytokines, in particular the TNF-α. Although A-317491 and TNP-ATP have reduced the TNF-α concentration, they did not block it. This finding suggests that the increased concentration of TNF-α induced by carrageenan may be mediated by distinct pathways and that only one of them depends on P2X3,2/3 activation. The mechanism by which the activation of P2X3,2/3 receptors by endogenous ATP induces the release of TNF-α is unknown; however, it is presently under investigation in our laboratory.

Surprisingly, the co-administration of A-317491 or TNP-ATP with carrageenan did not alter the concentration of IL-1β, suggesting 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 IL1-β but not of TNF-α is increased after the injection of carrageenan in the gastrocnemius muscle (Loram et al., 2007). However, we can not exclude the possibility that the residual concentration of TNF-α observed after the co-administration of A-317491 or TNP-ATP with carrageenan could be enough to keep the concentration of IL1-β elevated.

Induction of neutrophil migration

We confirmed that neutrophil migration contributes to carrageenan-induced inflammatory hyperalgesia (Jain et al., 2001) by showing that pre-treatment with the non specific selectin inhibitor fucoidan significantly reduced the neutrophil migration and the mechanical hyperalgesia induced by carrageenan. Therefore, the activation of P2X3,2/3 receptors by endogenous ATP may mediate the development of carrageenan-induced mechanical hyperalgesia, at least in part, through the induction of neutrophil migration. Co-administration of TNP-ATP with carrageenan completely inhibited the mechanical hyperalgesia, but slightly reduced the neutrophil migration. Because pro-inflammatory cytokines induce neutrophil migration (Ramos et al., 2003, Bombini et al., 2004, Oliveira et al., 2007b), the partial reduction of

neutrophils migration induced by TNP-ATP probably resulted from the inability of this P2X3,2/3 receptors antagonists in inhibiting the production of IL1-β in the site of inflammation.

Direct sensitization of the primary afferent nociceptor

Intrathecal administration of ODN antisense against P2X3 receptors during seven days significantly reduced the expression of these receptors in the saphenous nerve, as previously demonstrated (Barclay et al., 2002, Honore et al., 2002a), and significantly reduced the mechanical hyperalgesia induced by carrageenan. These findings suggest that the activation of neuronal P2X3 receptors by endogenous ATP contributes to the development of carrageenan-induced mechanical hyperalgesia and are consistent with the high expression of mRNA of the P2X3 receptors in nociceptive sensory neurons (Chen et al., 1995, Kennedy and Leff, 1995, Lewis et al., 1995). Thus, the essential role of P2X3,2/3 receptors in the development of carrageenan-induced mechanical hyperalgesia is mediated, at least in part, by a direct sensitization of the primary afferent nociceptor.

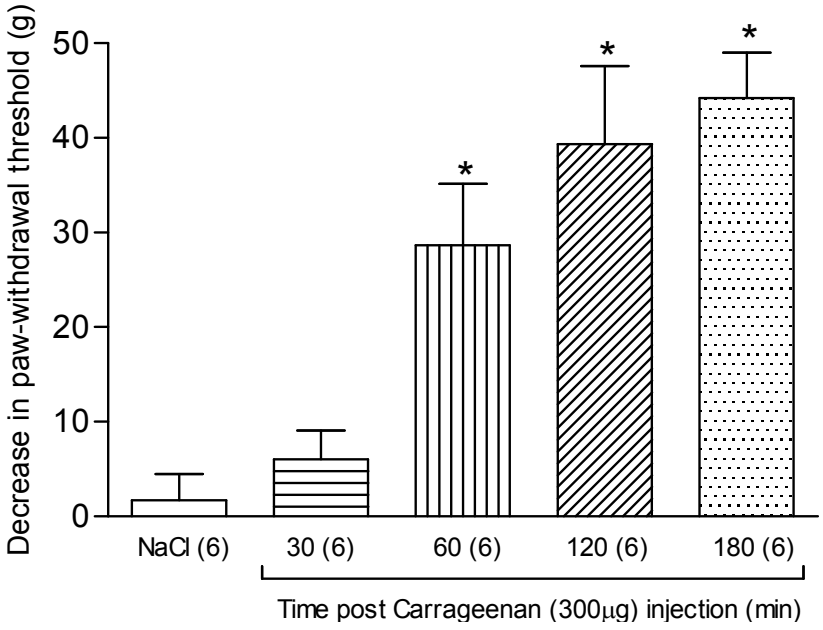
In summary, we concluded that activation of P2X3,2/3 receptors by endogenous ATP is essential to the development of the mechanical hyperalgesia induced by carrageenan. Furthermore, we showed that this essential role of P2X3,2/3 receptors in the development of carrageenan-induced mechanical hyperalgesia is mediated by an indirect sensitization of the primary afferent nociceptors dependent on the previous release of TNF-α and by a direct

sensitization of the primary afferent nociceptors. Finally, the finding that blockade of P2X3,2/3 receptors prevented the development of inflammatory hyperalgesia suggests that selective antagonists for the P2X3,2/3 receptors may be more effective than the currently available drugs in the treatment of inflammatory pain.

Figures and legends

Figure 1

A



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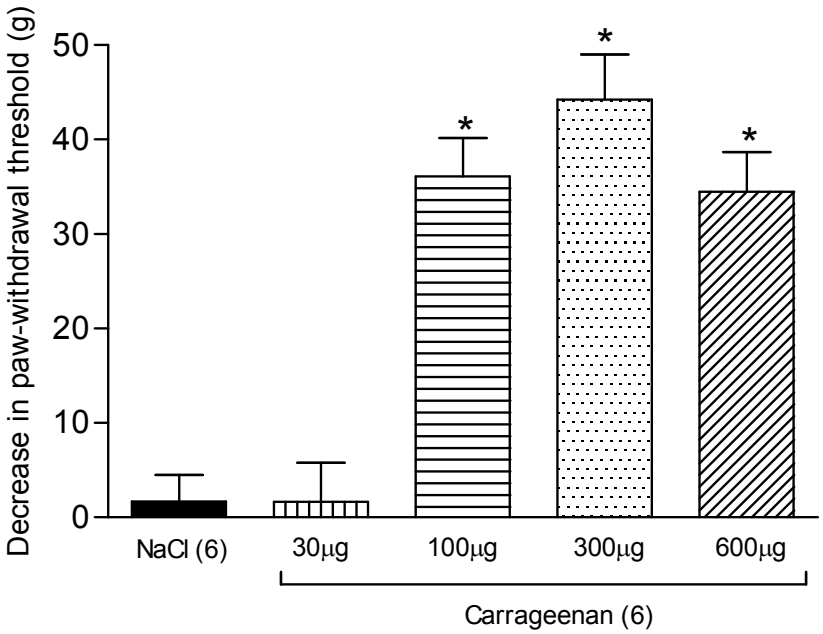
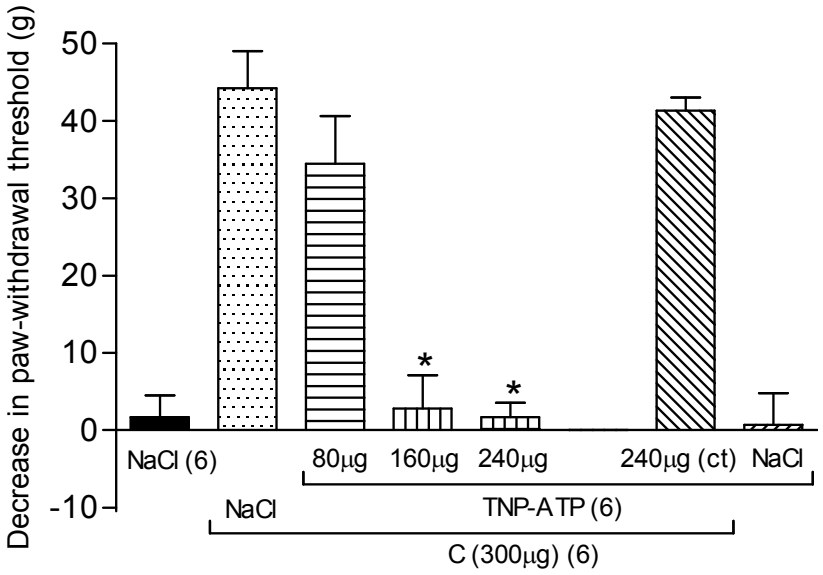


Fig. 1_ Hyperalgesic effect induced by carrageenan

Subcutaneous administration of carrageenan (300μg/paw) but not of 0.9% NaCl induced a significant mechanical hyperalgesia 60, 120 and 180 min. after its administration (**A**). Carrageenan (100, 300 or 600μg/paw) induced a dose-related mechanical hyperalgesia (**B**). In this and in the subsequent figures the mechanical hyperalgesia was measured 180 min. after 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


Decrease in paw-withdrawal threshold (g)
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40
30
20
10
0
-10
*
*
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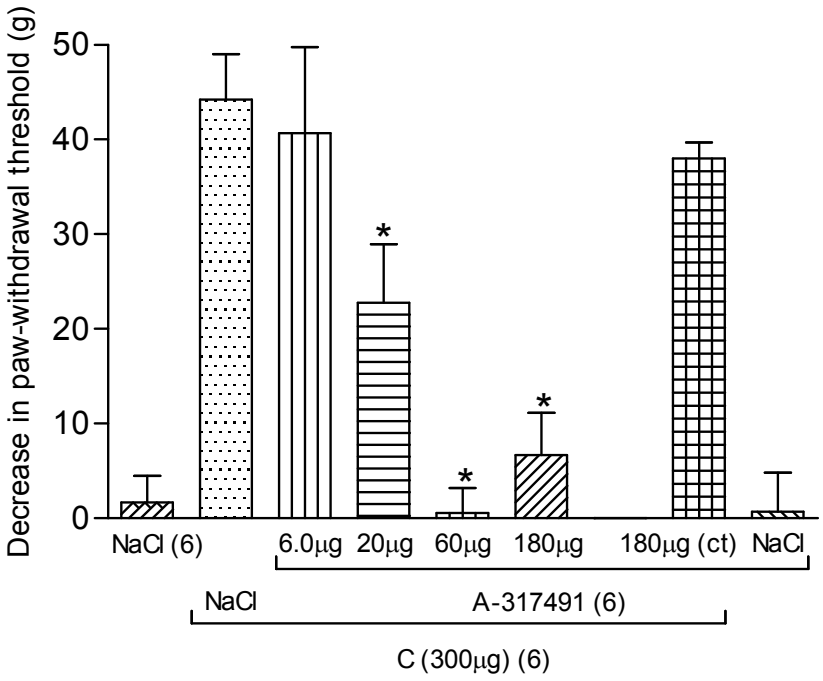
80μg
160μg
240μg
240μg (ct)
NaCl
NaCl
TNP-ATP (6)
C (300μg) (6)

B


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
*
*
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20μg
60μg
180μg
180μg (ct)
NaCl
NaCl
A-317491 (6)
C (300μg) (6)

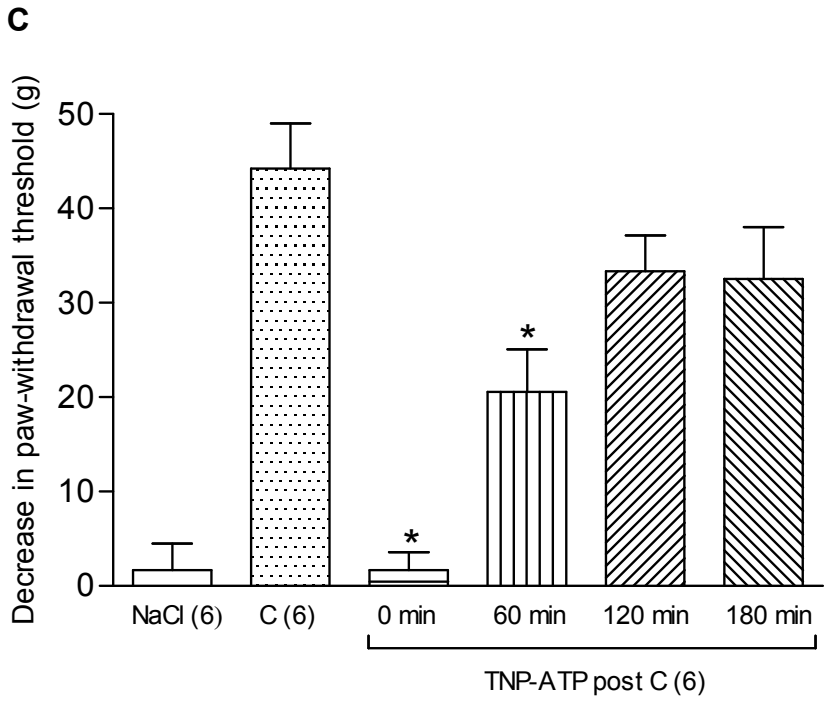


Fig. 2_Effect of P2X3,2/3 receptors antagonists on carrageenan-induced mechanical hyperalgesia

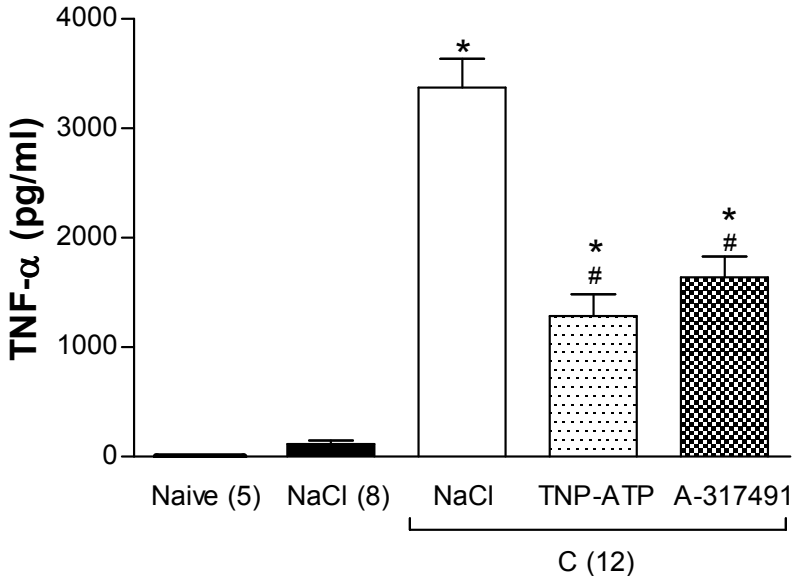
Co-administration of the P2X1,3,2/3,1/5 receptors antagonist TNP-ATP (160 or 240μg/paw, **A**), or of the P2X3,2/3 receptors antagonist A-317491 (60 or 180μg/paw, **B**) with carrageenan (C, 300μg/paw) blocked carrageenan-induced mechanical hyperalgesia. The highest doses of the antagonists applied on the contralateral paw (ct) did not affect carrageenan-induced mechanical hyperalgesia.

Co-administration of TNP-ATP (240μg/paw) with carrageenan or administration of this antagonist 60 min, but not 120 or 180 min after the carrageenan administration significantly reduced carrageenan-induced mechanical hyperalgesia (**C**). The

symbol “*” indicates a response significantly lower than that induced by carrageenan plus 0.9% NaCl ($p<0.05$, Tukey test).

Figure 3

A



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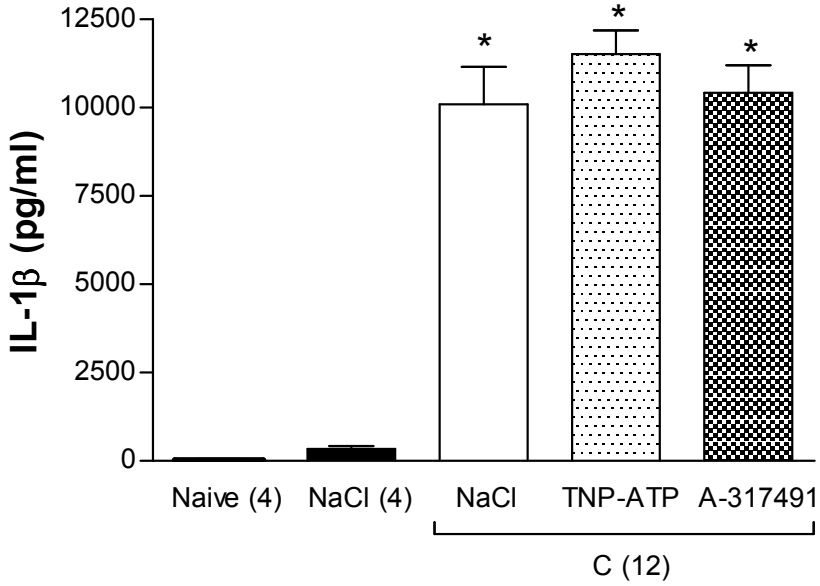


C.

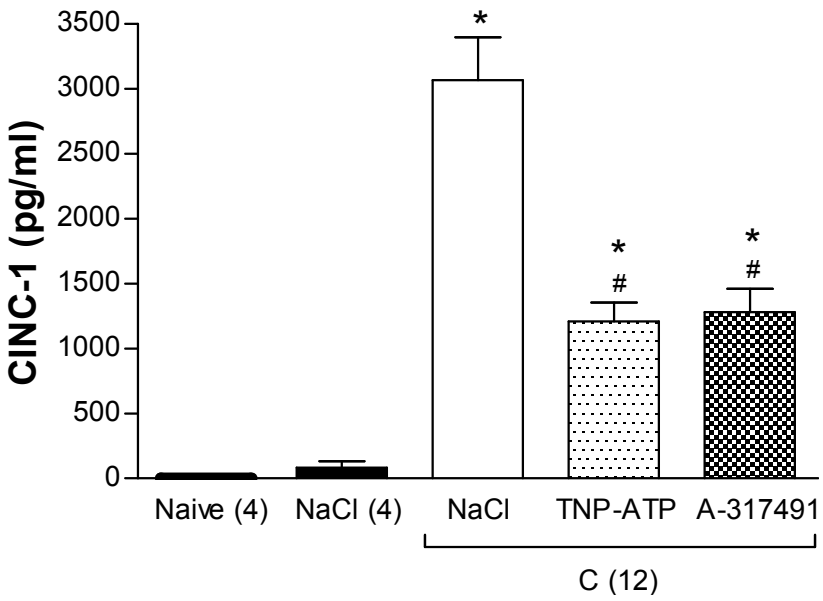
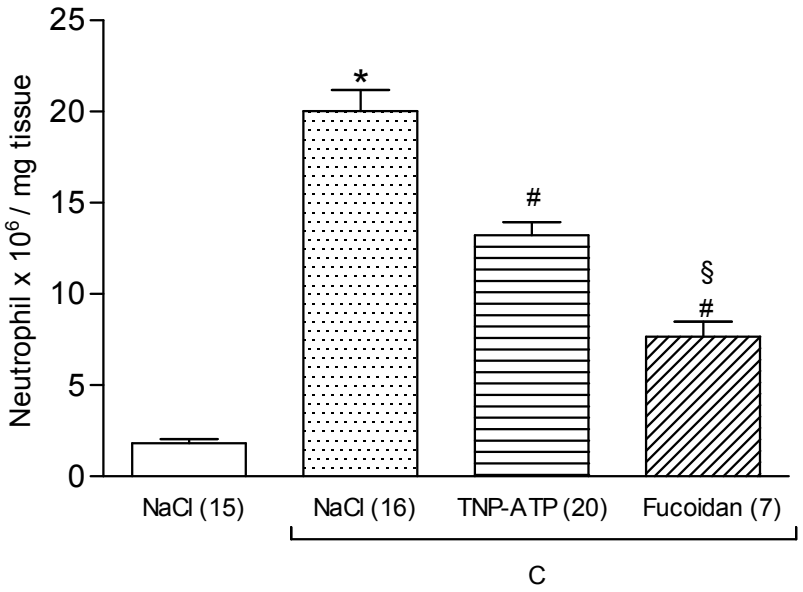


Fig. 3_ Effect of TNP-ATP or A-317491 on carrageenan-induced release of cytokines

Co-administration of TNP-ATP (240μg/paw) or A-317491 (60μg/paw) with carrageenan (C, 300 μg/paw) significantly reduced the increased concentration of TNF-α (**A**) and CINC-1 (**C**), but not that of IL-1β (**B**) induced by carrageenan 180 min. after its injection. The concentration of TNF-α and CINC-1 induced by the co-administration of TNP-ATP or A-317491 with carrageenan was significantly greater than that induced by 0.9% NaCl alone. The local administration of 0.9% NaCl did not induce the release of cytokines. The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test) and the symbol "#" indicates a response significantly lower than that induced by carrageenan plus 0.9% NaCl ($p<0.05$, Tukey test).

Figure 4

A.


Neutrophil x 10^6 / mg tissue
25
20
15
10
5
0
*
#
§
#
NaCl (15)
NaCl (16)
TNP-ATP (20)
Fucoidan (7)
C

B.

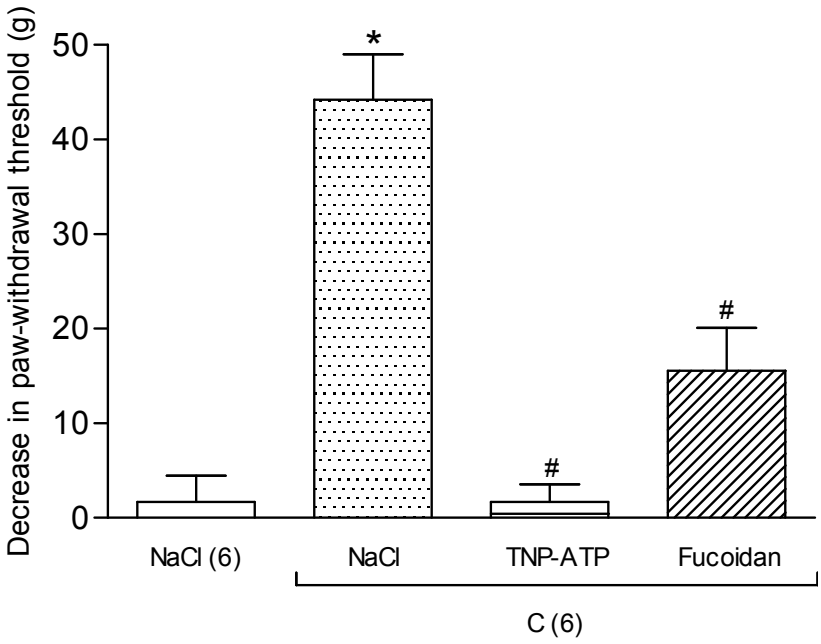
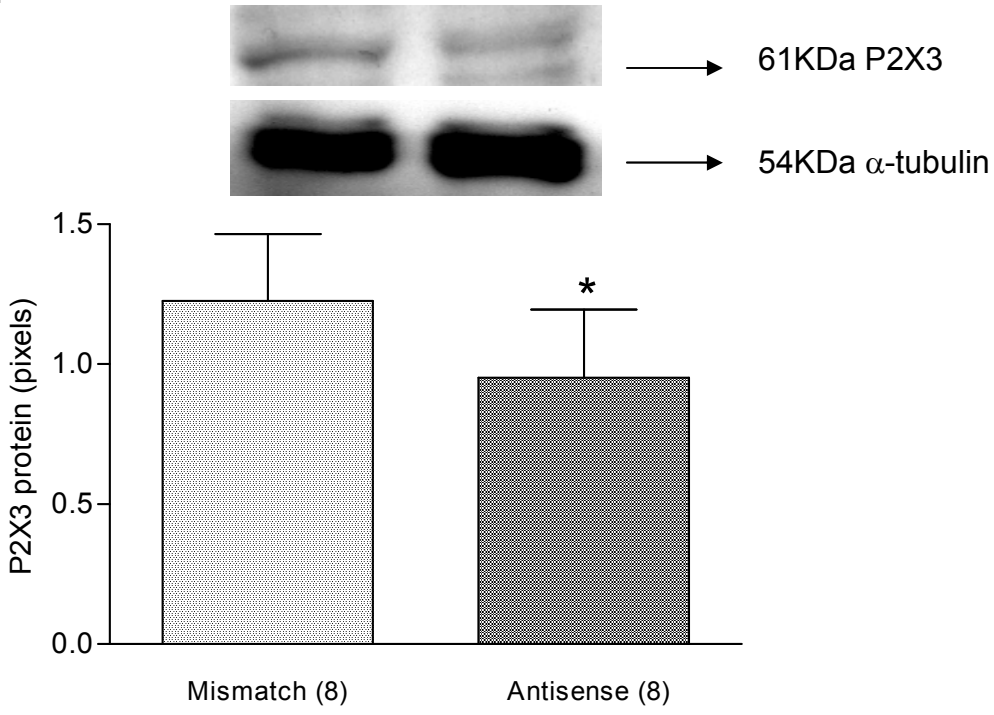

Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
#
#
NaCl (6)
NaCl
TNP-ATP
Fucoidan
C (6)

Fig.4_ Effect of TNP-ATP on carrageenan-induced neutrophils migration

Co-administration of TNP-ATP (240μg/paw) with carrageenan (C, 300μg/paw) or pre-treatment with fucoidan (25mg/Kg i.v.) 20 min. before the injection of carrageenan significantly reduced the MPO activity (**A**) and the mechanical hyperalgesia (**B**). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test); the symbols "#" and "§" indicate a response significantly lower than those induced by carrageenan plus 0.9% NaCl and carrageenan plus TNP-ATP, respectively ($p<0.05$, Tukey test).

Figure 5

A.



B.

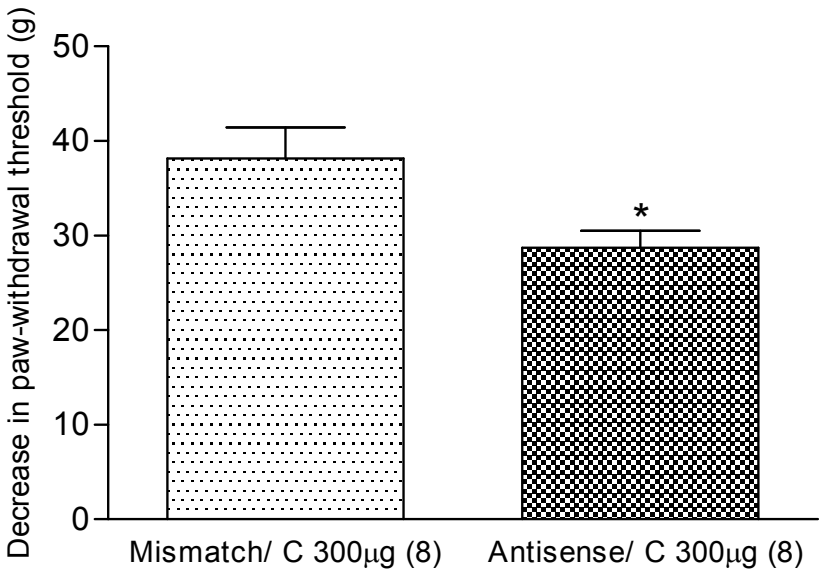


Fig. 5_ Effect of intrathecal treatment with ODN antisense against P2X3 receptor on carrageenan-induced mechanical hyperalgesia

Daily treatment with ODN antisense (80 μg/day, 7 days), but not mismatch, significantly reduced the P2X3 receptor expression on saphenous nerve (**A**) and the carrageenan (C, 300μg/paw)-induced mechanical hyperalgesia (**B**). Immunoblot for P2X3 receptor expression with the corresponding blot for α-tubulin is shown. The symbol "*" indicates a response significantly lower than that induced by mismatch ($p<0.05$, paired t-test).

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CAPÍTULO 2

O presente artigo será submetido ao periódico “Neuroscience”.

Endogenous ATP via P2X3,2/3 receptors mediates mechanical hyperalgesia induced by bradykinin, but not by pro-inflammatory cytokines, PGE_2 or dopamine

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Abstract

Activation of P2X3,2/3 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia. The aim of this study was to verify whether the activation of P2X3,2/3 receptors by endogenous ATP contributes to the mechanical hyperalgesia induced by bradykinin, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine. Co-administration of the selective P2X3,2/3 receptors antagonist A-317491 or the P2X1,3,2/3,1/5 receptors antagonist TNP-ATP with bradykinin, but not with TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine, prevented in a dose-response manner the mechanical hyperalgesia. We also verified whether neutrophil migration or cytokines released is involved in the role that P2X3,2/3 receptors activation plays in bradykinin-induced hyperalgesia. TNP-ATP or A-317491 did not affect either neutrophil migration or the release of TNF-α, IL-1β, IL-6 and CINC-1 induced by bradykinin. These findings demonstrated that endogenous ATP via activation of P2X3,2/3 receptors mediates bradykinin-induced mechanical hyperalgesia by a mechanism that is not dependent on neutrophil migration or cytokines release.

Keywords: Kinins, P2X3 receptor, ATP, inflammation, hyperalgesia, neutrophils, cytokines.

Introduction

ATP released from damaged tissues plays an important role in the development of inflammatory pain by activating P2X receptors. Recent reports using inflammatory pain models, such as local administration of carrageenan (McGaraughty et al., 2003), Complete Freund Adjuvant (Honore et al., 2002a, Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty et al., 2005) or formalin (Souslova et al., 2000, Honore et al., 2002a, McGaraughty et al., 2005) in the rat hind paw, and nerve injury (Honore et al., 2002a, Jarvis et al., 2002, McGaraughty et al., 2003, McGaraughty et al., 2005) indicate that the activation of P2X3,2/3 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia.

Following tissue injury, in addition to ATP other inflammatory mediators, that are released at the site of the injury, induce or/and maintain the inflammatory hyperalgesia (Verri et al., 2006). It has been proposed that the inflammatory mediator bradykinin, which is early released during inflammation, triggers the subsequent release of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 (Ferreira et al., 1993a, Ferreira et al., 1993b). These cytokines induce the synthesis of final inflammatory mediators, such as prostaglandins and sympathomimetic amines, which in turn, directly sensitize the primary afferent nociceptors (Gold et al., 1996, Rush and Waxman, 2004). Furthermore, these cytokines also induce neutrophils migration (Ramos et al., 2003, Bombini et al.,

2004, Oliveira et al., 2007b) that contributes to the development of inflammatory hyperalgesia (Jain et al., 2001, Tambeli et al., 2006, Oliveira et al., 2007a).

However, it is not known whether endogenous ATP via P2X3,2/3 receptors activation contributes to the hyperalgesia induced by inflammatory mediators and, if so, whether this contribution is mediated by an indirect sensitization of the primary afferent nociceptors. To test the hypothesis that ATP via P2X3,2/3 receptors activation contributes to the hyperalgesia induced by inflammatory mediators, we explored the ability of P2X3,2/3 receptors antagonists to reduce the mechanical hyperalgesia induced by bradykinin, TNF-α, IL-1β, IL-6, CINC-1 (rat IL-8 related chemokine), PGE_2 and dopamine. To test the hypothesis that ATP and P2X3,2/3 receptors activation contributes to the hyperalgesia induced by inflammatory mediators by an indirect mechanism, we explored the ability of P2X3,2/3 receptors antagonists to reduce the neutrophil migration and release of endogenous cytokines induced by the inflammatory mediators whose hyperalgesia is mediated by endogenous ATP.

Experimental procedures

Drugs and doses

The follow drugs were used: the P2X1,3,2/3,1/5 receptor antagonist, 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP; 9.6, 48 and 240μg/paw); the selective P2X3/2/3 receptor antagonist, 5-([(3-Phenoxybenzyl) [(1S)-1,2,3,4-tetrahydro-1naphthalenyl] [amino] carbonyl)-1,2,4-benzene-tricarboxylic acid (A-317491; 6.0, 20 and 60μg/paw); bradykinin (0.15, 0.5 and 1.5μg/paw), prostaglandin (0.05, 0.1 and 0.2μg/paw) and dopamine (1.0, 3.0, 10 and 30μg/paw) were obtained from Sigma Chemicals (St Louis, Missouri, USA). TNF-α (0.25 and 0.8pg/paw); IL-1β (0.01, 0.05 and 0.15pg/paw); IL-6 (0.03, 0.1and 0.3ng/paw); CINC-1 (0.3, 1.0 and 3.0pg/paw) were obtained from R&D Systems (Minneapolis, USA). All drugs were dissolved in PBS (Sigma Chemicals, St Louis, Missouri, USA).

Subjects

Male albino Wistar rats weighing 150–250g were used, and the experiments were conducted in accordance with the IASP guidelines on using laboratory animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas-Unicamp. Animal suffering and number of animals per group were kept at a minimum. 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 on a temperature-controlled room test (± 23ºC) for a 1-hour habituation period prior to the test.

Subcutaneous Injections

Drugs or their vehicle were subcutaneously injected in the dorsum of the 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., 2007a). 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 50μl.

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., 2007a). 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.

Measurement of myeloperoxidase activity (MPO)

The neutrophil migration in the skin of rat's hind paw was evaluated by the myeloperoxidase (MPO) kinetic-colorimetric assay as previously described (Bradley et al., 1982). Approximately 0.5 cm^2 of cutaneous tissue was harvested 3 hours after the subcutaneous injection of stimuli. The samples were homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 1.015 M NaEDTA) followed by centrifugation at 3000 rpm for 15 min. The pellet was subjected to hypotonic lyses (1.5 mL of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). After that, the tissue was snap-frozen in liquid nitrogen three times and was centrifuged at 10,000 rpm for 15 min and was re-homogenized. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM). Results were calculated by comparing the optical density of hind paw tissue supernatant with a standard curve of neutrophil (> 95% purity) numbers. The results were presented as number of neutrophils x 10^6/mg tissue. All procedures were repeated two times to guarantee the authenticity of the results.

ELISA procedure

The subcutaneous tissues of dorsum of the rat's hind paw were collected 3-hour post the subcutaneous injection of Bradykinin 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-methyl-sulfonyl fluoride, 0.1mM benzotonic chloride, 10mM EDTA, and 20Kl/ml aprotinine (Sigma, USA). The samples were centrifuged at 10000 rpm 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 TNF-alpha/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. Data are expressed in figures by the decrease with paw-withdrawal threshold and presented as means ± S.E.M.

Results

Effect of P2X3,2/3 receptors antagonists on mechanical hyperalgesia induced by *bradykinin, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine*

Bradykinin (0.5 and 1.5µg/paw, Fig.1A), TNF-α (0.8pg/paw, Fig.1C), IL-1β (0.05 and 0.15pg/paw, Fig. 1D), IL-6 (0.1, 0.3 and 1.0ng/paw, Fig. 1E), CINC-1 (1.0 and 3.0pg/paw, Fig. 1F), PGE_2 (0.1and 0.2µg/paw, Fig. 1G) or dopamine (3.0, 10 and 30µg/paw, Fig 1H) induced mechanical hyperalgesia in a dose-dependent manner 3 h after their injection in subcutaneous tissue of rat's hind paw.

To verify whether ATP via activation of P2X3,2/3 receptors mediates the mechanical hyperalgesia induced by bradykinin, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine, TNP-ATP or A-317491 was co-administered with each one of these mediators. Co-administration of TNP-ATP (240µg/paw) or A-317491 (20 and 60µg/paw) significantly reduced ($p<0.05$, Tukey test) the mechanical hyperalgesia induced by bradykinin (1.5µg/paw, Fig. 1B) when compared with PBS (control group), but did not affect the mechanical hyperalgesia when administered on contralateral rat hind paw ($p>0.05$, Tukey test, Fig. 1B), rulling out its systemic effect. Co-administration of the TNP-ATP (240µg/paw) or A-317491 (60µg/paw) did not reduce ($p>0.05$, Tukey test) the hyperalgesic response induced by TNF-α (0.8pg/paw, Fig.1C), IL-1β (0.15pg/paw, Fig. 1D), IL-6 (0.1ng/paw, Fig. 1E), CINC-1 (1.0pg/paw, Fig. 1F), PGE_2 (0.1µg/paw, Fig. 1G) or Dopamine (10µg/paw, Fig. 1H) when compared with PBS control group.

Effect of P2X3,2/3 receptors antagonists on local increase of neutrophil migration induced by bradykinin

Because only the hyperalgesia induced by bradykinin was reduced by P2X3,2/3 receptors antagonists, we verify whether endogenous ATP via activation of P2X3,2/3 receptors mediates neutrophils migration induced by bradykinin. TNP-ATP, A-317491 or PBS was co-administrated with bradykinin and the MPO activity in subcutaneous tissue of rat's hind paw was quantified 3h after their injections. TNP-ATP (240μg/paw) or A-317491 (60μg/paw) did not reduce ($p>0.05$, Tukey test) the MPO activity induced by bradykinin (1.5μg/paw, Fig. 2) when compared with PBS control group.

Effect of P2X3,2/3 receptors antagonists on local increase in cytokines concentration induced by bradykinin

To verify whether endogenous ATP via activation of P2X3,2/3 receptors mediates the release of pro-inflammatory cytokines induced by bradykinin, TNP-ATP, A-317491 or PBS was co-administrated with bradykinin in subcutaneous tissue of rat's hind paw and the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 were quantified 3h after the administration of bradykinin. TNP-ATP (240μg/paw) or A-317491 (60μg/paw) did not affect ($p>0.05$, Tukey test) the increase in concentration of TNF-α (Fig. 3A), IL-1β (Fig.3B), IL-6 (Fig. 3C) and CINC-1 (Fig. 3D) induced by bradykinin (1.5μg/paw) when compared with PBS control group.

Discussion

In this study we demonstrated that the selective P2X3,2/3 receptors antagonist A-317491 (Jarvis et al., 2002) or the P2X1,3,2/3,1/5 receptors antagonist TNP-ATP (Jarvis et al., 2001) prevented the bradykinin-induced mechanical hyperalgesia, whereas the mechanical hyperalgesia induced by TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine was not affect. These findings suggest that bradykinin in the subcutaneous tissue, as well as in culture cells (Chopra et al., 2005, Zhao et al., 2007), induces the release of ATP, which mediates bradykinin-induced hyperalgesia through P2X3,2/3 receptor activation.

Considering that bradykinin is an inflammatory mediator released at the early phase of inflammatory hyperalgesia (Ferreira et al., 1993a, Ferreira et al., 1993b), our results that A-317491 or TNP-ATP prevented the hyperalgesic response induced by bradykinin suggest that endogenous ATP via activation of P2X3,2/3 receptors has a role in the beginning of the development of inflammatory hyperalgesia.

It has been described that bradykinin induces hyperalgesia by two distinct pathways that ultimately result in the local production of prostaglandins and in the local release of sympathomimetic amines (Ferreira et al., 1993a, Ferreira et al., 1993b), which directly sensitize the primary afferent nociceptor (Gold et al., 1996, Rush and Waxman, 2004). Therefore, the prevention of bradykinin-induced inflammatory hyperalgesia by the co-administration of P2X3,2/3 receptor antagonists suggests that the activation of the P2X3,2/3 receptors must be crucial to prostaglandin- and sympathomimetic amines-mediated sensitization of the

primary afferent nociceptor. It has been proposed that bradykinin triggers the synthesis of prostaglandins and sympathomimetic amines through the release of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 (Ferreira et al., 1993a, Ferreira et al., 1993b). Therefore, the findings that the co-administration of A-317491 or TNP-ATP with bradykinin did not affect the endogenous release of cytokines induced by bradykinin indicates that the mechanism by which the endogenous ATP via activation of P2X3,2/3 receptors mediates the mechanical hyperalgesia induced by bradykinin is not dependent on cytokines release. It is important to point out that it has been described that bradykinin-induced hyperalgesia depends on pro-inflammatory cytokines (Ferreira et al., 1993a, Ferreira et al., 1993b) and, as demonstrated in this study, bradykinin induces the release of cytokines. However, bradykinin but not pro-inflammatory cytokines-induced hyperalgesia depends on P2X3,2/3 receptors. In addition, the release of cytokines by bradykinin does not depend on the endogenous release of ATP and the activation of P2X3,2/3 receptors. This apparent contradiction can be explained by the fact that the mechanism involved in the hyperalgesia induced by exogenous TNF-α, IL-1β, IL-6 or CINC-1 may differ from the mechanisms involved in the hyperalgesia induced by these cytokines when endogenously released by bradykinin.

We also demonstrated that co-administration of A-317491 or TNP-ATP with bradykinin did not affect the neutrophil migration induced by bradykinin, indicating that the mechanism by which the endogenous ATP via activation of P2X3,2/3

receptors mediates the mechanical hyperalgesia induced by bradykinin is not dependent on neutrophil migration.

The findings of this study also demonstrated that the hyperalgesia induced by PGE_2 or dopamine was not affected by P2X3,2/3 receptors antagonists. These data suggest that, similarly to pro-inflammatory cytokines, the hyperalgesia induced by these final inflammatory mediators does not depend on the release of ATP and P2X3,2/3 receptor activation.

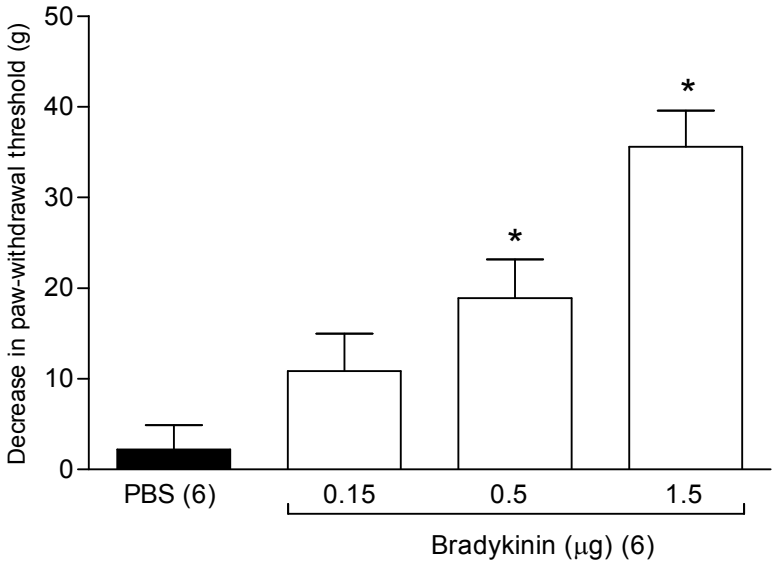
Considering that the mRNA distribution of P2X3 receptors is restricted to primary afferent neurons (Chen et al., 1995, Kennedy and Leff, 1995, Lewis et al., 1995, Collo et al., 1996), it is plausible to hypothesize that the mechanism by which endogenous ATP contributes to mechanical hyperalgesia induced by bradykinin is by a direct action on P2X3 receptors expressed on primary afferent nociceptors. Studies that supports this hypothesis demonstrated that the treatment with oligonucleotide antisense against P2X3 receptors significantly reduced inflammatory hyperalgesia induced by Complete Adjuvant Freund (Barclay et al., 2002, Honore et al., 2002a), carrageenan (data not published) or nerve injury (Barclay et al., 2002, Honore et al., 2002a). Recent studies have demonstrated that TNF-α acting on primary afferent nociceptor can increase its susceptibility to the development of inflammatory hyperalgesia induced by PGE_2 (Parada et al., 2003a, Parada et al., 2003b). A similar sensitizing effect of the P2X3 neuronal receptor activation by ATP could be involved in the mechanism underlying bradykinin-induced hyperalgesia.

In summary, we demonstrated that endogenous ATP via activation of P2X3,2/3 receptors mediates the mechanical hyperalgesia induced by bradykinin but not by TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or dopamine. The mechanism by which endogenous ATP via activation of P2X3,2/3 receptors mediates bradykinin-induced mechanical hyperalgesia does not depend on the release of cytokines and on neutrophil migration. We suggest that endogenous ATP contributes to the mechanical hyperalgesia induced by bradykinin by a direct action on P2X3 receptors expressed on the primary afferent nociceptors.

Figures and legends

Figure 1

A



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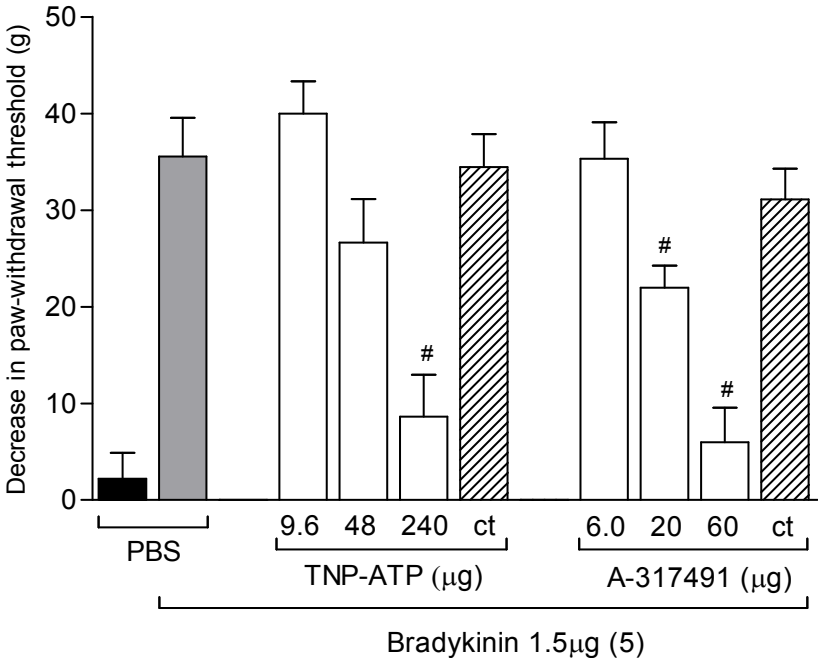
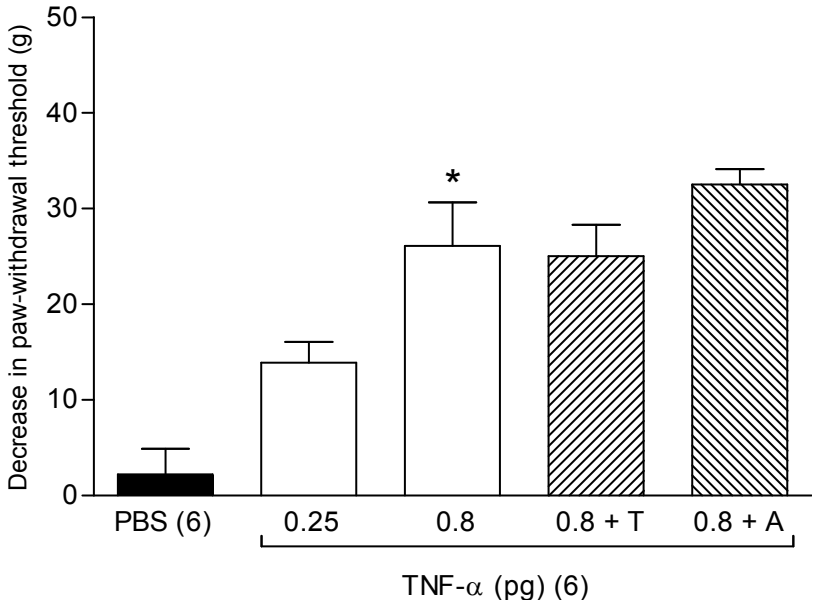
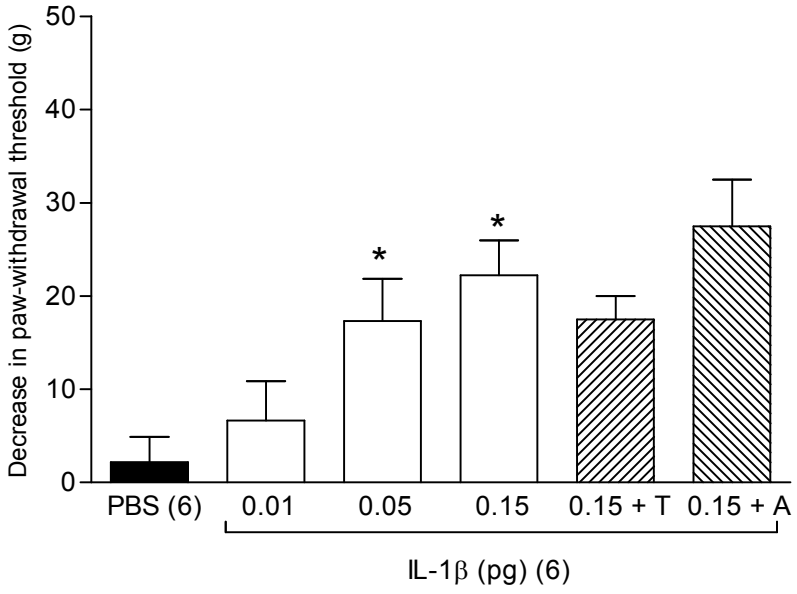


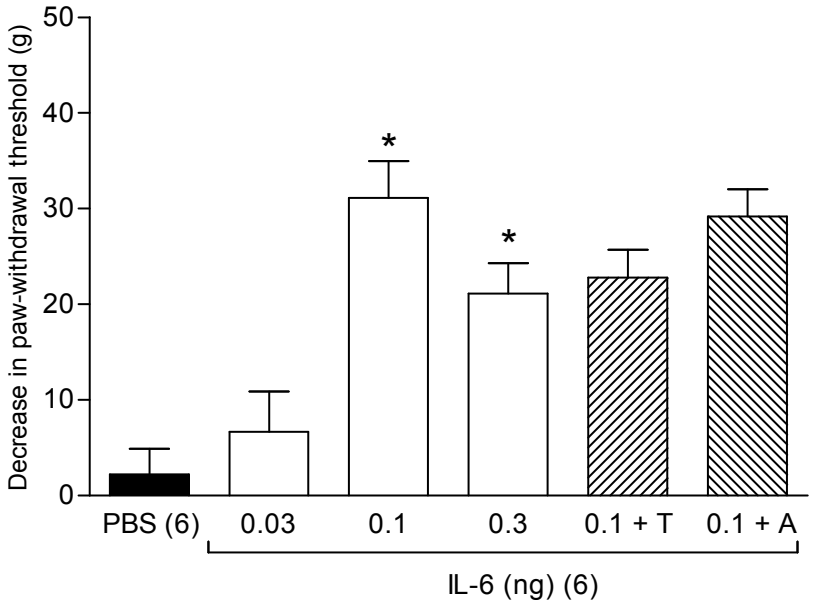
C


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
PBS (6)
0.25
0.8
0.8 + T
0.8 + A
TNF-α (pg) (6)

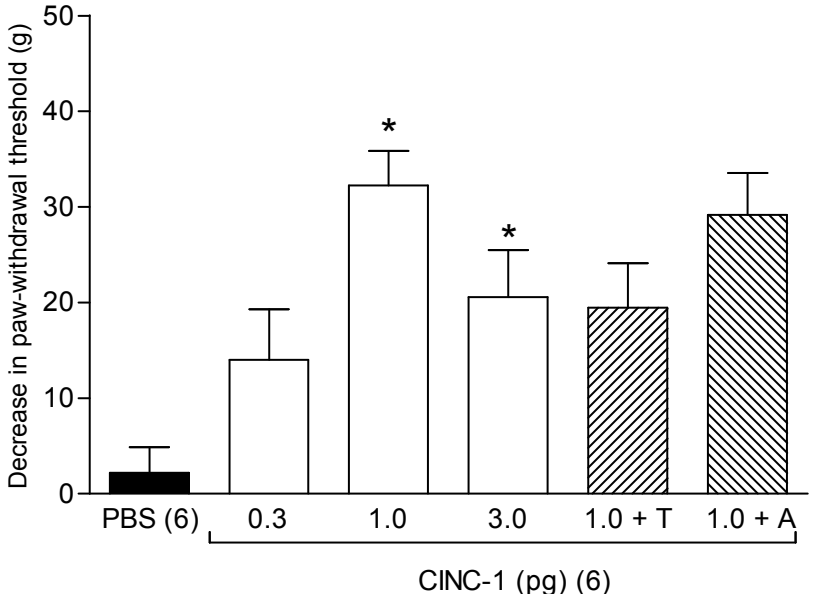
D


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
*
PBS (6)
0.01
0.05
0.15
0.15 + T
0.15 + A
IL-1β (pg) (6)

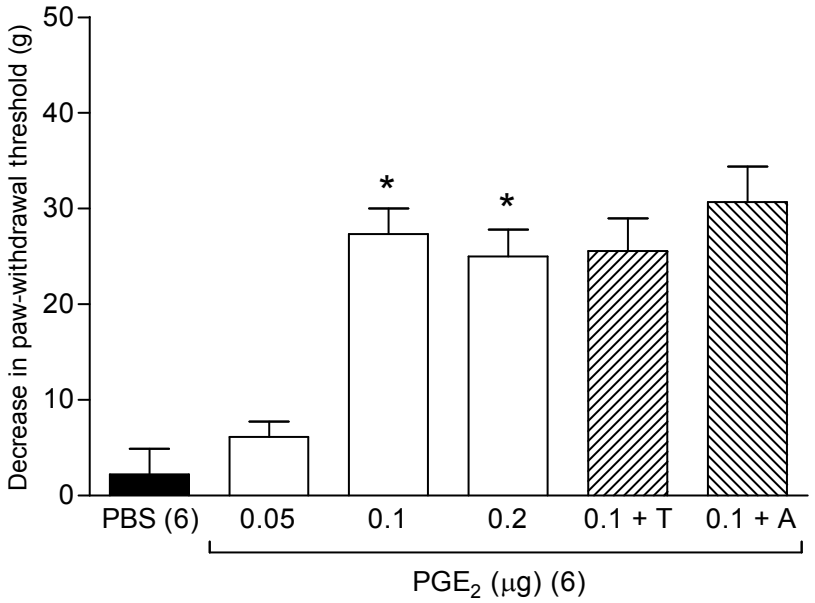
E


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
*
PBS (6)
0.03
0.1
0.3
0.1 + T
0.1 + A
IL-6 (ng) (6)

F


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
*
PBS (6)
0.3
1.0
3.0
1.0 + T
1.0 + A
CINC-1 (pg) (6)

G


Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
*
PBS (6)
0.05
0.1
0.2
0.1 + T
0.1 + A
PGE2 (μg) (6)

H

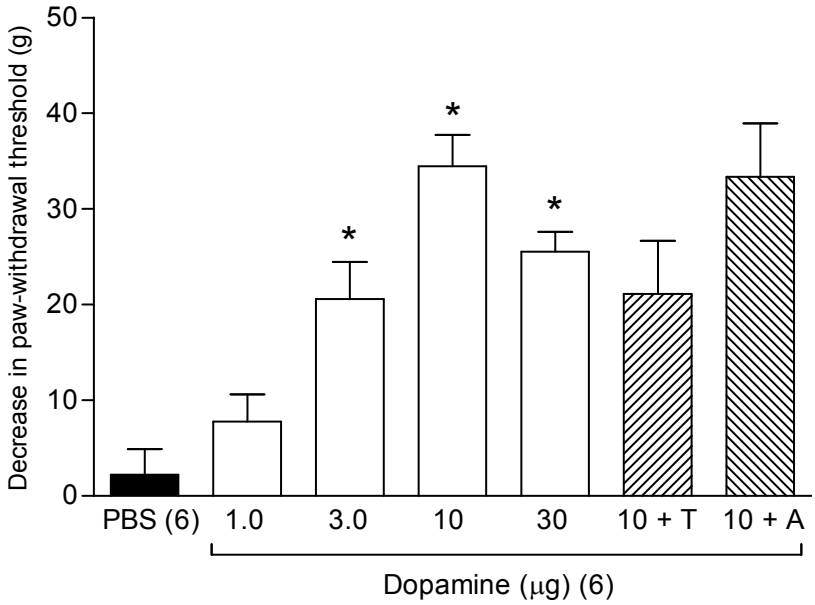

Decrease in paw-withdrawal threshold (g)
50
40
30
20
10
0
*
*
*
PBS (6)
1.0
3.0
10
30
10 + T
10 + A
Dopamine (μg) (6)

Fig_1.Effect of P2X3,2/3 receptors antagonists on mechanical hyperalgesia induced by TNF-α, IL-1β, IL-6, CINC-1, PGE_2 or Dopamine

The subcutaneous injection of Bradykinin (0.5 and 1.5μg/paw, **A**), TNF-α (0.8pg/paw, **C**), IL-1β (0.05 and 0.15pg/paw, **D**), IL-6 (0.1, 0.3 and 1.0ng/paw, **E**), CINC-1 (1.0 and 3.0pg/paw, **F**), PGE_2 (0.1 and 0.2μg/paw, **G**) or dopamine (3.0, 10 and 30μg/paw, **H**) induced mechanical hyperalgesia in a dose-dependent manner 3h after each injection on subcutaneous tissue of rat's hind paw ($p<0.05$, Tukey test). The co-administration of the P2X1,3,2/3,1/5 receptors antagonist TNP-ATP (T, 240μg/paw) or P2X3,2/3 receptors antagonist A-317491 (A, 20 and 60μg/paw) significantly reduced ($p<0.05$, ANOVA with pos hoc Tukey test) the mechanical hyperalgesia induced by bradykinin (1.5μg/paw, **B**), but not by TNF-α (0.8pg/paw, **C**), IL-1β (1.5pg/paw, **D**), IL-6 (0.1ng/paw, **E**), CINC-1 (1.0pg/paw, **F**), PGE_2 (0.1μg/paw, **G**) or Dopamine (10μg/paw, **H**). The maximal dose of TNP-ATP or A-317491 applied on the contralateral paw (c.t.) did not affect ($p>0.05$, t-test) bradykinin-induced hyperalgesia. The symbol "*" indicates statistically significant when compared with PBS. The symbol "#" indicates statistically significant when compared with PBS co-administered with bradykinin.

Figure 2

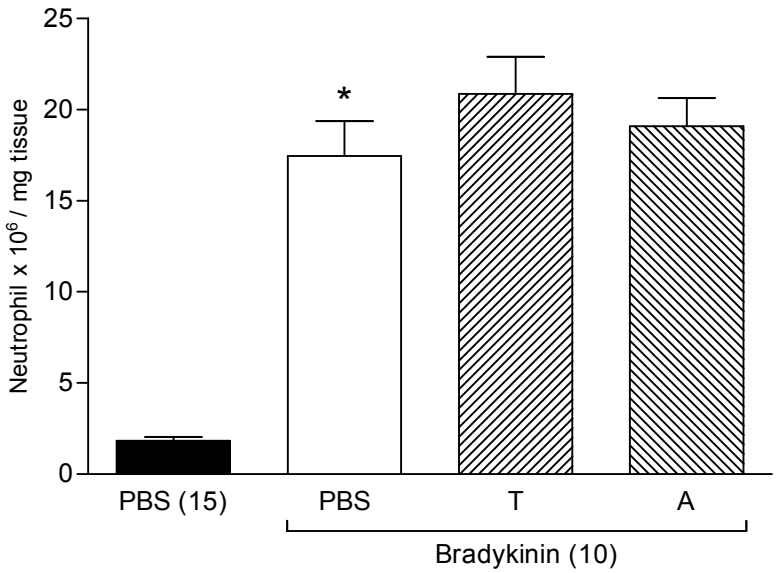
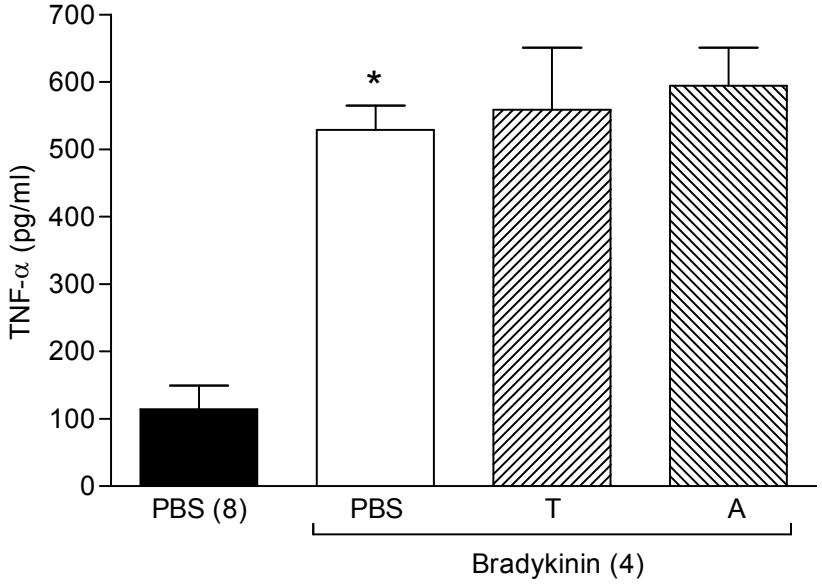


Fig.2_ Effect of P2X3,2/3 receptors antagonists on neutrophil migration induced by bradykinin

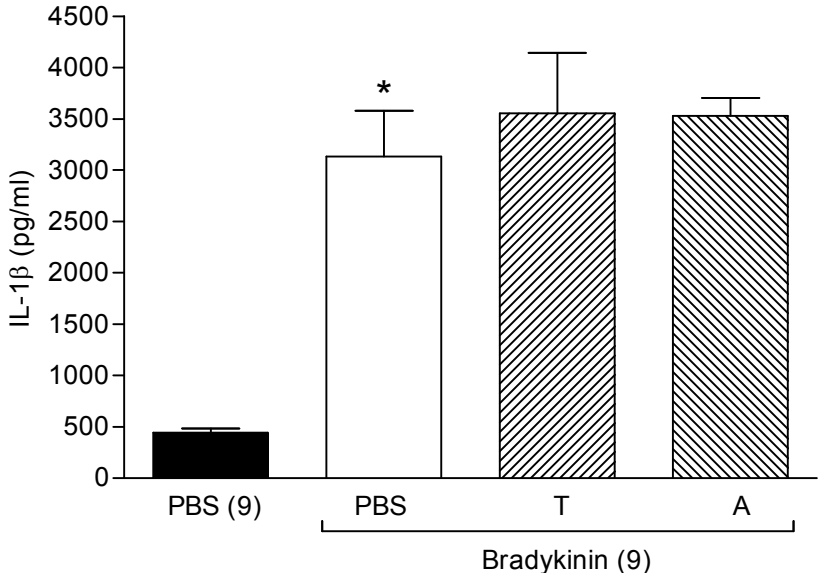
Bradykinin (BK, 1.5μg/paw) induced significantly increase ($p<0.05$, t-test) on MPO activity on subcutaneous tissue of rats hind paw when compared with PBS control group. Co-administration of TNP-ATP (T, 240μg/paw) or A-317491 (A, 60μg/paw) with bradykinin did not affect ($p>0.05$, t-test) the MPO activity induced by bradykinin. The symbol "*" indicates statistically significant when compared with PBS

Figure 3

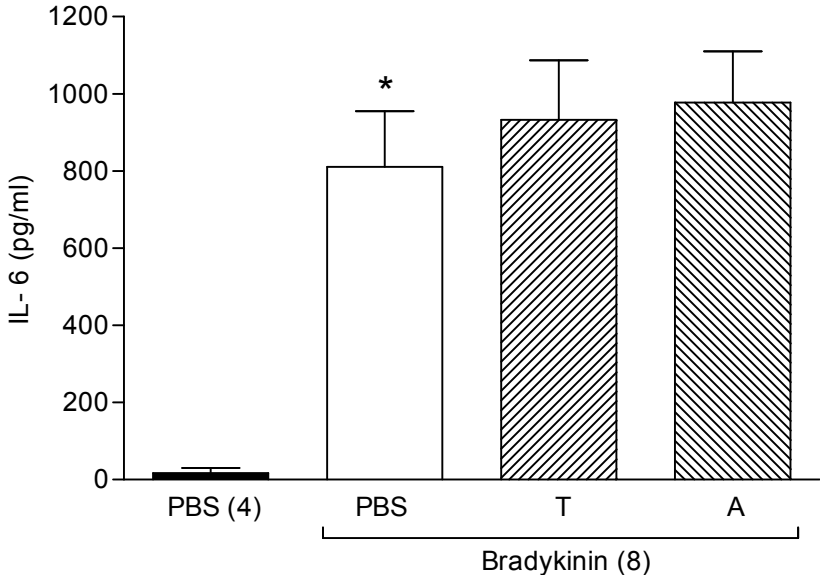
A.


700
600
500
400
300
200
100
0
TNF-α (pg/ml)
*
PBS (8)
PBS
T
A
Bradykinin (4)

B.


4500
4000
3500
3000
2500
2000
1500
1000
500
0
IL-1β (pg/ml)
*
PBS (9)
PBS
T
A
Bradykinin (9)

C.


1200
1000
800
600
400
200
0
IL- 6 (pg/ml)
*
PBS (4)
PBS
T
A
Bradykinin (8)

D.

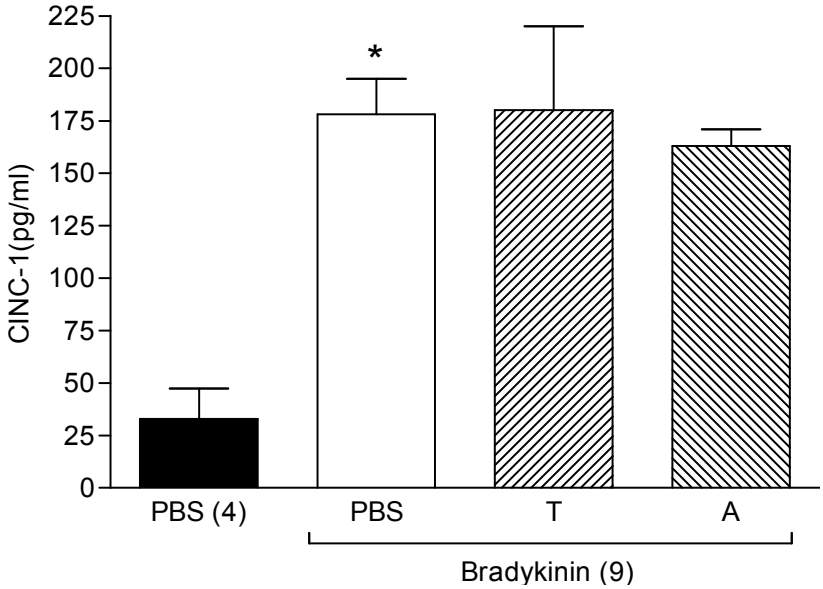

225
200
175
150
125
100
75
50
25
0
CINC-1(pg/ml)
*
PBS (4)
PBS
T
A
Bradykinin (9)

Fig.3_ Effect of P2X3,2/3 receptors antagonists on release of cytokines induced by bradykinin

The administration of TNP-ATP (T, 240μg/paw) or A-317491 (A, 60μg/paw) did not affect ($p>0.05$, ANOVA with *pos hoc* Tukey test) the bradykinin (1.5μg/paw)-induced local increase in the concentration of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D**).The symbol "*" indicates statistically significant when compared with PBS co-administered with bradykinin.

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CAPÍTULO 3

O presente artigo será submetido ao periódico “Neuroscience”.

Activation of P2X1,3,2/3 receptors induces mechanical hyperalgesia by indirect mechanisms

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Abstract

The aim of this study was to verify whether the P2X1,3,2/3 receptors agonist α,β-methylene ATP (α,β-meATP) induces mechanical hyperalgesia in the subcutaneous tissue of the rat's hind paw by an indirect sensitization of the primary afferent nociceptors. Subcutaneous injection of α,β-meATP induced a dose-dependent mechanical hyperalgesia. The α,β-meATP-induced mechanical hyperalgesia was significantly reduced by the selective P2X3,2/3 receptors antagonist A-317491, by the selective B1- or B2-receptor antagonist DALBK and bradyzide, respectively, by the cyclo-oxygenase inhibitor indomethacin, by the β_1- or β_2-adrenoceptor antagonist atenolol and ICI 118,551, respectively, and by the nonspecific selectin inhibitor fucoidan. α,β-meATP also induced release of the cytokines TNF-α, IL-1β, IL-6 and CINC-1 and neutrophil migration. Taken together, these findings suggest that α,β-meATP induces mechanical hyperalgesia by an indirect action on the primary afferent nociceptor of the subcutaneous tissue of the rat's hind paw mediated by release of bradykinin, prostaglandin, sympathomimetic amines, pro-inflammatory cytokines and by neutrophil migration.

Key words: α,β-meATP, hyperalgesia, cytokines, neutrophil migration, P2X3,2/3 receptors.

Introduction

P2X receptors are a family of ligand-gated ion channels activated by extracellular ATP that are involved in pain mechanisms. Recent reports using behavioral nociceptive models with gene knockout methods (Cockayne et al., 2005), antisense oligonucleotide technologies (Barclay et al., 2002, Honore et al., 2002a) and selective P2X3,2/3 receptors antagonist (Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty et al., 2005, Sharp et al., 2006) indicate that the activation of P2X3,2/3 receptors by extracellular ATP contributes to the development of inflammatory hyperalgesia. It has also been demonstrated that the administration of the selective P2X1,3,2/3 receptor agonist α,β-methylene ATP (α,β-meATP) in rat's hind paw induces mechanical hyperalgesia (Barclay et al., 2002), mechanical allodynia (Tsuda et al., 2000, Wang et al., 2007) and thermal hyperalgesia (Hamilton et al., 1999, Waldron and Sawynok, 2004).

Following tissue injury, an inflammatory response is generated by local release of inflammatory mediators which induce and/or maintain the inflammatory hyperalgesia (Verri et al., 2006). It has been proposed that the release of inflammatory mediators, such as bradykinin, TNF-α, IL-1β, IL-6, IL-8, prostaglandins and sympathomimetic amines (Cunha et al., 1992, Ferreira et al., 1993a, Loram et al., 2007) and the neutrophils migration (Jain et al., 2001, Tambeli et al., 2006, Oliveira et al., 2007a) participates of the development of inflammatory hyperalgesia.

Despite the role of extracellular ATP and P2X3,2/3 activation on inflammatory hyperalgesia, the mechanism by which the selective P2X1,3,2/3 receptor agonist α,β-meATP induces hyperalgesia is unknown. Therefore, the aim of this study was to verify whether the selective P2X1,3,2/3 receptor agonist α,β-meATP induces mechanical hyperalgesia by an indirect action on primary afferent nociceptors. To this end, we tested the hypothesis that the cyclo-oxygenase inhibitor indomethacin, the β_1- or β_2-adrenoceptor antagonist atenolol and ICI 118,551, respectively, the selective B1- or B2-receptor antagonist DALBK and bradyzide, respectively, reduce the mechanical hyperalgesia induced by α,β-meATP in the subcutaneous tissue of the rat's hind paw. Also, we tested the hypothesis that α,β-meATP induces the release of the inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 (rat IL-8 related chemokine) and neutrophil migration, which participate in the development of P2X1,3,2/3 activation-induced hyperalgesia.

Experimental procedures

Drugs and doses

The follow drugs were used: the agonist of P2X1,3,2/3 receptors α,β-methyleneATP lithium salt (α,β-meATP; 0.5, 10, 25, 50 and 100μg/paw), the antagonist of P2X3,2/3 receptors 5-([(3-Phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl)-1,2,4-benzenetricarboxylic acid (A-317491; 6, 20 and 60μg/paw); the bradykinin B1 receptor antagonist Des-Arg^{8}-Leu^{9}-BK (DALBK – 0.5, 1.5 and 3.0μg/paw); the bradykinin B2 receptor antagonist Bradyzide (0.15, 0.5 and 1.5μg/paw); the β_1 receptor antagonist atenolol (2.0 and 6.0μg/paw); the β_2 receptor antagonist ICI 118,551 (0.5, 1.0 and 1.5μg/paw); the cyclooxygenase inhibitor indomethacin (10, 25 and 50μg/paw) and the nonspecific selectin inhibitor fucoidan [25mg/Kg, i.v., (Zhang et al., 2001)] were all obtained from Sigma Chemicals, St Louis, Missouri, USA. All drugs were dissolved in saline (0.9% NaCl).

Subjects

Male albino Wistar rats weighing 200 – 350g were used. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of IASP on using laboratory animals (Zimmermann, 1983). Experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas - Unicamp. Animal suffering and the

number of rats per group were kept at a minimum. Rats 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 on a temperature-controlled room test (± 23ºC) for a 1-hour habituation period prior to the test.

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., 2007a). 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 50μl.

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., 2007a). 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

The subcutaneous tissues of dorsum of the rat's hind paw were collected 1-hour post the subcutaneous administration of α,β-meATP 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-methyl-sulfonyl 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 TNF-alpha/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.

Measurement of myeloperoxidase activity (MPO)

The neutrophil migration to the site of α,β-meATP administration in the skin of rat's hind paw was evaluated by the myeloperoxidase (MPO) kinetic-colorimetric assay as previously described (Bradley et al., 1982). Approximately 0.5 cm^2 of cutaneous tissue was harvested 60 minutes after the subcutaneous injection of α,β-meATP. The samples were homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 1.015 M NaEDTA) followed by centrifugation at 3000 rpm for 15 min. The pellet was subjected to hypotonic lyses (1.5 mL of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). After that, the tissue was snap-frozen in liquid nitrogen three times and was centrifuged at 10,000 rpm for 15 min and was re-homogenized. Myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM). Results were calculated by comparing the optical density of hind paw tissue supernatant with a standard curve of neutrophil (> 95% purity) numbers. The results were presented as number of neutrophils x 10^6/mg tissue. All procedures were repeated three 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. Data are expressed in figures by the decrease with paw-withdrawal threshold and presented as means ± S.E.M.

Results

α,β-meATP induced mechanical hyperalgesia

Subcutaneous administration of α,β-meATP (50μg/paw) in the dorsum of the rat's hind paw induced a significant mechanical hyperalgesia 60 min., but not 120 or 180 min. after its administration (Fig. 1A, $p<0.05$, Tukey test). Therefore, in further experiments, the mechanical hyperalgesia was evaluated only 60 min. after the administration of α,β-meATP.

Subcutaneous administration of α,β-meATP (50 and 100μg/paw) induced a dose-related mechanical hyperalgesia (Fig.1B, $p<0.05$, Tukey test). The sub-maximal dose of 50μg/paw was used in following experiments.

To verify whether the mechanical hyperalgesia induced by α,β-meATP was mediated by P2X3,2/3 receptors, the selective P2X3,2/3 receptor antagonist A-317491 was co-administered with α,β-meATP. A-317491 (20 and 60μg/paw) significantly reduced (Fig. 1C, $p<0.05$, Tukey test) the mechanical hyperalgesia induced by α,β-meATP and did not affect ($p>0.05$, T test) the hyperalgesic response when administered on contralateral hind paw, rulling out a systemic effect. Co-administration of A-317491 (60μg/paw) with 0.9% NaCl did not change the mechanical withdrawal threshold.

Effect of cyclo-oxygenase inhibitor on mechanical hyperalgesia induced by α,β-meATP

To verify whether mechanical hyperalgesia induced by α,β-meATP was mediated by prostaglandins, rats were treated with local administration of indomethacin 30 min. before and the mechanical hyperalgesia was evaluated 60 min. after α,β-meATP administration. Indomethacin (25 and 50μg/paw) significantly reduced (Fig.2, $p<0.05$, Tukey test) the mechanical hyperalgesia induced by α,β-meATP when administered on the ipsilateral but not on the contralateral paw ($p>0.05$, Tukey test). Co-administration of indomethacin (50μg/paw) with 0.9% NaCl did not change the mechanical withdrawal threshold.

Effect of β_1- or β_2- adrenoceptor antagonists on mechanical hyperalgesia induced by α,β-meATP

To verify whether mechanical hyperalgesia induced by α,β-meATP was mediated by sympathomimetics amines, the β_1- or β_2- adrenoceptor antagonists atenolol and ICI 118,551, respectively, was co-administered with α,β-meATP. Atenolol (6.0μg/paw, Fig. 3A) or ICI 118,551 (1.0 and 1.5μg/paw, Fig. 3B) significantly reduced ($p<0.05$, Tukey test) the mechanical hyperalgesia induced by α,β-meATP and did not affect ($p>0.05$, Tukey test) the hyperalgesic response when administered on contralateral hind paw, rulling out a systemic effect. Co-administration of atenolol (6.0μg/paw) or ICI 118,551 (1.5μg/paw) with 0.9% NaCl did not change the mechanical withdrawal threshold.

Effect of the bradykinin B1 or B2 receptor antagonists on mechanical hyperalgesia induced by α,β-meATP

To verify whether mechanical hyperalgesia induced by α,β-meATP was mediated by bradykinin, the bradykinin B1 or B2 receptor antagonists, DALBK and Bradyzide, respectively, was co-administered with α,β-meATP. DALBK (1.5 and 3.0μg/paw, Fig. 4A) or bradyzide (0.5 and 1.5μg/paw, Fig. 4B) significantly reduced ($p<0.05$, Tukey test) the mechanical hyperalgesia induced by α,β-meATP and did not affect ($p>0.05$, Tukey test) the hyperalgesic response when administered on contralateral hind paw. Co-administration of DALBK (3.0μg/paw) or bradyzide (0.5μg/paw) with 0.9% NaCl did not change the mechanical withdrawal threshold.

α,β-meATP induced increase in cytokines concentration in the subcutaneous tissue

To verify whether α,β-meATP induces the release of pro-inflammatory cytokines, α,β-meATP or 0.9% NaCl was administrated in subcutaneous tissue of rat's hind paw and the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 were quantified 60 min. after stimuli. Local administration of α,β-meATP induced significant increase ($p<0.05$, Tukey test) in concentration of TNF-α (Fig.5A), IL-1β (Fig. 5B), IL-6 (Fig. 5C) and CINC-1 (Fig. 5D) when compared with 0.9% NaCl administration. The subcutaneous administration of 0.9% NaCl did not alter ($p>0.05$, Tukey test) the concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naïve group.

α,β-meATP induced neutrophil migration

To verify whether α,β-meATP induces neutrophils migration, α,β-meATP or 0.9% NaCl was locally administrated and the MPO activity in subcutaneous tissue of rat's hind paw was quantified 60 min. after stimuli. The administration of α,β-meATP induced significant increase ($p<0.05$, Tukey test) in the MPO activity when compared with 0.9% NaCl administration (Fig. 6A). Also, pre-treatment with fucoidan (25mg/Kg, i.v.), 20 min before α,β-meATP significantly reduced ($p<0.05$, Tukey test) the MPO activity (Fig. 6A).

To verify whether neutrophils migration contributes to mechanical hyperalgesia induced by α,β-meATP, rats were treated with fucoidan 20 min. before and the mechanical hyperalgesia was evaluated 60 min. post α,β-meATP administration. Pre-treatment with fucoidan (25 mg/kg, i.v.) significantly reduced ($p<0.05$, Tukey test) the hyperalgesia induced by α,β-meATP (Fig 6B).

Discussion

α,β-meATP induced mechanical hyperalgesia

The α,β-meATP induced mechanical hyperalgesia in the subcutaneous tissue of the rat's hind paw. Although α,β-meATP is a P2X1,3,2/3 receptors agonist, the involvement of P2X1 seems to be unlikely, because the hyperalgesia induced by α,β-meATP was completely reversed by A-317491, a selective P2X3,2/3 receptor antagonist in a dose response manner. In addition, IP5I, a potent and selective P2X1 receptor antagonist, was ineffective at reducing inflammatory pain (Honore et al., 2002b) or mechanical hypersensitivity (Dai et al., 2004). Recent reports using different inflammatory pain models, such as local administration in the hind paw tissue of carrageenan (McGaraughty et al., 2003), Complete Freund Adjuvant (Honore et al., 2002a, Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty et al., 2005) or formalin (Souslova et al., 2000, Honore et al., 2002a, McGaraughty et al., 2005) indicate that the activation of P2X3,2/3 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia. Therefore, we suggest that the hyperalgesic response induced by α,β-meATP is mediated by activation of P2X3,2/3 receptors expressed on the subcutaneous tissue of rat's hind paw.

Indirect mechanisms underlying the α,β-meATP induced mechanical hyperalgesia

The findings of this study demonstrated that the cyclo-oxygenase inhibitor, β receptor antagonists or bradykinin receptor antagonists reduced the mechanical hyperalgesia induced by α,β-meATP. It has been described that the development of inflammatory hyperalgesia depends on local production of prostaglandins and local release of sympathomimetic amines that ultimately sensitizes the primary afferent nociceptors (Gold et al., 1996, Rush and Waxman, 2004). Although in carrageenan model of inflammation the hyperalgesia is the summation of the partial hyperalgesia induced by prostaglandin and sympathomimetic amines (Cunha et al., 1991, Cunha et al., 1992), our data demonstrated that in α,β-meATP-induced hyperalgesia both prostaglandin and sympathomimetic act synergically to sensitize primary afferent nociceptor. The findings of this study also demonstrated that B2 receptor antagonist, bradyzide reduced the α,β-meATP-induced hyperalgesia while the B1 receptor antagonist, DALBK blocked it. In contrast to B2 receptor, B1 receptors are generally absent in healthy tissues but its expression increases during an inflammatory process (Steranka et al., 1988, Davis and Perkins, 1994, Marie et al., 1999). Considering that, it is plausible to suggest that α,β-meATP induced an up-regulation of B1 receptors.

α,β-meATP induced cytokines release and neutrophils migration

Considering that the synthesis of prostaglandins and release of sympathomimetic amines depend on previous formation of cytokines (Cunha et al.,

1991, Cunha et al., 1992, Ferreira et al., 1993a), this study also investigated whether local administration of α,β-meATP induces an increase on cytokines concentration in the subcutaneous tissue. The findings of this study demonstrated that α,β-meATP increases the concentration of TNF-α, IL-1β, IL-6 and CINC-1 (rat IL-8 related chemokine). Because it has been proposed that bradykinin induces release of the pro-inflammatory cytokines TNF-α, IL-1β/IL-6 and CINC-1 (Ferreira et al., 1993a, Ferreira et al., 1993b), it is plausible to hypothesize that α,β-meATP induced the release of bradykinin that, in turn, triggers the release of TNF-α, IL-1β, IL-6 and CINC-1.

Although previous studies demonstrate that ATP induces the release of TNF-α (Hide et al., 2000, Suzuki et al., 2004), IL-1β (Perregaux and Gabel, 1998, Perregaux et al., 2000, Mehta et al., 2001), IL-6 (Inoue, 2002, Seiffert et al., 2006) and IL-8 (Idzko et al., 2003, Seiffert et al., 2006) via P2X7 receptor activation, the findings of this study demonstrated that the activation of P2X1,3,2/3 receptors by its exogenous agonist also induced cytokines release. However, because α,β-meATP induces endogenous release of ATP (Kirkpatrick and Burnstock, 1994), it is possible that the release of cytokines induced by α,β-meATP depends on an indirect activation of P2X7 receptor by endogenous released ATP.

The findings of this study also demonstrated that α,β-meATP induces neutrophils migration. Indeed, the release of pro-inflammatory cytokines and, in particular the chemokine CINC-1, induces neutrophils migration (Canetti et al., 2001, Bochenska-Marciniak et al., 2003, Ramos et al., 2003). Therefore, the

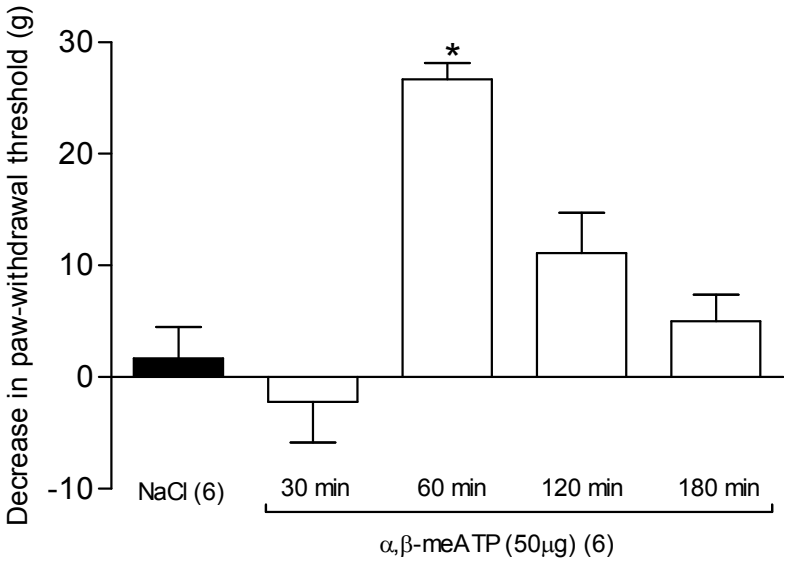
neutrophil migration induced by α,β-meATP probably results from its ability to release TNF-α, IL-1β, IL-6 and CINC-1. It has been demonstrated that the release of cytokines (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993a) and neutrophils migration (Jain et al., 2001, Tambeli et al., 2006, Oliveira et al., 2007a) participate of the development of inflammatory hyperalgesia. Although our data do not permit correlate the release of cytokines with the development of hyperalgesia induced by α,β-meATP, the findings of this study demonstrated that fucoidan, which inhibits the neutrophil migration also inhibited α,β-meATP-induced hyperalgesia.

In summary, this study suggest that the mechanical hyperalgesia induced by α,β-meATP via P2X3,2/3 receptors activation is mediated by an indirect action on the primary afferent nociceptor, which involves synthesis of prostaglandins, release of sympathomimetic amines, release of bradykinin, cytokines formation and neutrophil migration.

Figures and legends

Figure 1

A



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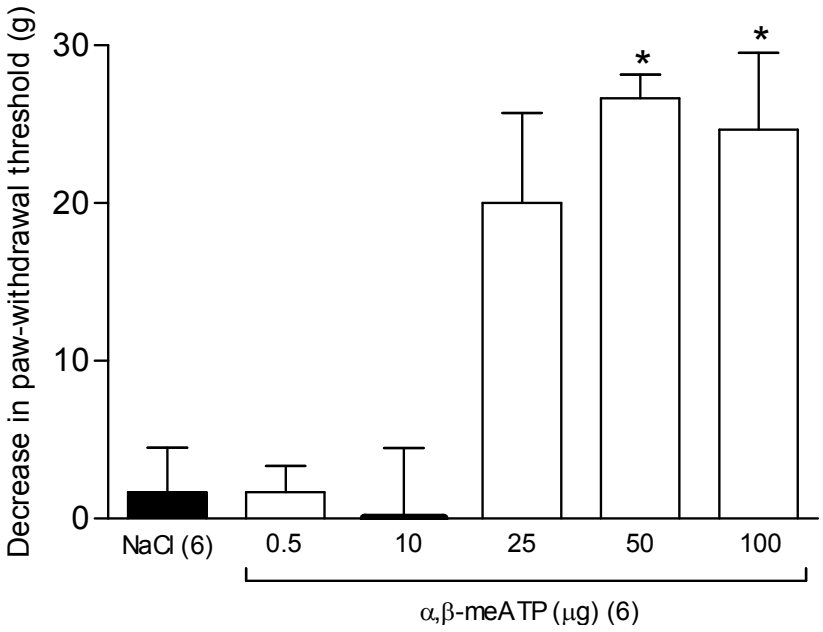

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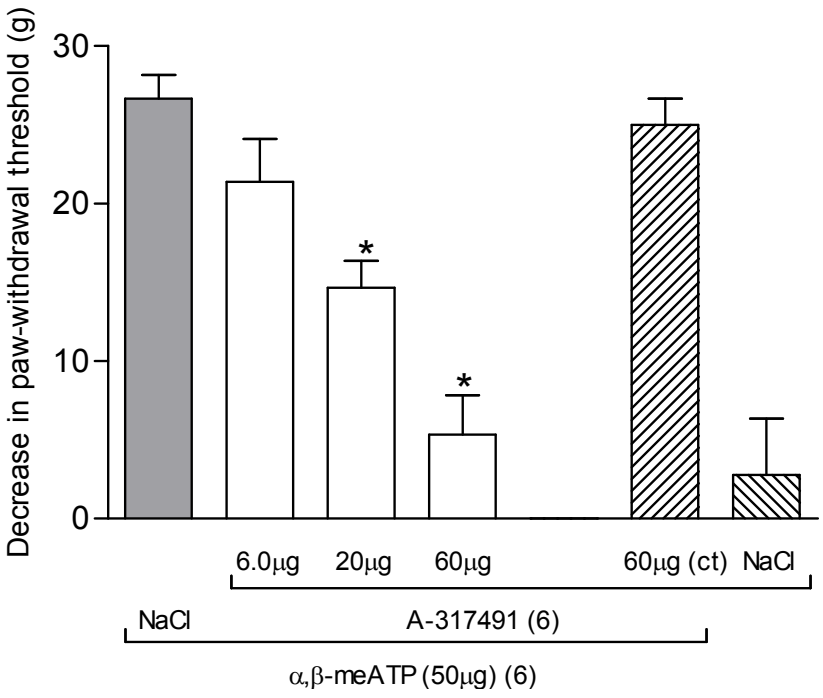


Fig.1_ α,β-meATP- induced mechanical hyperalgesia

Subcutaneous administration of α,β-meATP (50μg/paw) induced a significant mechanical hyperalgesia 60 min. after its administration when compared with 0.9% NaCl administration (**A**). α,β-meATP (0.5-100μg/paw) induced a dose-related mechanical hyperalgesia (**B**). Co-administration of the P2X3,2/3 receptors antagonist A-317491 (60μg/paw, **B**) with α,β-meATP (50μg/paw) blocked α,β-meATP -induced mechanical hyperalgesia. The highest doses of the antagonist applied on the contralateral paw (ct) did not affect α,β-meATP -induced mechanical hyperalgesia (**C**).In this and subsequent figures hyperalgesia was measured 60 min after α,β-meATP administration and the number of rats used are in parentheses.

The symbol "*" indicates statistically significant when compared with 0.9% NaCl group ($p<0.05$, ANOVA with pos hoc Tukey test).

Figure 2

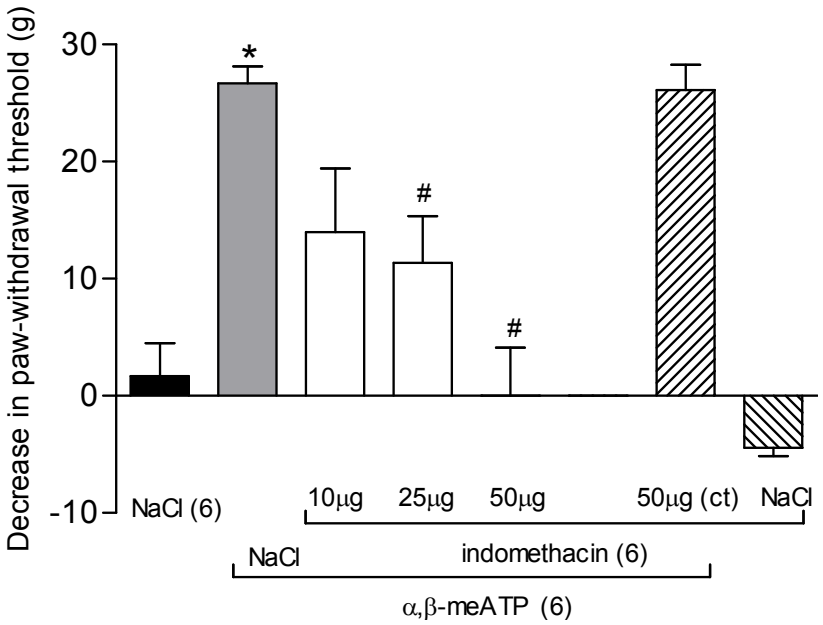
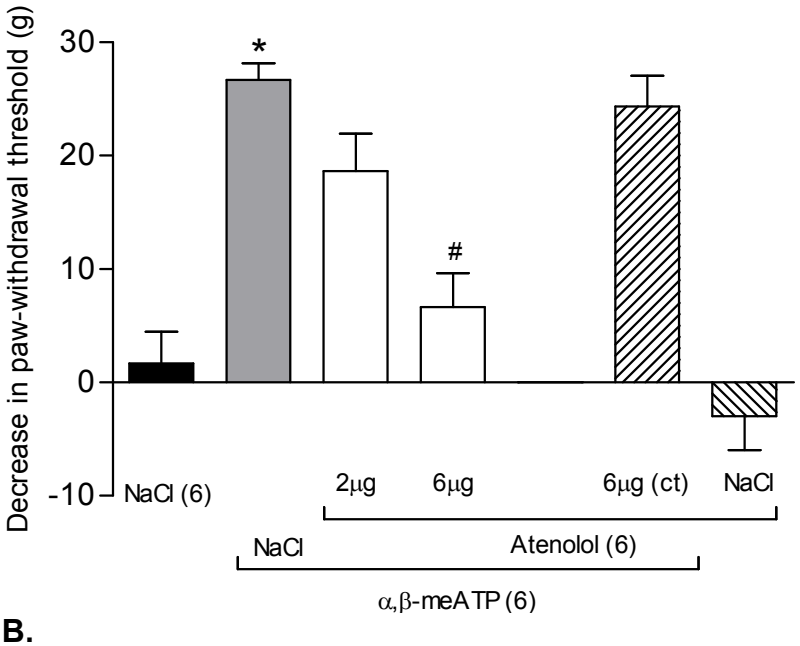


Fig. 2_ Effect of indomethacin on mechanical hyperalgesia induced by α,β-meATP

Local treatment with indomethacin 30 min. before prevented the mechanical hyperalgesia induced by α,β-meATP (50μg/paw) in a dose-related manner. Indomethacin (50μg/paw) administrated in contralateral (c.t.) hind paw or co-administrated with 0.9% NaCl did not affect the the hyperalgesic response or the mechanical nociceptive threshold, respectively. The symbol "*" indicates statistically significant when compared with 0.9% NaCl group ($p<0.05$, ANOVA with *pos hoc* Tukey test). The symbol "#" indicates statistically significant when compared with 0.9% NaCl co-administered with α,β-meATP group ($p<0.05$, ANOVA with *pos hoc* Tukey test).

Figure 3

A.


Decrease in paw-withdrawal threshold (g)
30
20
10
0
-10
*
#
NaCl (6)
2μg
6μg
6μg (ct)
NaCl
NaCl
Atenolol (6)
α,β-meATP (6)

B.

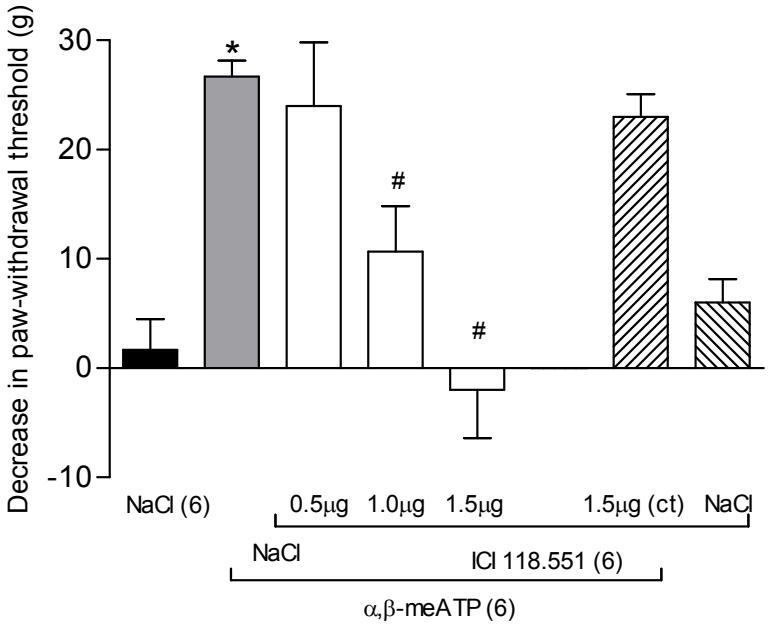
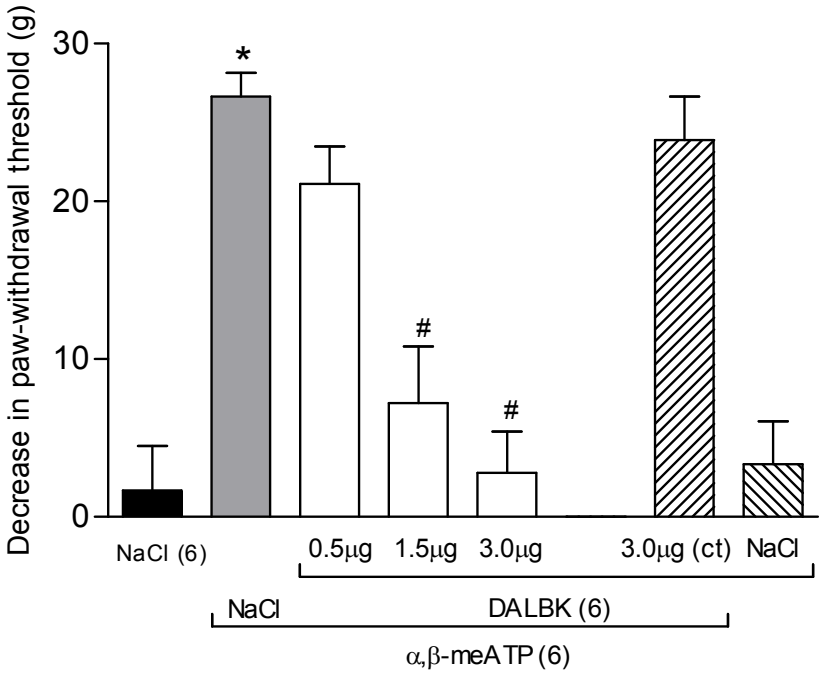

Decrease in paw-withdrawal threshold (g)
30
20
10
0
-10
*
#
#
NaCl (6)
0.5μg
1.0μg
1.5μg
1.5μg (ct)
NaCl
NaCl
ICI 118.551 (6)
α,β-meATP (6)

Fig. 3_Effect of β_1- or β_2- adrenoceptor antagonist on mechanical hyperalgesia induced by α,β-meATP

Atenolol (**A**) or ICI 118,551 (**B**) completely prevented the mechanical hyperalgesia induced by α,β-meATP (50μg/paw) in dose-related manner. Atenolol (6.0μg/paw) or ICI 118,551 (1.5μg/paw) administrated in contralateral (c.t.) hind paw or co-administrated with 0.9% NaCl did not affect the the hyperalgesic response or the mechanical nociceptive threshold, respectively. The symbol "*" indicates statistically significant when compared with 0.9% NaCl group ($p<0.05$, ANOVA with *pos hoc* Tukey test). The symbol "#" indicates statistically significant when compared with 0.9% NaCl co-administered with α,β-meATP group ($p<0.05$, ANOVA with *pos hoc* Tukey test).

Figure 4

A.


Decrease in paw-withdrawal threshold (g)
30
20
10
0
*
#
#
NaCl (6)
0.5μg
1.5μg
3.0μg
3.0μg (ct)
NaCl
NaCl
DALBK (6)
α,β-meATP (6)

B.

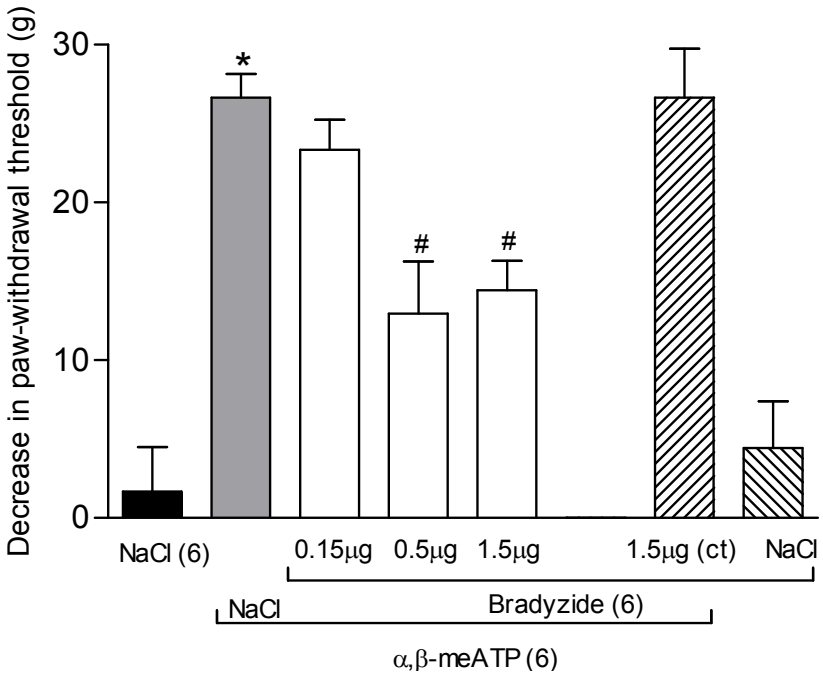
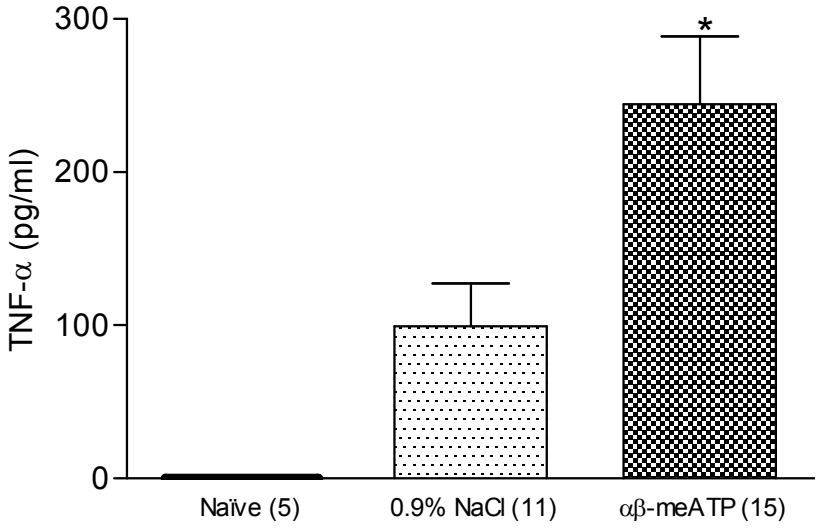

Decrease in paw-withdrawal threshold (g)
30
20
10
0
*
#
#
NaCl (6)
0.15μg
0.5μg
1.5μg
1.5μg (ct)
NaCl
NaCl
Bradyzide (6)
α,β-meATP (6)

Fig. 4_Effect of bradykinin B1 or B2 receptors antagonist on mechanical hyperalgesia induced by α,β-meATP

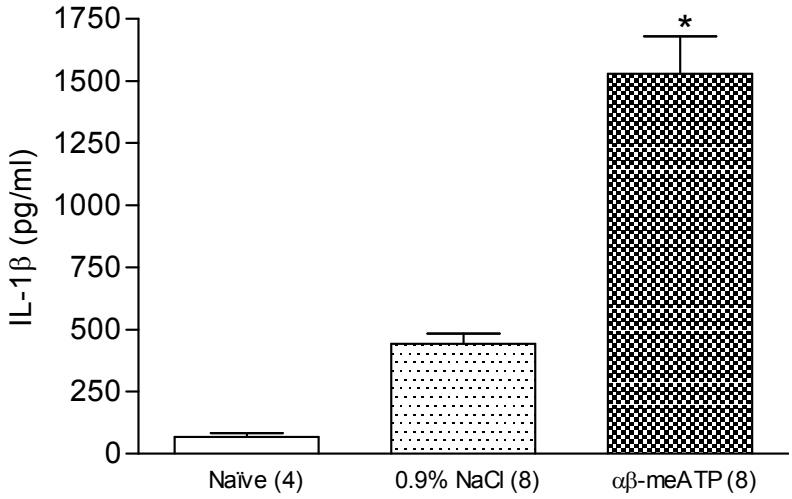
DALBK (**A**) or bradyzide (**B**) prevented and significantly reduced, respectively, the mechanical hyperalgesia induced by α,β-meATP (50μg/paw) in a dose-related manner. DALBK (3.0μg/paw) or bradyzide (1.5μg/paw) administrated in contralateral (c.t.) hind paw or co-administrated with 0.9% NaCl did not affect the the hyperalgesic response or the mechanical nociceptive threshold, respectively. The symbol "*" indicates statistically significant when compared with 0.9% NaCl group ($p<0.05$, ANOVA with *pos hoc* Tukey test). The symbol "#" indicates statistically significant when compared with 0.9% NaCl co-administered with α,β-meATP group ($p<0.05$, ANOVA with *pos hoc* Tukey test).

Figure 5

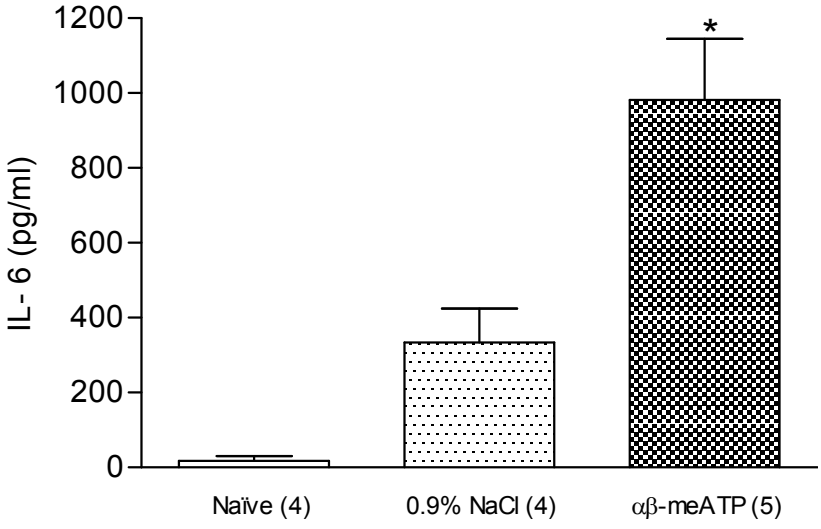
A.



B.


1750
1500
1250
1000
750
500
250
0
IL-1β (pg/ml)
*
Naïve (4)
0.9% NaCl (8)
αβ-meATP (8)

C.


1200
1000
800
600
400
200
0
IL- 6 (pg/ml)
*
Naïve (4)
0.9% NaCl (4)
αβ-meATP (5)

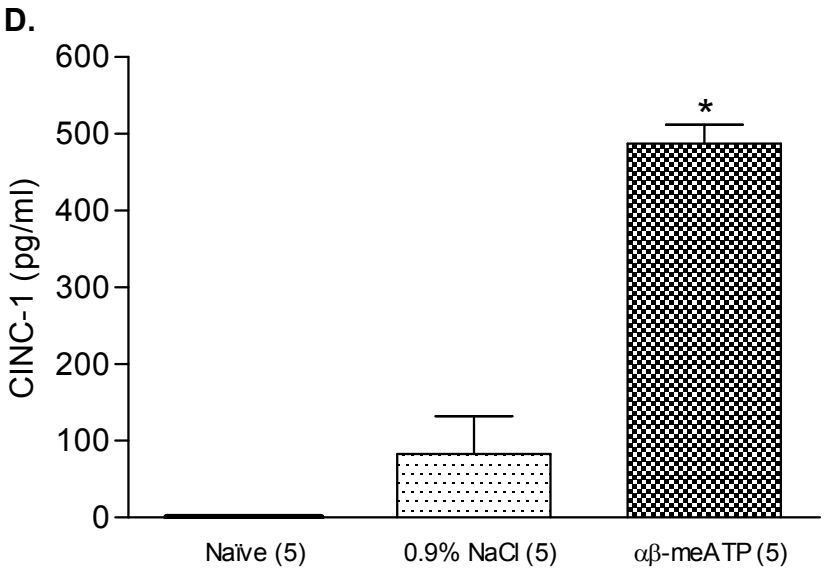
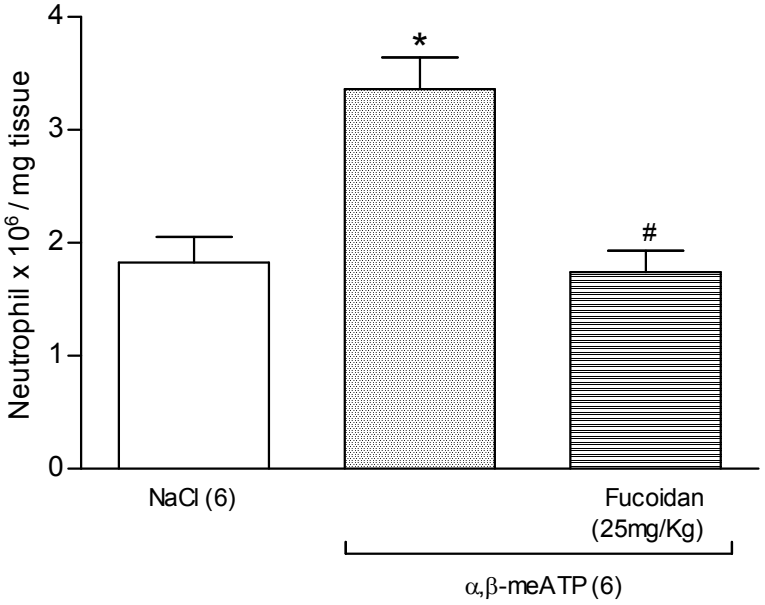


Fig. 5_ α,β-meATP- induced release of cytokines

Local administration of α,β-meATP (50μg/paw) induced significant local increase ($p<0.05$, Tukey test) of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D**) concentration when compared with 0.9% NaCl administration. The subcutaneous injection of 0.9% NaCl did not alter the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naïve group. The symbol "*" indicates statistically significant when compared with 0.9% NaCl group ($p<0.05$, ANOVA with *pos hoc* Tukey test).

Figure 6

A.


Neutrophil x 10^6 / mg tissue
4
3
2
1
0
*
#
NaCl (6)
Fucoidan
(25mg/Kg)
α,β-meATP (6)

B.

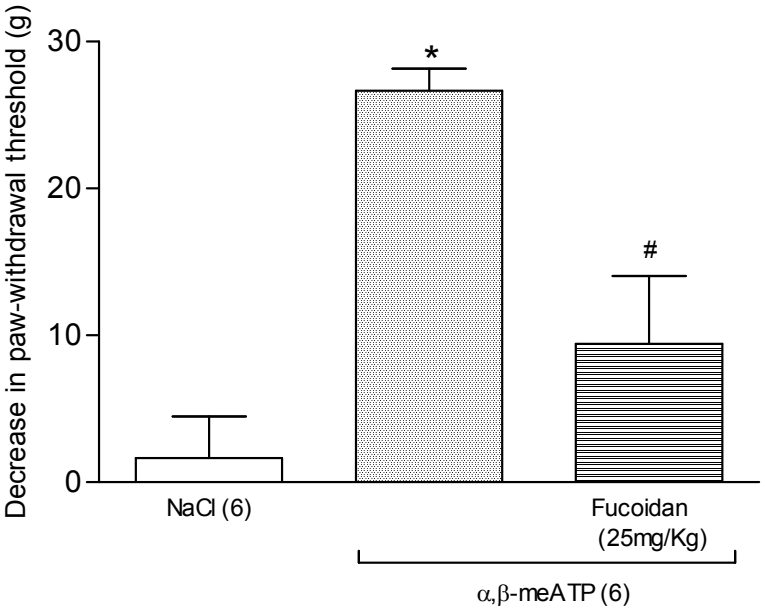

Decrease in paw-withdrawal threshold (g)
30
20
10
0
*
#
NaCl (6)
Fucoidan
(25mg/Kg)
α,β-meATP (6)

Fig. 6_ α,β-meATP -induced neutrophils migration

Local administration of α,β-meATP (50μg/paw) induced significant increase in the MPO activity when compared with 0.9% NaCl administration (**A**). Treatment with fucoidan (25mg/Kg, i.v.) 20 min before α,β-meATP significantly reduced the MPO activity (**A**) and the mechanical hyperalgesia (**B**). The symbol "*" indicates statistically significant when compared with 0.9% NaCl group ($p<0.05$, ANOVA with *pos hoc* Tukey test). The symbol "#" indicates statistically significant when compared with α,β-meATP group ($p<0.05$, ANOVA with *pos hoc* Tukey test).

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CONCLUSÕES

O presente trabalho demonstrou que (1) o ATP endógeno via ativação dos receptores P2X3,2/3 contribui para a hiperalgesia mecânica induzida pela carragenina através de uma sensibilização indireta dos nociceptores aferentes primários, mediada pela liberação prévia de TNF-α, e através da sensibilização direta dos nociceptores aferentes primários; (2) o mecanismo pelo qual a ativação dos receptores P2X3,2/3 pelo ATP endógeno contribui para a hiperalgesia induzida pela bradicinina independe da liberação de citocinas e migração de neutrófilos; (3) o α,β-meATP induz hiperalgesia mecânica através de uma sensibilização indireta dos nociceptores aferentes primários mediada pela síntese de prostaglandinas, liberação de aminas simpatomiméticas, liberação de citocinas e migração de neutrófilos.

CONSIDERAÇÕES GERAIS

Em uma perspectiva clínica-terapêutica, os resultados obtidos sugerem que, como a ativação dos receptores P2X3,2/3 pelo ATP endógeno é fundamental para o desenvolvimento da hiperalgesia inflamatória, os receptores P2X3,2/3 podem ser alvos farmacológicos interessantes para o desenvolvimento de medicamentos usados no controle da dor inflamatória. Ressalta-se ainda que a magnitude do efeito analgésico dos antagonistas de receptores P2X3,2/3 na inibição da hiperalgesia é comparável à dos antiinflamatórios esteroidais. Do ponto de vista do uso dos antagonistas de receptores P2X3,2/3 como uma ferramenta farmacológica, os resultados deste trabalho, vistos de uma maneira global, demonstram que os mecanismos envolvidos na hiperalgesia induzida por mediadores inflamatórios não são necessariamente os mesmos mecanismos envolvidos no desenvolvimento da hiperalgesia induzida por um agente inflamatório. A ação sinérgica dos vários mediadores inflamatórios, aumentando a susceptibilidade dos neurônios nociceptivos aferentes primários à ação de mediadores finais como as prostaglandinas e aminas simpatomiméticas, parece ser um novo mecanismo envolvido no desenvolvimento da hiperalgesia inflamatória que deve ser melhor investigado a partir dos resultados deste trabalho.

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CERTIFICADO

Certificamos que o Protocolo nº 707-1, sobre "MECANISMOS ENVOLVIDOS NA AÇÃO HIPERALGÉSICA DO ATP EM RATOS" sob a responsabilidade de Profa. Dra. Claudia Herrera Tambeli / Maria Claudia Gonçalves de Oliveira 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)-IB-UNICAMP em reunião de 05 de Agosto de 2004.

CERTIFICATE

We certify that the protocol nº 707-1, entitled "MECHANISMS FOR HYPERALGESIC ACTIONS OF ATP", 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 August 5, 2004.

Campinas, 05 e Agosto de 2004.

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Presidente

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Secretária

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