Jalile Garcia Schiavuzzo

"Mecanismos envolvidos na ação hiperalgésica induzida pela ativação dos receptores P2X3 e P2X2/3 no músculo gastrocnêmio de ratos"

Limeira

2013

UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS APLICADAS

FCA
UNICAMP • LIMEIRA

Jalile Garcia Schiavuzzo

"Mecanismos envolvidos na ação hiperalgésica induzida pela ativação dos receptores P2X3 e P2X2/3 no músculo gastrocnêmio de ratos"

Dissertação apresentada à Faculdade de Ciências Aplicadas da UNIVERSIDADE ESTADUAL DE CAMPINAS para obtenção do Título de Mestra em Ciências da Nutrição e do Esporte e Metabolismo, na área de **Biodinâmica do Movimento Humano e Esporte**.

Orientadora: Profa. Dra. Maria Cláudia Gonçalves de Oliveira Fusaro

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELA ALUNA JALILE GARCIA SCHIAVUZZO E ORIENTADA PELA PROF. DRA. MARIA CLÁUDIA GONÇALVES DE OLIVEIRA FUSARO

Profa. Dra. Maria Cláudia Gonçalves de Oliveira Fusaro

Limeira
2013

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Aplicadas
Sueli Ferreira Júlio de Oliveira - CRB 8/2380

Schiavuzzo, Jalile Garcia, 1980-
Sch31m Mecanismos envolvidos na ação hiperalgésica induzida pela ativação de receptores P2X3 e P2X2/3 no músculo gastrocnêmio de ratos / Jalile Garcia Schiavuzzo. – Campinas, SP : [s.n.], 2013.

Orientador: Maria Cláudia Gonçalves de Oliveira Fusaro.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Ciências Aplicadas.

1. Hiperalgesia. 2. Receptor P2X3. 3. Receptor P2X2/3. 4. Músculo. 5. Prostaglandinas. I. Oliveira-Fusaro, Maria Cláudia Gonçalves. II. Universidade Estadual de Campinas. Faculdade de Ciências Aplicadas. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Mechanisms underling the role of P2X3 and P2X2/3 receptors in mechanical hyperalgesia in gastrocnemius muscle of rats
Palavras-chave em inglês:
Hyperalgesia
P2X3 receptor
P2X2/3 receptor
Muscle
Prostaglandins
Área de concentração: Biodinâmica do Movimento Humano e Esporte
Titulação: Mestra em Ciências da Nutrição e do Esporte e Metabolismo
Banca examinadora:
Maria Cláudia Gonçalves de Oliveira Fusaro [Orientador]
Dionéia Araldi
Hosana Gomes Rodrigues
Data de defesa: 22-07-2013
Programa de Pós-Graduação: Ciências da Nutrição e do Esporte e Metabolismo

BANCA EXAMINADORA

Limeira, 22 de julho de 2013

BANCA EXAMINADORA

Profa. Dra. Maria Cláudia Gonçalves de Oliveira Fusaro
Presidente da Comissão Julgadora

Profa. Dra. Rosana Gomes Rodrigues

Profa. Dra. Dionéia Araldi

Resumo

Existem evidências do envolvimento do ATP via ativação do receptor P2X3 na dor muscular. Portanto, o objetivo deste estudo foi verificar se a ativação do receptor P2X3 no músculo gastrocnêmio de ratos induz hiperalgesia mecânica, e em caso afirmativo, analisar os mecanismos inflamatórios pelo qual os receptores P2x3 induzem hiperalgesia mecânica. O Antagonista não seletivo para o receptor P2X3 α,βmeATP foi administrado no músculo gastrocnêmio de ratos, induzindo hiperalgesia, a qual foi significativamente reduzida pelo antagonista seletivo do receptor P2X3 e P2X2/3 - A-317491. A hiperalgesia mecânica induzida pelo α,βmeATP foi reduzida pelo inibidor de ciclooxigenase Indometacina, pelo antagonista seletivo do receptor de Bradicinina B1 e B2- Dalbk e Bradyzide, respectivamente, antagonista dos adrenoceptores β1 e β2 – Atenolol e ICI 118,551 respectivamente, e inibidor não específico de selectinas Fucoidan. O α,βmeATP também induziu o aumento da concentração local de citocinas pro inflamatórias TNF-α, IL-1β, IL-6 e CIN e migração de neutrófilos. Juntos estes achados sugerem que o α,βmeATP induz hiperalgesia mecânica no músculo gastrocnêmio via ativação de receptor periférico P2X3, o qual envolve bradicinina, prostaglandinas e aminas simpatomiméticas e migração de neutrófilos. Portanto, nós sugerimos que os receptores P2X3 sejam um importante alvo no controle da dor muscular.

Abstract

There is evidence of the involvement of endogenous ATP via activation of P2X3 in muscle pain. Therefore, the aim of this study was to verify whether the activation of P2X3 receptors in the gastrocnêmio muscle of rats induces mechanical hyperalgesia and, if so, to analyze the inflammatory mechanisms by which P2X3 receptors induce mechanical hyperalgesia. Intramuscular administration of the non-selective P2X3 receptor agonist α,β-meATP in the gastrocnemius muscle of rats induced mechanical hyperalgesia, which was significantly reduced by the selective P2X3 and P2X2/3 receptors antagonist A-317491. The α,β-meATP-induced mechanical hyperalgesia was prevented by the indomethacin cyclooxygenase inhibitor, the selective bradykinin B1- or B2- receptor antagonist DALBK and bradyzide, respectively, the β_1- or β_2-adrenoceptor antagonist atenolol and ICI 118,551, respectively, and the nonspecific selectin inhibitor fucoidan. α,β-meATP also induced increase in the local concentration of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 and the neutrophil migration. Together, these findings suggest that α,β-meATP induced mechanical

hyperalgesia in the gastrocnemius muscle of rats via activation of peripheral P2X3 receptors, which involves bradykinin, prostaglandins, sympathetic amines, pro-inflammatory cytokines and neutrophil migration. Therefore, we suggest that P2X3 receptors are important targets to control muscle inflammatory pain.

Sumário

Agradecimentos

*À **Deus**, cuja presença constante me faz manter a fé, a perseverança e a força em todos os momentos da vida.*

*Aos meus pais **Roberto** e **Estér**, pelo amor e pela dedicação à formação intelectual e pessoal de seus filhos.*

*Aos meus irmãos **Jacqueline** e **Roberto Júnior**, aos meus sobrinhos **Vítor, Caio e Roberto Neto**, pelo apoio, incentivo e por adoçarem a da minha vida.*

*Ao meu marido **Ricardo**, pelo apoio integral e fundamental, sem o qual eu não poderia realizar este trabalho, pelo amor, carinho e por estar ao meu lado lutando com todas as forças para nossa eterna evolução.*

Agradecimentos

Á Faculdade de Ciências Aplicadas, FCA-Limeira, e a todos os professores do Curso de Ciências da Nutrição Esporte e Metabolismo, pelo trabalho incansável e intensa dedicação na formação de um programa de Pós Graduação de excelência, do qual me orgulho em fazer parte da primeira turma.

Á Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – processo 2011/13884-9) pelo apoio financeiro nesta pesquisa.

Agradecimentos especiais:

À minha orientadora Prof. **Maria Cláudia G. de Oliveira Fusaro**, pela confiança, dedicação, respeito e por dividir comigo seus preciosos conhecimentos.

Ao prof. **Carlos Almilcar Parada**, pela colaboração e apoio em todo o desenvolvimento deste trabalho.

Aos amigos **Juliana Maia Teixeira, Dionéia Araldi, Elayne Vieira, César Sartori, Maria Carolina Athié, André Vieira, Lilian Calili, Lilian Rocha, Gilson Gonçalvez, Filipe Prado, Ivan Bonet, Bruna Melo e Diogo Francisco**, pela companhia, ensinamentos e por dividirem comigo não só o entusiasmo, mas a "dor e a delícia" de ser um pesquisador.

Epígrafe.

“ Tudo aquilo que o homem ignora, não existe para ele. Por isso o universo de cada um, se resume ao tamanho de seu saber”

Albert Einstein

Abreviações:

ATP, Trifosfato 5´de Adenosina

BSA, Albumina Bovina Sérica

CINC-1, cytokine-induced neutrophil chemoattractant-1

DALBK, Des-Arg^{8}-Leu^{9}-BK

EDTA, Ácido etilenodiaminotetracético

ELISA, Ensaio Imuno Enzimático

IASP, Associação International de Estudos da Dor

IL-1β, interleucina 1β

IL-6, interleucina 6

IL-8, interleucina 8

PBS, Tampão Fosfato Salina

TNFα, Fator de necrose tumoral-α

Introdução

Durante o processo evolutivo, a sensação dolorosa, associada a uma percepção desagradável e a um comportamento aversivo, foi um dos principais determinantes da sobrevivência das espécies. A Associação Internacional para Estudos da Dor - IASP (Internacional Association for Study of Pain) define a dor como uma experiência sensorial e emocional desagradável que se relaciona com uma lesão real ou potencial dos tecidos. De forma mais abrangente, a dor pode ser definida como uma percepção de uma sensação nociceptiva, apresentando aspectos anatômicos e neurofisiológicos. De maneira simplificada, pode-se dizer que a dor é uma espécie de alarme fisiológico, que alerta o organismo sobre uma ameaça a sua integridade. De modo a evitar danos maiores causados por estímulos deletérios, há necessidade da presença de estruturas nervosas especializadas para detecção e transmissão da informação nociceptiva. Esse processo envolve a ativação de vias sensoriais responsáveis por detectar a presença de estímulos nocivos ou potencialmente nocivos; fenômenos iônicos, que consistem na transdução de um estímulo de alta intensidade em sinais elétricos, via influxo do íon sódio (Na^+) por canais específicos, e a condução dessa informação ao longo das vias nociceptivas, desencadeando, finalmente, a reação e/ou percepção. Além disso, alterações metabólicas nestas vias sensoriais também podem induzir mudanças fazendo com que essas fibras detectem e conduzam a informação dolorosa.

Os estímulos indutores da sensação dolorosa (nocicepção) podem ser tanto de natureza mecânica ou térmica quanto química ou elétrica, externos ou internos ao organismo. Os estímulos ambientais internos ou externos ao organismo são detectados por estruturas receptoras localizadas por todo o corpo e conhecidas como receptores sensoriais. Estes receptores transduzem a informação produzida pelos diferentes tipos de estímulo, os quais serão transmitidos até o sistema nervoso central (SNC) por meio das fibras nervosas aferentes (ou primárias). Estas fibras aferentes estabelecem contato com os neurônios de segunda ordem (ou secundários) que, por sua vez, conduzem a informação até os centros superiores para seu processamento (Bonica, 1990). Tais células nervosas periféricas possuem seus corpos celulares

localizados nos gânglios das raízes dorsais (GRDs) ou nos gânglios trigeminais, no caso dos neurônios sensoriais que inervam a região orofacial. Destes gânglios saem prolongamentos em direção à medula espinal (ou tronco encefálico, no caso dos neurônios orofaciais), onde estão localizados os neurônios secundários. A ativação dos neurônios sensoriais primários leva à liberação de aminoácidos excitatórios, como o glutamato, e peptídeos, como a substância P e o peptídeo relacionado ao gene da calcitonina, nos terminais pré-sinápticos (Millan, 1999). Estas substâncias, chamadas neurotransmissores e neuromoduladores, atuarão em receptores pós-sinápticos, estimulando os neurônios secundários. A partir de sua transmissão para o neurônio secundário, a informação nociceptiva ascenderá para as áreas supraespinais através de tratos neuronais específicos (espinotalâmico, espinoreticular, espinomesencefálico, espinocervicotalâmico, espinoparabraquial, espinoparabraquio (trigêmio) hipotalâmico, via pós-sináptica da coluna dorsal (Prado, Araldi *et al.*; Besson e Chaouch, 1987; Millan, 1999), até a convergência em populações de neurônios no núcleo posterior ventral do tálamo (núcleo ventrobasal) (MILNE *et al*., 1981). Nesse nível, a informação será conduzida para as áreas sensoriais do córtex cerebral e suas várias características serão integradas, ou seja, aspectos como qualidade, intensidade, localização, duração e os componentes afetivo e emocional serão interpretados, diferenciando a sensação dolorosa da percepção (Noback, Terpstra *et al.*, 1996). Ainda, no que se refere às fibras aferentes primárias nociceptivas, elas são de pequeno diâmetro e estão ligadas a terminações livres, ou seja, não estão associadas a receptores sensoriais especializados. Possuem alto limiar de ativação e são diretamente relacionadas às vias nociceptivas (Aguggia, 2003). Por isso, genericamente são denominadas nociceptores, podendo ser encontradas em dois tipos: fibras A-delta (Aδ), de médio diâmetro, finamente mielinizadas, com velocidade de condução média, entre 12 e 30 m/s (correspondentes a 20% das fibras de dor e responsáveis pela dor rápida, aguda e lancinante que sentimos após estimulação nociva), e fibras C não mielinizadas, de pequeno diâmetro, com velocidade de condução menor (0,5 a 2 m/s) (correspondentes a 80% das fibras condutoras da informação dolorosa e responsáveis pela dor com característica lenta e difusa) (Millan, 1999; Julius e Basbaum, 2001). Levando-se em conta o critério funcional, as fibras Aδ respondem à estimulação mecânica, porém podem ser sensibilizadas pelo calor, enquanto as fibras do tipo C respondem tanto a estímulos térmicos quanto mecânicos e químicos, sendo classificadas, por isso, como nociceptores polimodais.

Em determinadas situações a dor é acompanhadas por outros fenômenos, como a hiperalgesia, que é o resultado da sensibilização das fibras aferentes primárias nociceptivas. Essa sensibilização, caracterizada eletrofisiologicamente pela redução do limiar de excitabilidade neuronal (Riedel e Neeck, 2001), ocorre por ação de mediadores produzidos pelo processo inflamatório (Huang, Zhang *et al.*, 2006; Verri, Cunha *et al.*, 2006). Estes mediadores atuarão em seus respectivos receptores, induzindo, como resultado final, alterações metabólicas que facilitarão a produção de potenciais de ação pelos neurônios nociceptores. Em outras palavras, essas fibras poderão ser ativadas mais facilmente frente à estimulação, e, devido a essa nova condição, estímulos que antes não eram capazes de ativá-las, passam a sê-lo, ou a fazê-lo mais intensamente. Assim, o processo inflamatório pode ser, de modo geral, diretamente associado à hiperalgesia (dor aumentada em resposta a um estímulo que já era doloroso).

A inflamação pode ser definida como um processo bioquímico e celular que acontece no tecido vascularizado, envolvendo o plasma, as células circulantes, os vasos e os constituintes celulares e extracelulares (Rote, Vogt *et al.*, 1998). As características principais são exsudação dos líquidos e proteínas plasmáticas (edema), migração celular e sensibilização ou ativação dos nociceptores. Pode ser provocada por um trauma mecânico, privação de oxigênio ou nutrientes, alterações imunológicas ou genéticas, agentes químicos, micro-organismos, temperaturas extremas ou radiação (Rote, Vogt *et al.*, 1998; Sherwood e Toliver-Kinsky, 2004). Após uma lesão tecidual, uma resposta inflamatória é gerada por macrófagos locais e amplificada por células sanguíneas migratórias, como os neutrófilos (Van Furth, Nibbering *et al.*, 1985; Laskin e Pendino, 1995). Tem sido sugerido que durante esse processo ocorra liberação de mediadores inflamatórios, tais como a bradicinina, TNF-α, IL-1β, IL-6, CINC-1 (Cunha, Poole *et al.*, 1992; Ferreira, Lorenzetti *et al.*, 1993), que estimulam a síntese das prostaglandinas e liberação das aminas simpatomiméticas, as quais sensibilizam diretamente os nociceptores aferentes primários (Gold, Shuster *et al.*, 1996; Rush e Waxman, 2004). Além desses mediadores inflamatórios, recentes estudos demonstram o importante papel do nucleotídeo adenosina 5'-trifosfato (ATP) como mediador da hiperalgesia inflamatória (Wu, Whiteside *et al.*, 2004; Mcgaraughty, Honore *et al.*, 2005; Oliveira, Parada *et al.*, 2005; Wang, Li *et al.*, 2007).

O ATP está presente em concentrações milimolares em todas as células do corpo (Mccleskey e 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 e 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 e 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 e, especialmente nas células musculares sua concentração é particularmente alta ((Reinohl, Hoheisel *et al.*, 2003), 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, Akopian *et al.*, 1995; Kennedy e Leff, 1995; Lewis, Neidhart *et al.*, 1995), especialmente nos neurônios sensoriais de pequeno diâmetro, particularmente as fibras C (Chen, Akopian *et al.*, 1995). Recentes estudos, que utilizaram modelos comportamentais nociceptivos (Bland-Ward e Humphrey, 2000; Jarvis, Burgard *et al.*, 2002; Mcgaraughty, Wismer *et al.*, 2003; Wu, Whiteside *et al.*, 2004; Mcgaraughty, Honore *et al.*, 2005; Oliveira, Parada *et al.*, 2005), animais knockout para receptor P2X3 (Cockayne, Hamilton *et al.*, 2000; Souslova, Cesare *et al.*, 2000), oligonucleotideos antisense P2X3 (Barclay, Patel *et*

al., 2002; Honore, Kage *et al.*, 2002) e antagonistas seletivos de receptores P2X3 e P2X2/3 (Jarvis, Burgard *et al.*, 2002; Mcgaraughty, Wismer *et al.*, 2003; Wu, Whiteside *et al.*, 2004; Mcgaraughty, Honore *et al.*, 2005; Sharp, Reeve *et al.*, 2006), demonstraram que o ATP endógeno e os receptores P2X3 e P2X2/3 estão envolvidos com o desenvolvimento da dor em diferentes condições inflamatórias. Além disso, demonstrou-se que a administração do agonista não seletivo de receptores P2X3, α,β-metileno ATP (α,β-meATP), induz hiperalgesia inflamatória (Hamilton, Wade *et al.*, 1999; Tsuda, Koizumi *et al.*, 2000; Barclay, Patel *et al.*, 2002; Waldron e Sawynok, 2004; Wang, Li *et al.*, 2007) no tecido subcutâneo da pata de ratos.

Recentemente, nosso grupo de pesquisa demonstrou que a ativação dos receptores P2X3 e P2X2/3 pelo ATP endógeno é essencial para o desenvolvimento da dor inflamatória induzida pela carragenina no tecido subcutâneo da pata de ratos, uma vez que o antagonista seletivo de receptores P2X3 e P2X2/3, A-317491 (Oliveira, Pelegrini-Da-Silva *et al.*, 2009), bloqueou a hiperalgesia mecânica induzida pela carragenina. Neste estudo, demonstramos que a participação dos receptores P2X3 e P2X2/3 na hiperalgesia mecânica induzida pela carragenina é mediada pela sensibilização direta e indireta dos nociceptores aferentes primários. A sensibilização direta envolve a ativação dos receptores P2X3 expressos nas fibras aferentes primárias, enquanto que a sensibilização indireta envolve a liberação prévia da citocina pró-inflamatória TNF-α (Oliveira, Pelegrini-Da-Silva *et al.*, 2009). Em outro estudo também realizado por nosso grupo de pesquisa demonstramos que o agonista não seletivo de receptores P2X3, α,β-meATP, induz hiperalgesia mecânica no tecido subcutâneo da pata de ratos mediado por bradicinina, prostaglandinas, aminas simpatomiméticas e migração de neutrófilos (dados não publicados). Entretanto, apesar de termos demonstrado alguns dos mecanismos inflamatórios envolvidos da hiperalgesia induzida pela ativação dos receptores P2X3 no tecido subcutâneo, pouco se sabe sobre o envolvimento desses receptores na dor muscular.

As desordens do sistema musculoesquelético são uma das maiores causas de incapacidades funcionais das sociedades ocidentais. Especificamente a dor muscular é um grande problema de saúde. Dados epidemiológicos demonstram que 70 a 80% da população apresentou dor muscular na região das costas em algum momento das suas vidas (Krismer e Van Tulder, 2007).

Sabe-se que a dor muscular pode estar associada a um processo inflamatório mediado por citocinas pró-inflamatórias, tais como TNF-α (Schafers, Sorkin *et al.*, 2003) , IL-1β, IL-6 e CINC-1 (Dessem, Ambalavanar *et al.*; Loram, Fuller *et al.*, 2007) 5-hydroxytrypamine (Christidis, Kopp *et al.*, 2005), bradicinina (Boix, Roe *et al.*, 2005) e prostaglandina E_2 (PGE_2) (Hedenberg-Magnusson, Ernberg *et al.*, 2001) . Além disso, semelhante a outros tecidos, tem sido descrito o envolvimento do ATP via ativação de receptores P2X3 na dor muscular (Mense, 2009). A contração de máxima intensidade ou de padrão excêntrico do músculo masseter de ratos induz hiperalgesia mecânica mediada por receptores P2X3 e aumento na expressão de receptores P2X3 no gânglio trigeminal (Dessem, Ambalavanar *et al.*; Noma, Shinoda *et al.*; Shinoda, Ozaki *et al.*, 2005); administração de ATP no músculo trapézio de indivíduos saudáveis induz dor muscular intensa e sensibilidade local (Mork, Ashina *et al.*, 2003); administração de ATP (Makowska, Panfil *et al.*, 2006) ou de αβ-meATP (Reitz et al., 2009, Ristic et al.,2010 , Ellrich et al., 2010) nos músculos do pescoço de camundongos induz nocicepção; e os nociceptores aferentes primários do músculo gastrocnêmio de ratos (Reinohl, Hoheisel *et al.*, 2003) e gatos (Hanna e Kaufman, 2004) são ativados por αβ-meATP ou ATP em concentrações equivalentes àquelas encontradas nas células musculares.

Objetivos

Considerando-se a relevância clínica das dores musculares e as evidências do envolvimento de mediadores inflamatórios e do ATP na dor muscular, o objetivo desse estudo foi verificar se a ativação de receptores P2X3 no músculo gastrocnêmio de ratos induzia hiperalgesia muscular. Em caso positivo, analisar os mecanismos inflamatórios envolvidos nesse processo.

Objetivos específicos:

1. Verificar se a administração local do agonista não seletivo de receptor P2X3, αβ-meATP, induzia hiperalgesia mecânica no músculo gastrocnêmio de ratos;

2. Em caso positivo, avaliar se a hiperalgesia mecânica induzida pelo αβ-meATP é mediada por bradicinina, prostaglandinas e/ou aminas simpatomiméticas;

3. Verificar se o αβ-meATP induzia aumento na concentração local das citocinas pró-inflamatórias TNF-α, IL-1β, IL-6 e CINC-1.

4. Avaliar o envolvimento da migração de neutrófilos na hiperalgesia muscular induzida pelo αβ-meATP.

MECHANISMS UNDERLYING THE ROLE OF P2X3 RECEPTORS IN MECHANICAL HYPERALGESIA IN THE GASTROCNEMIUS MUSCLE OF RATS

Jalile Garcia Schiavuzzo[1]

Juliana Maia Teixeira[2]

Bruna Melo[1]

Diogo Francisco da Silva dos Santos[1]

Maria Cláudia G. Oliveira-Fusaro*[1]

Carlos Amílcar Parada

1- Laboratory of Studies of Pain and Inflammation, School of Applied Sciences- UNICAMP- Limeira-SP, Brazil

2- Department of Structural and Functional Biology, Institute of Biology, State University of Campinas- UNICAMP, SP, Brazil.

* Corresponding author:

School of Applied Sciences, State University of Campinas - UNICAMP

Pedro Zaccaria, 1300, Jd. Sta Luiza, Zip Code: 13484-350, Limeira, São Paulo - Brazil

Tel: + 55-19-37016716 Fax: +55-19-37016680

E-mail address: maria.fusaro@fca.unicamp.br (Oliveira-Fusaro MC)

Abstract

There is evidence of the involvement of endogenous ATP via activation of P2X3 in muscle pain. Therefore, the aim of this study was to verify whether the activation of P2X3 receptors in the gastrocnemius muscle of rats induces mechanical hyperalgesia and, if so, to analyze the inflammatory mechanisms by which P2X3 receptors induce mechanical hyperalgesia. Intramuscular administration of the non-selective P2X3 receptor agonist α,β-meATP in the gastrocnemius muscle of rats induced mechanical hyperalgesia, which was significantly reduced by the selective P2X3 and P2X2/3 receptors antagonist A-317491. The α,β-meATP-induced mechanical hyperalgesia was prevented by the indomethacin cyclooxygenase inhibitor, the selective bradykinin B1- or B2- receptor antagonist DALBK and bradyzide, respectively, the β_1- or β_2-adrenoceptor antagonist atenolol and ICI 118,551, respectively, and the nonspecific selectin inhibitor fucoidan. α,β-meATP also induced increase in the local concentration of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 and the neutrophil migration. Together, these findings suggest that α,β-meATP induced mechanical hyperalgesia in the gastrocnemius muscle of rats via activation of peripheral P2X3 receptors, which involves bradykinin, prostaglandins, sympathetic amines, pro-inflammatory cytokines and neutrophil migration. Therefore, we suggest that P2X3 receptors are important targets to control muscle inflammatory pain.

Keywords: α,β-meATP, P2X3 receptor, muscle, hyperalgesia, prostaglandins, cytokines

1. Introduction

Disorders of the musculoskeletal system are the leading causes of disability in western societies. Specifically, muscle pain is one of the most significant health problems. Seventy to Eighty-five percent of all people have back pain at some time in their life (Krismer and van Tulder, 2007) and more people around the world experience muscle pain rather than any other type of pain. It is well known that muscle pain can be associated with an inflammatory process mediated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (Schafers et al., 2003), interleukin 1β (IL-1β), interleukin 6 (IL-6) and cytokine-induced neutrophil chemoattractant 1 (CINC-1) (Loram et al., 2007, Dessem et al.2010), 5-hydroxytrypamine (Christidis et al., 2005), bradykinin (Boix et al., 2005) and prostaglandin E_2 (PGE_2) (Hedenberg-Magnusson et al., 2001, Tegeder et al., 2002). Moreover, it has been described that the molecule adenosine 5'-triphosphate (ATP) has an important role in muscle pain (Mense, 2009).

ATP is an energy-rich molecule present in all body cells and, in muscle cells, its concentration is particularly high and sufficient for activating group IV muscle units (Reinohl et al., 2003). Any damage of tissue injury is accompanied by a release of ATP (Burnstock, 2007). Therefore, there are almost unlimited circumstances where ATP might be released as a peripheral sensory mediator by activating the purinergic P2X3 receptor. It has been described that the P2X3 receptor is expressed on primary afferent neurons and, in the muscle tissue, it is preferentially expressed on slowly conducting muscle afferent units (Ambalavanar et al., 2005). The activation of P2X3 receptor by ATP or by its agonists initiates depolarization, Ca^{2+} influx through the ionic channel coupled to P2X3 receptor and Ca^{2+} influx via the co-expressed voltage-dependent Ca^{2+}channels (Kennedy et al., 2003). 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, Oliveira et al., 2009) and selective P2X3 and P2X2/3 receptors antagonist (Jarvis et al., 2002, McGaraughty et al., 2003, Wu et al., 2004, McGaraughty and Jarvis, 2005, Oliveira et al., 2009, de Oliveira Fusaro et al., 2010) indicate that the activation of P2X3 and P2X2/3 receptors by endogenous ATP contributes to the development of inflammatory hyperalgesia in the subcutaneous tissue. Also, we have recently demonstrated that activation of P2X3 receptors by the non-selective agonist of P2X3 αβ-meATP in the subcutaneous tissue of the rat paw induces mechanical hyperalgesia mediated by bradykinin, prostaglandins and sympathetic amines (data not published).

In the muscle tissue, there is also evidence of the role of endogenous ATP on muscle pain. It has been described that maximal or eccentric contraction of masseter muscle of rats induces mechanical hyperalgesia mediated by P2X3 receptors and the increase of P2X3 receptors expression on trigeminal ganglia (Shinoda et al., 2008, Dessem et al., 2010, Noma et al., 2013); administration of ATP in the trapezius muscle of health humans induces intense muscle pain and mild tenderness (Mork et al., 2003); administration of ATP (Makowska et al., 2006) or αβ-meATP (Reitz et al., 2009, Ristic et al.,2010 , Ellrich et al., 2010) on the neck muscle of mice induced nociception; and the primary afferent nociceptors of the gastrocnemius muscle of rats (Reinohl et al., 2003), and cats (Hanna and Kaufman, 2004) are activated by αβ-meATP or ATP in concentrations equivalent to those found in muscle cells. However, despite the clinical relevance of muscle pain, the mechanism by which endogenous ATP via activation of P2X3 contributes to the development of muscle pain is poorly understood.

Considering the involvement of inflammatory mediators and ATP in muscle pain, the aim of this study was to verify whether the activation of P2X3 receptors in the gastrocnemius muscle of rats induces mechanical hyperalgesia and, if so, to analyze the inflammatory mechanisms involved in this process.

2. Experimental Procedures

2.1. Animals

Male albino Wistar rats weighing 150g to 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-hour light/dark cycle (lights on at 6:00 AM) with food and water available *ad libitum*. They were maintained on a temperature-controlled room test (±23°C) and handled for one week prior to the experiments.

2.2. Drugs and Doses

The following drugs were used: non-selective P2X3 receptor agonist, α,β-methyleneATP lithium salt (α,β-meATP, 10, 100 and 1000µg/muscle), the selective P2X3 and P2X2/3 receptors antagonist, 5-([(3-Phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl)-1,2,4-benzenetricarboxylic acid (A-317491, 10 and 100µg/muscle); bradykinin B1 receptors antagonist, Des-Arg 8-Leu9-BK (DALBK, 0.03, 0.3 and 3.0µg/muscle); bradykinin B2 receptors antagonist, Bradyzide (0.15 and 1.5µg/muscle); β_1 adrenoreceptor antagonist, Atenolol (24 and 72µg/muscle), β_2 adrenoreceptor antagonist, ICI 118,551 (0.015, 0.15 and 1,5µg/muscle), cyclooxygenase inhibitor, indomethacin (1.0, 10 and 100µg/muscle), non-specific selectin inhibitor, fucoidan (25mg/kg, i.v., Oliveira et al., 2009). All drugs were dissolved in sterile saline (0.9% NaCl) and obtained from Sigma Aldrich (Brazil).

2.3. Intramuscular Injections

Drugs or their vehicle were injected into the belly of the gastrocnemius muscle of rats with a 30-gauge needle, as previously described (Gautam and Benson, 2013). The needle was connected to a polyethylene catheter and also to a Hamilton syringe (50µl). The animals were briefly restrained and the total volume administered was 50µl.

2.4. Mechanical Nociceptive Threshold Test

Testing session took place during light phase (between 9:00 A.M. and 5:00 P.M.) in a quiet room maintained at 23°C (Rosland, 1991). The Randall-Selitto nociceptive paw-withdrawal flexion reflex test (Randall and Selitto, 1957) was performed using a Ugo-Basile analgesymeter (Stoelting, Chicago, IL, USA), which applies a linearl mechanical force to the belly of the gastrocnemius muscle of rats (Fujii et al., 2008). The baseline muscle-withdrawal threshold was defined as the mean of three tests performed at five-minute 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.

2.5. ELISA Procedures

An adaptation of ELISA (Safieh-Garabedian et al., 1995) was used to determine whether α,β-meATP was able to induce the release of TNF-α, IL-1β, IL-6 and CINC-1 in the gastrocnemius muscle of rats. The muscle tissues were collected two hours after the muscle 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 10000 rpm for 15 minutes 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 gastrocnemius muscle of rats. The cytokines were quantified by the following kits: TNF-α: Rat TNF-α/ TNFSF1A DuoSet ELISA Kit (R&D Systems, catalog number DY510); IL-1β: Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, catalog number DY501), IL-6: Rat IL-6 DuoSet ELISA Kit (R&D Systems, catalog number: DY506) and CINC-1: Rat CXCL1/CINC-1 DuoSet ELISA Kit (R&D Systems, catalog number DY515). All procedures were performed following the instructions of the manufacturer (R&D Systems). All procedures were repeated twice to guarantee the authenticity of the results.

2.6. Measurement of Myeloperoxidase Activity MPO

The myeloperoxidase kinetic-colorimetric assay was conducted as previously described (Torres-Chavez et al., 2012). Two hours after the muscle administration of α,β-meATP, the muscle tissue was dissected into a standard sample size, weighed and quickly frozen with liquid isopentane in dry ice. Tissues were stored at -70°C. In the assay procedure, each sample was homogenized in 500µl of buffer (0.1M NaCl, 0,02M $NaPo_4$, 1.015M Na EDTA, pH 4.7) followed by centrifugation at 2500 g for 15 minutes. The pellet was resuspended in 500µl of buffer and subjected to hypotonic lyses by the addition of 500µl of 0.2% NaCl and followed, 30 seconds later, by the addition of 500µl of 1.6% NaCl in glucose. After further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% headecyl-trimethilammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10000 g for 15 min. Fifty microliters of each sample (supernatant) and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate.

Twenty-five microliters of 3,3',5,5'-tetranethylbezidine were added in each well. The reaction was initiated by the addition of 100µl of H_2O_2. The reaction was interrupted five minutes later by adding 50µl of 4 M H_2SO_4. The optical density was read at 450 nm using an Anthos 2020. Results were calculated by comparing the optical density of muscle tissue supernatant with a standard curve of neutrophil (>95% purity). The results were presented as number of neutrophils $x10^8$ /mg tissue.

2.7. Statistical Analysis

For data shown in Fig. 1C, a two-way repeated measures ANOVA, with one between-subject factor (i.e., treatment) and one within-subject factor (i.e., time) were used to determine whether there were significant ($p < 0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. For data of other figures, a one-way ANOVA or t-test was performed to determine whether there were significant differences ($p < 0.05$) between the groups. 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 the figures by the decrease in paw-withdrawal threshold and are presented as means ± SEM

3. Results

3.1. α,β-meATP Induced Mechanical Hyperalgesia in the Gastrocnemius Muscle of Rats

Administration of α,β-meATP (1000μg, but not 10μg and 100μg) in the gastrocnemius muscle of rats induced significant behavioral hyperalgesic response when compared with NaCl 0.9% ($P < 0.05$, Tukey test, Fig 1A). To verify whether the α,β-meATP-induced mechanical muscle hyperalgesia was mediated by P2X3 and P2X2/3 receptors, the selective P2X3 and P2X2/3 receptor antagonist A-317491 was co-administered with α,β-meATP. A-317491 (100μg/muscle, but not 10μg) prevented ($P < 0.05$, Tukey test, Fig. 1B) the α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the contralateral gastrocnemius muscle, confirming its local peripheral action. Co-administration of A-317491 (100□g/muscle) with 0.9% NaCl did not affect the mechanical withdrawal threshold ($P > 0.05$, Tukey test, Fig. 1B). The mechanical muscle hyperalgesia occurred ½, 1, 2, 3 and 6 hours after the administration of α,β-meATP (1000μg) ($P < 0.05$, Two Way ANOVA, Bonferroni test, Fig 1C), with the greatest response after 2 hours, and co-administration of A-317491 (100□g/muscle) prevented the α,β-meATP-induced mechanical muscle hyperalgesia at all time points ($P < 0.05$, Two Way ANOVA, Bonferroni test, Fig 1C). Therefore, in further experiments, the mechanical hyperalgesia was evaluated only 2 hours after the administration of α,β-meATP (1000μg). The baselines were not significantly different among groups ($P > 0.05$, Tukey test, data not shown).

Intradermal or subcutaneous administration of the quaternary lidocaine derivate QX134 (4%) five minutes before intramuscular administration of α,β-meATP did not affect ($P > 0.05$, Tukey test, Fig. 1D) the mechanical muscle hyperalgesia, ruling out the involvement of dermal or subcutaneous tissue on the mechanical hyperalgesia induced by α,β-meATP in the gastrocnemius muscle.

3.2. Effect of the Bradykinin B1 or B2 Receptor Antagonists in α,βmeATP-induced Mechanical Muscle Hyperalgesia

To verify whether α,β-meATP-induced mechanical muscle hyperalgesia was mediated by bradykinin, the bradykinin B1 or B2 receptor antagonists, DALBK and Bradyzide, respectively, were co-administered with α,β-meATP. DALBK (0.3 and 3.0μg/muscle, Fig. 2A) or bradyzide (0,015 and 1.5μg/muscle, Fig. 2B) significantly reduced ($P < 0.05$, Tukey test) the α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the

contralateral gastrocnemius muscle ($P > 0.05$, Tukey test), confirming its local peripheral action. Co-administration of DALBK (3.0µg/muscle) or bradyzide (1.5µg/muscle) with 0.9% NaCl did not affect the mechanical withdrawal threshold.

3.3. Effect of Cyclooxygenase Inhibitor in α,β-meATP-induced Mechanical Muscle Hyperalgesia

To verify whether α,β-meATP-induced mechanical hyperalgesia was mediated by prostaglandins, the indomethacin cyclooxygenase inhibitor was administered 30 minutes before α,β-meATP in the gastrocnemius muscle of rats. Indomethacin (10 and 100µg/muscle) significantly reduced ($P < 0.05$, Tukey test, Figure 3) the α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the contralateral gastrocnemius muscle ($P > 0.05$, Tukey test), confirming its local peripheral action. Co-administration of indomethacin (100□g/muscle) with 0.9% NaCl did not affect the mechanical withdrawal threshold.

3.4. Effect of β_1- or β_2- adrenoceptor Antagonists in α,β-meATP-induced Mechanical Muscle Hyperalgesia

To verify whether α,β-meATP-induced mechanical hyperalgesia was mediated by sympathetic amines, the β_1- or β_2- adrenoceptor antagonists atenolol and ICI 118,551, respectively, were co-administered with α,β-meATP. Atenolol (6.0µg/muscle, Fig. 4A) or ICI 118,551 (1.5µg/muscle, Fig. 4B) significantly reduced ($P < 0.05$, Tukey test) α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the contralateral gastrocnemius muscle ($P > 0.05$, Tukey test), confirming its local peripheral action. Co-administration of atenolol (6.0µg/muscle) or ICI 118,551 (1.5µg/muscle) with 0.9% NaCl did not affect the mechanical withdrawal threshold.

3.5. α,β-meATP-induced Increase in Cytokines Concentration in the Gastrocnemius Muscle of Rats

To verify whether α,β-meATP induces the local release of pro-inflammatory cytokines, α,β-meATP (1000µg/muscle) or 0.9% NaCl was administrated in the gastrocnemius muscle of rats and the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 were quantified 2 hours later.

Local administration of α,β-meATP (1000µg/muscle) significantly increased ($P < 0.05$, Tukey test) the concentrations of TNF-α (Figure 5A), IL-1β (Figure 5B) IL-6 (Figure 5C) and CINC-1 (Fig. 5D) when compared with 0.9% NaCl administration. The muscle administration of 0.9% NaCl did not alter ($P > 0.05$, Tukey test) the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive group.

3.6. α,β-meATP Induced Neutrophil Migration in the Gastrocnemius Muscle of Rats

To verify whether α,β-meATP induces local neutrophil migration, α,β-meATP or 0.9% NaCl was administrated in the gastrocnemius muscle tissue of rats and the MPO activity was quantified 2 hous later. The administration of α,β-meATP (1000µg/muscle) significantly increased ($P < 0.05$, Tukey test) the MPO activity when compared with 0.9% NaCl administration (Fig. 6A).

To verify whether neutrophil migration contributes to α,β-meATP-induced mechanical muscle hyperalgesia, rats were pretreated with fucoidan 20 minutes before α,β-meATP administration. Pre-treatment with fucoidan (25 mg/kg, i.v.) significantly reduced ($P < 0.05$, Tukey test) the α,β-meATP-induced mechanical muscle hyperalgesia (Fig 6B).

4. Discussion

The findings of the present study have demonstrated, for the first time, that activation of P2X3 receptors of the gastrocnemius muscle of rats by the non-selective P2X3 receptors agonist, α,β-meATP, induced mechanical muscle hyperalgesia mediated by bradykinin, prostaglandins, sympathetic amines and neutrophil migration. Also, the activation of P2X3 receptors in the gastrocnemius muscle of rats induced the release of pro-inflammatory cytokines.

The findings that the selective P2X3 and P2X2/3 receptors antagonist A317491 blocked the mechanical muscle hyperalgesia induced by the non-selective P2X3 receptors agonist α,β-meATP in the gastrocnemius muscle of rats demonstrate the role of peripheral P2X3 receptors in muscle hyperalgesia. Although α,β-meATP is a non-selective P2X1, P2X3 and P2X2/3 receptors agonist, the involvement of P2X1 seems to be unlikely, because α,β-meATP-induced mechanical hyperalgesia was completely reversed by A-317491. In addition, it has been demonstrated that IP5I, a potent and selective P2X1 receptor antagonist, is ineffective in reducing inflammatory pain (Honore et al., 2002b). Our data that activation of P2X3 receptors by α,β-meATP induces hyperalgesia is supported by other studies in muscle (Shinoda et al., 2008) and subcutaneous (Barclay et al., 2002, Waldron and Sawynok, 2004, Wang et al., 2007, Prado et al., 2013) tissues.

The α,β-meATP-induced mechanical muscle hyperalgesia was prevented by the selective bradykinin B1 or B2 receptor antagonists, suggesting that the mechanical hyperalgesia induced by the activation of P2X3 receptors in the gastrocnemius muscle of rats is mediated, at least in part, by bradykinin. Our findings are supported by studies demonstrating that chronic muscle pain (Gerdle et al., 2008) and sustained isometric contraction (Boix et al., 2005) of the trapezius muscle of humans is mediated by bradykinin and kallidin, and that administration of bradykinin in health humans induces muscle pain (Babenko et al., 1999). Considering that bradykinin is an inflammatory mediator released at the early phase of inflammatory hyperalgesia (Ferreira et al., 1993a, Ferreira et al., 1993b), it is plausible to suggest that bradykinin is one of the first inflammatory mediators released by α,β-meATP in muscle tissue.

Bradykinin has been reported to induce hyperalgesia by two distinct pathways that ultimately result in the local production of prostaglandins and in the local release of sympathetic amines (Ferreira et al., 1993a, Ferreira et al., 1993b), which directly sensitize the primary afferent nociceptor (Gold et al., 1996, Rush and Waxman, 2004). Also, it has been described that PGE_2 levels are related to muscle pain in patients with fibromyalgia (Hedenberg-Magnusson et

al., 2001, 2002) and delayed onset muscle soreness (Tegeder et al., 2002). Considering the findings of the present study that indomethacin cyclooxygenase inhibitor as well as β_1- or β_2-adrenoceptor antagonists atenolol and ICI 118,551, respectively, reduced the α,β-meATP-induced mechanical muscle hyperalgesia, it is plausible to suggest that α,β-meATP induced the release of bradykinin, which induced the local production of prostaglandins and the local release of sympathetic amines.

It has been proposed that bradykinin triggers the synthesis of prostaglandins and sympathetic amines through the release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 (Ferreira et al., 1993a, Ferreira et al., 1993b) to induce inflammatory hyperalgesia. Considering that the findings of the present study have demonstrated that α,β-meATP also induces cytokines release, it is plausible to hypothesize that α,β-meATP induced the release of bradykinin which, in turn, triggered the release of TNF-α, IL-1β, IL-6 and CINC-1. It is interesting to point out that successive IL-1β injections into the masseter muscle of rats increase the expression of P2X3 receptors in the trigeminal ganglia. Moreover, successive IL1ra injections attenuate the muscle hyperalgesia induced by excessive contraction of masseter muscle and the increased expression of P2X3 receptors in the trigeminal ganglia (Noma et al., 2013).

It has been recently demonstrated that the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 induce neutrophil migration and that the mechanical hyperalgesia induced by each of them is prevented by the inhibition of neutrophil migration by fucoidan (Cunha et al., 2008). Therefore, our findings that α,β-meATP induced neutrophil migration in the gastrocnemius tissue and that the inhibition of neutrophil migration by fucoidan prevented the α,β-meATP-induced mechanical muscle hyperalgesia, suggest that the neutrophil migration induced by α,β-meATP probably results from its ability to induce the release of TNF-α, IL-1β, IL-6 and CINC-1. It is interesting to point out that, in the subcutaneous tissue, the role that P2X3 receptors play on carrageenan (Oliveira et al., 2009) and bradykinin (de Oliveira Fusaro et al., 2010) -induced hyperalgesia does not depend on neutrophil migration, suggesting a mechanism tissue-dependent.

Although we have demonstrated the involvement of some inflammatory mechanisms in mechanical muscle hyperalgesia induced by α,β-meATP, it can exclude the involvement of

others mechanisms. It has been described that inhibition of nitric oxide synthases prevents α,β-meATP -induced neck muscle nociception in mice (Ellrich et al., 2010, Ristic et al., 2010).

4.1. Conclusion

The findings of the present study suggest that α,β-meATP induced mechanical hyperalgesia in the gastrocnemius muscle of rats via activation of peripheral P2X3 receptors, which involves bradykinin, prostaglandins, sympathetic amines, pro-inflammatory cytokines and neutrophil migration. Therefore, we suggest that P2X3 receptors are important targets to control muscle inflammatory pain.

5. Conflicts of interest

The authors have no conflicts to report.

6. Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; BRAZIL).

7. Figure Legends

7.1. α,β-meATP Induced Mechanical Hyperalgesia in the Gastrocnemius Muscle of Rats

Administration of α,β-meATP (1000µg) in the gastrocnemius muscle of rats induced significant behavioral hyperalgesic response when compared with NaCl 0.9% ($P < 0.05$, Tukey test, A) and co-administration of A-317491 (100µg) prevented ($P < 0.05$, Tukey test, B) this response when administered in the ipsilateral but not in the contralateral gastrocnemius muscle. Co-administration of A-317491 (100µg) with 0.9% NaCl did not affect the mechanical withdrawal threshold ($P > 0.05$, Tukey test, C). The mechanical muscle hyperalgesia occurred ½, 1, 2, 3 and 6 hours after administration of α,β-meATP (1000µg) ($P < 0.05$, Two Way ANOVA, Bonferroni test, C) and co-administration of A-317491 (100µg/muscle) prevented the α,β-meATP-induced mechanical muscle at all time points ($P < 0.05$, Two Way ANOVA, Bonferroni test, C). Intradermal or subcutaneous administration of the QX134 (4%) five minutes before intramuscular administration of α,β-meATP did not affect α,β-meATP-induced mechanical muscle hyperalgesia ($P > 0.05$, Tukey test, E). The symbol "*" indicates responses significantly greater than the one induced by the other groups. The symbol "#" indicates responses significantly lower than the one induced by α,β-meATP.

7.2. Effect of the Bradykinin B1 or B2 Receptor Antagonists in α,βmeATP-Induced Mechanical Muscle Hyperalgesia

DALBK (0.3 and 3.0µg, A) or bradyzide (0,015 and 1.5µg, B) significantly reduced ($P < 0.05$, Tukey test) the α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the contralateral gastrocnemius muscle. Co-administration of DALBK (3.0µg) or bradyzide (1.5µg) with 0.9% NaCl did not affect the mechanical withdrawal threshold. The symbol indicates responses significantly lower than the one induced by α,β-meATP.

7.3. Effect of Cyclooxygenase Inhibitor in α,β-meATP-induced Mechanical Muscle Hyperalgesia

Indomethacin (10 and 100µg) significantly reduced ($P < 0.05$, Tukey test) the α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the contralateral gastrocnemius muscle ($P > 0.05$, Tukey test). Co-administration of

indomethacin (100µg) with 0.9% NaCl did not affect the mechanical withdrawal threshold. The symbol indicates responses significantly lower than the one induced by α,β-meATP.

7.4. Effect of β_1- or β_2- adrenoceptor Antagonists in α,β-meATP-induced Mechanical Muscle Hyperalgesia

Atenolol (6.0µ, A) or ICI 118,551 (1.5µg, B) significantly reduced ($P < 0.05$, Tukey test) α,β-meATP-induced mechanical muscle hyperalgesia when administered in the ipsilateral but not in the contralateral gastrocnemius muscle ($P > 0.05$, Tukey test). Co-administration of atenolol (6.0µg) or ICI 118,551 (1.5µg) with 0.9% NaCl did not affect the mechanical withdrawal threshold. The symbol indicates responses significantly lower than the one induced by α,β-meATP. The symbol indicates responses significantly lower than the one induced by α,β-meATP.

7.5. α,β-meATP-induced Increase in Cytokines Concentration in the Gastrocnemius Muscle of Rats

Local administration of α,β-meATP (1000µg) significantly increased ($P < 0.05$, Tukey test) the concentrations of TNF-α (A), IL-1β (B) IL-6 (C) and CINC-1 (D) when compared with 0.9% NaCl. The muscle administration of 0.9% NaCl did not alter ($P > 0.05$, Tukey test) the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with the naïve group. The symbol "*" indicates responses significantly greater than the one induced by NaCl 0.9%.

7.6. α,β-meATP Induced Neutrophil Migration in the Gastrocnemius Muscle of Rats

The administration of α,β-meATP (1000µg) significantly increased ($P < 0.05$, Tukey test, A) the MPO activity when compared with 0.9% NaCl. Pre-treatment with fucoidan (25 mg/kg, i.v.) significantly reduced ($P < 0.05$, Tukey test, B) the α,β-meATP-induced mechanical muscle hyperalgesia. The symbol "*" indicates responses significantly greater that the one induced by the other groups. The symbol "#" indicates responses significantly lower than the one induced by α,β-meATP.

8. References

Ambalavanar R, Moritani M, Dessem D (2005) Trigeminal P2X3 receptor expression differs from dorsal root ganglion and is modulated by deep tissue inflammation. Pain 117:280-291.

Babenko VV, Graven-Nielsen T, Svensson P, Drewes AM, Jensen TS, Arendt-Nielsen L (1999) Experimental human muscle pain induced by intramuscular injections of bradykinin, serotonin, and substance P. Eur J Pain 3:93-102.

Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, Eunson L, Abdel'al S, Natt F, Hall J, Winter J, Bevan S, Wishart W, Fox A, Ganju P (2002) Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. J Neurosci 22:8139-8147.

Boix F, Roe C, Rosenborg L, Knardahl S (2005) Kinin peptides in human trapezius muscle during sustained isometric contraction and their relation to pain. J Appl Physiol 98:534-540.

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87:659-797.

Christidis N, Kopp S, Ernberg M (2005) The effect on mechanical pain threshold over human muscles by oral administration of granisetron and diclofenac-sodium. Pain 113:265-270.

Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G, Ford AP (2005) P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. J Physiol 567:621-639.

Cunha TM, Verri WA, Jr., Schivo IR, Napimoga MH, Parada CA, Poole S, Teixeira MM, Ferreira SH, Cunha FQ (2008) Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. J Leukoc Biol 83:824-832.

de Oliveira Fusaro MC, Pelegrini-da-Silva A, Araldi D, Parada CA, Tambeli CH (2010) P2X3 and P2X2/3 receptors mediate mechanical hyperalgesia induced by bradykinin, but not by pro-inflammatory cytokines, PGE(2) or dopamine. Eur J Pharmacol 649:177-182.

Dessem D, Ambalavanar R, Evancho M, Moutanni A, Yallampalli C, Bai G (2010) Eccentric muscle contraction and stretching evoke mechanical hyperalgesia and modulate CGRP and P2X(3) expression in a functionally relevant manner. Pain 149:284-295.

Ellrich J, Fischer A, Gilsbach JM, Makowska A, Spangenberg P (2010) Inhibition of nitric oxide synthases prevents and reverses alpha,beta-meATP-induced neck muscle nociception in mice. Cephalalgia 30:1225-1232.

Ferreira SH, Lorenzetti BB, Cunha FQ, Poole S (1993a) Bradykinin release of TNF-alpha plays a key role in the development of inflammatory hyperalgesia. Agents Actions 38 Spec No:C7-9.

Ferreira SH, Lorenzetti BB, Poole S (1993b) Bradykinin initiates cytokine-mediated inflammatory hyperalgesia. Br J Pharmacol 110:1227-1231.

Fujii Y, Ozaki N, Taguchi T, Mizumura K, Furukawa K, Sugiura Y (2008) TRP channels and ASICs mediate mechanical hyperalgesia in models of inflammatory muscle pain and delayed onset muscle soreness. Pain 140:292-304.

Gautam M, Benson CJ (2013) Acid-sensing ion channels (ASICs) in mouse skeletal muscle afferents are heteromers composed of ASIC1a, ASIC2, and ASIC3 subunits. Faseb J 27:793-802.

Gerdle B, Hilgenfeldt U, Larsson B, Kristiansen J, Sogaard K, Rosendal L (2008) Bradykinin and kallidin levels in the trapezius muscle in patients with work-related trapezius myalgia, in patients with whiplash associated pain, and in healthy controls - A microdialysis study of women. Pain 139:578-587.

Gold MS, Shuster MJ, Levine JD (1996) Role of a Ca(2+)-dependent slow afterhyperpolarization in prostaglandin E2-induced sensitization of cultured rat sensory neurons. Neurosci Lett 205:161-164.

Hanna RL, Kaufman MP (2004) Activation of thin-fiber muscle afferents by a P2X agonist in cats. J Appl Physiol 96:1166-1169.

Hedenberg-Magnusson B, Ernberg M, Alstergren P, Kopp S (2001) Pain mediation by prostaglandin E2 and leukotriene B4 in the human masseter muscle. Acta Odontol Scand 59:348-355.

Hedenberg-Magnusson B, Ernberg M, Alstergren P, Kopp S (2002) Effect on prostaglandin E2 and leukotriene B4 levels by local administration of glucocorticoid in human masseter muscle myalgia. Acta Odontol Scand 60:29-36.

Honore P, Kage K, Mikusa J, Watt AT, Johnston JF, Wyatt JR, Faltynek CR, Jarvis MF, Lynch K (2002a) Analgesic profile of intrathecal P2X(3) antisense oligonucleotide treatment in chronic inflammatory and neuropathic pain states in rats. Pain 99:11-19.

Honore P, Mikusa J, Bianchi B, McDonald H, Cartmell J, Faltynek C, Jarvis MF (2002b) TNP-ATP, a potent P2X3 receptor antagonist, blocks acetic acid-induced abdominal constriction in mice: comparison with reference analgesics. Pain. 96:99-105.

Jarvis MF, Burgard EC, McGaraughty S, Honore P, Lynch K, Brennan TJ, Subieta A, Van Biesen T, Cartmell J, Bianchi B, Niforatos W, Kage K, Yu H, Mikusa J, Wismer CT, Zhu CZ, Chu K, Lee CH, Stewart AO, Polakowski J, Cox BF, Kowaluk E, Williams M, Sullivan J, Faltynek C (2002) A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. Proc Natl Acad Sci U S A 99:17179-17184.

Kennedy C, Assis TS, Currie AJ, Rowan EG (2003) Crossing the pain barrier: P2 receptors as targets for novel analgesics. J Physiol 553:683-694.

Krismer M, van Tulder M (2007) Strategies for prevention and management of musculoskeletal conditions. Low back pain (non-specific). Best Pract Res Clin Rheumatol 21:77-91.

Le Bars D, Dickenson AH, Besson JM (1979) Diffuse noxious inhibitory controls (DNIC). I. Effects on dorsal horn convergent neurones in the rat. Pain 6:283-304.

Loram LC, Fuller A, Fick LG, Cartmell T, Poole S, Mitchell D (2007) Cytokine profiles during carrageenan-induced inflammatory hyperalgesia in rat muscle and hind paw. J Pain 8:127-136.

Makowska A, Panfil C, Ellrich J (2006) ATP induces sustained facilitation of craniofacial nociception through P2X receptors on neck muscle nociceptors in mice. Cephalalgia 26:697-706.

McGaraughty S, Jarvis MF (2005) Antinociceptive properties of a non-nucleotide P2X3/P2X2/3 receptor antagonist. Drug News Perspect 18:501-507.

McGaraughty S, Wismer CT, Zhu CZ, Mikusa J, Honore P, Chu KL, Lee CH, Faltynek CR, Jarvis MF (2003) Effects of A-317491, a novel and selective P2X3/P2X2/3 receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. Br J Pharmacol 140:1381-1388.

Mense S (2009) Algesic agents exciting muscle nociceptors. Exp Brain Res 196:89-100.

Mork H, Ashina M, Bendtsen L, Olesen J, Jensen R (2003) Experimental muscle pain and tenderness following infusion of endogenous substances in humans. Eur J Pain 7:145-153.

Noma N, Shinoda M, Honda K, Kiyomoto M, Dezawa K, Nakaya Y, Komiyama O, Imamura Y, Iwata K (2013) Interaction of IL-1beta and P2X(3) receptor in pathologic masseter muscle pain. J Dent Res 92:456-460.

Oliveira MC, Pelegrini-da-Silva A, Tambeli CH, Parada CA (2009) Peripheral mechanisms underlying the essential role of P2X3,2/3 receptors in the development of inflammatory hyperalgesia. Pain 141:127-134.

Papir-Kricheli D, Frey J, Laufer R, Gilon C, Chorev M, Selinger Z, Devor M (1987) Behavioural effects of receptor-specific substance P agonists. Pain 31:263-276.

Prado FC, Araldi D, Vieira AS, Oliveira-Fusaro MC, Tambeli CH, Parada CA (2013) Neuronal P2X3 receptor activation is essential to the hyperalgesia induced by prostaglandins and sympathomimetic amines released during inflammation. Neuropharmacology 67:252-258.

Randall LO, Selitto JJ (1957) A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 111:409-419.

Reinohl J, Hoheisel U, Unger T, Mense S (2003) Adenosine triphosphate as a stimulant for nociceptive and non-nociceptive muscle group IV receptors in the rat. Neurosci Lett 338:25-28.

Reitz M, Makowska A, Ellrich J (2009) Excitatory and inhibitory purinergic control of neck muscle nociception in anaesthetized mice. Cephalalgia 29:58-67.

Ristic D, Spangenberg P, Ellrich J (2010) Inhibition of nNOS prevents and inhibition of iNOS reverses alpha,beta-meATP-induced facilitation of neck muscle nociception in mice. Eur J Pharmacol 647:55-61.

Rosland JH (1991) The formalin test in mice: the influence of ambient temperature. Pain 45:211-216.

Rush AM, Waxman SG (2004) PGE2 increases the tetrodotoxin-resistant Nav1.9 sodium current in mouse DRG neurons via G-proteins. Brain Res 1023:264-271.

Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995) Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. Br J Pharmacol 115:1265-1275.

Schafers M, Sorkin LS, Sommer C (2003) Intramuscular injection of tumor necrosis factor-alpha induces muscle hyperalgesia in rats. Pain 104:579-588.

Shinoda M, Ozaki N, Sugiura Y (2008) Involvement of ATP and its receptors on nociception in rat model of masseter muscle pain. Pain 134:148-157.

Tegeder L, Zimmermann J, Meller ST, Geisslinger G (2002) Release of algesic substances in human experimental muscle pain. Inflamm Res 51:393-402.

Torres-Chavez KE, Sanfins JM, Clemente-Napimoga JT, Pelegrini-Da-Silva A, Parada CA, Fischer L, Tambeli CH (2012) Effect of gonadal steroid hormones on formalin-induced temporomandibular joint inflammation. Eur J Pain 16:204-216.

Waldron JB, Sawynok J (2004) Peripheral P2X receptors and nociception: interactions with biogenic amine systems. Pain 110:79-89.

Wang C, Li GW, Huang LY (2007) Prostaglandin E2 potentiation of P2X3 receptor mediated currents in dorsal root ganglion neurons. Mol Pain 3:22.

Wu G, Whiteside GT, Lee G, Nolan S, Niosi M, Pearson MS, Ilyin VI (2004) A-317491, a selective P2X3/P2X2/3 receptor antagonist, reverses inflammatory mechanical hyperalgesia through action at peripheral receptors in rats. Eur J Pharmacol 504:45-53.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109-110.

Figura 1

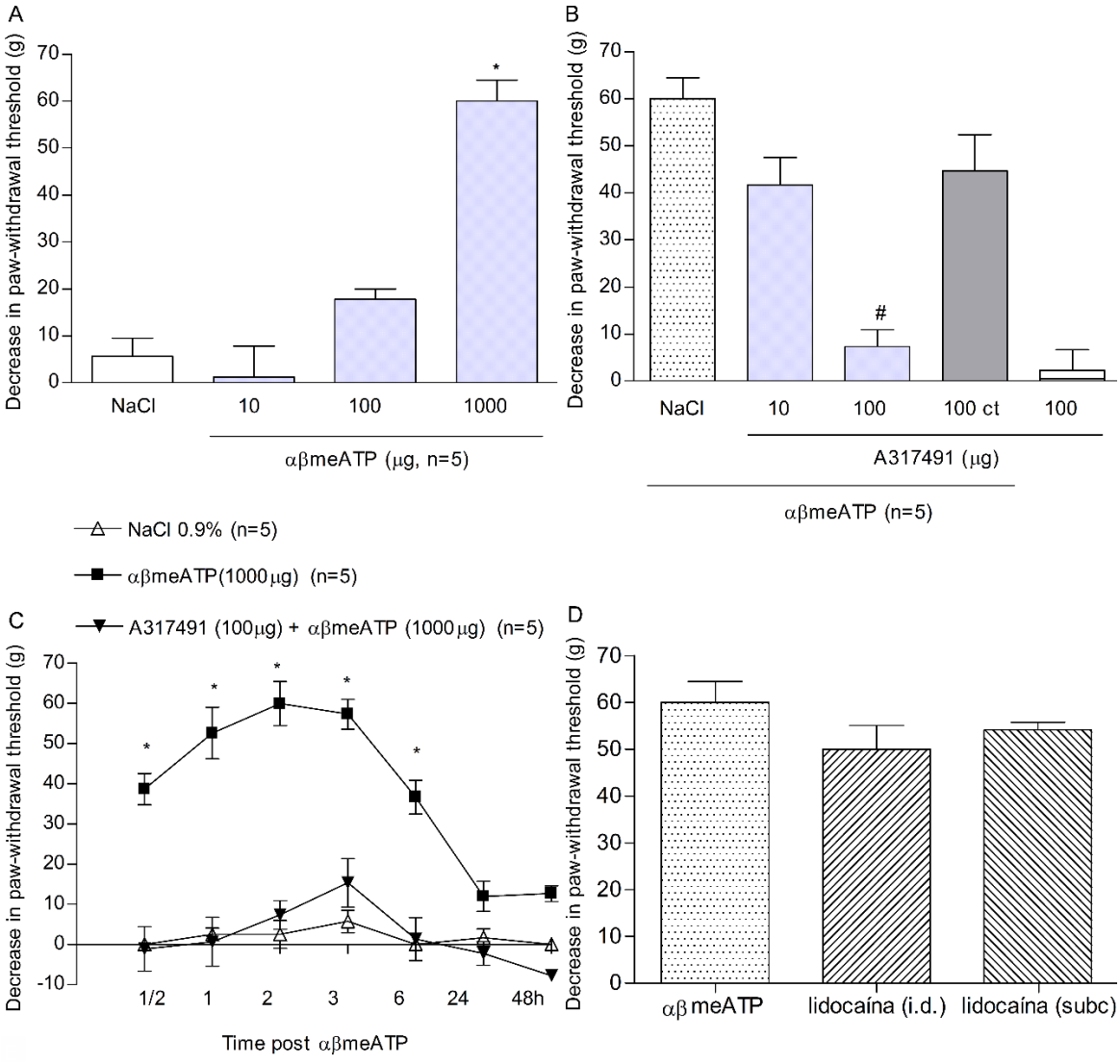

A
Decrease in paw-withdrawal threshold (g)
NaCl
10
100
1000
αβmeATP (μg, n=5)
B
Decrease in paw-withdrawal threshold (g)
NaCl
10
100
100 ct
100
A317491 (μg)
αβmeATP (n=5)
NaCl 0.9% (n=5)
αβmeATP(1000μg) (n=5)
A317491 (100μg) + αβmeATP (1000μg) (n=5)
C
Decrease in paw-withdrawal threshold (g)
1/2
1
2
3
6
24
48h
Time post αβmeATP
D
Decrease in paw-withdrawal threshold (g)
αβ meATP
lidocaína (i.d.)
lidocaína (subc)

Figura 2

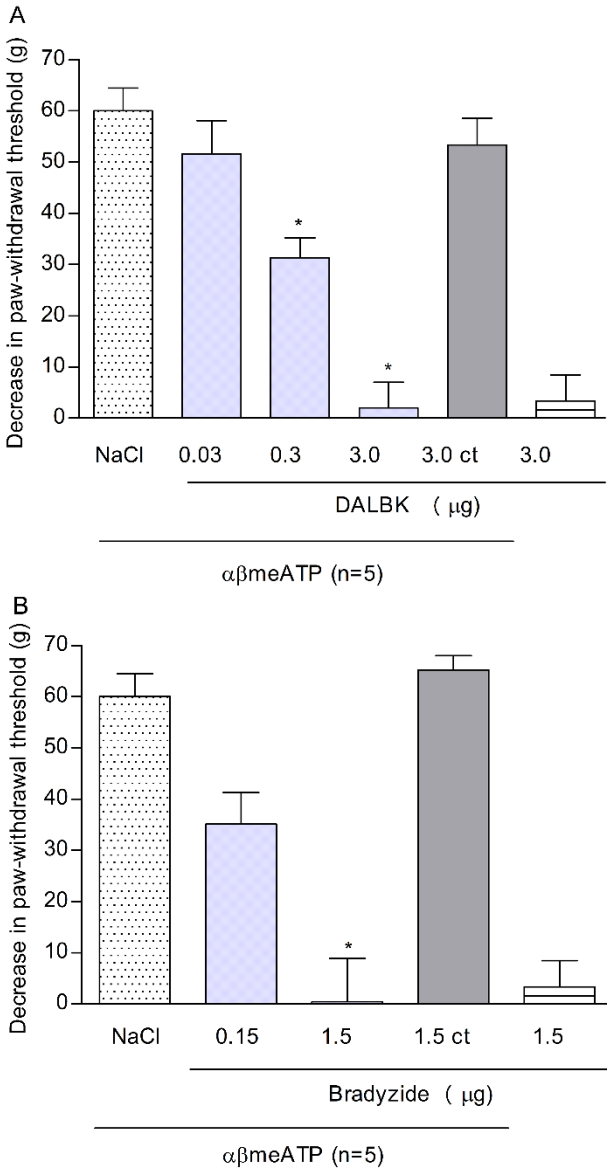

A
Decrease in paw-withdrawal threshold (g)
70
60
50
40
30
20
10
0
*
*
NaCl
0.03
0.3
3.0
3.0 ct
3.0
DALBK (μg)
αβmeATP (n=5)
B
Decrease in paw-withdrawal threshold (g)
70
60
50
40
30
20
10
0
*
NaCl
0.15
1.5
1.5 ct
1.5
Bradyzide (μg)
αβmeATP (n=5)

Figura 3

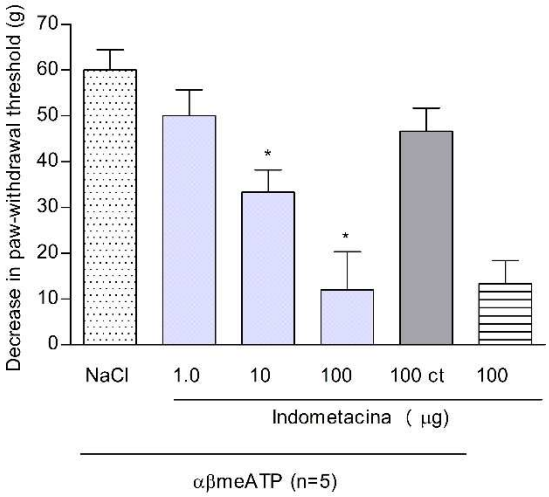


Figura 4

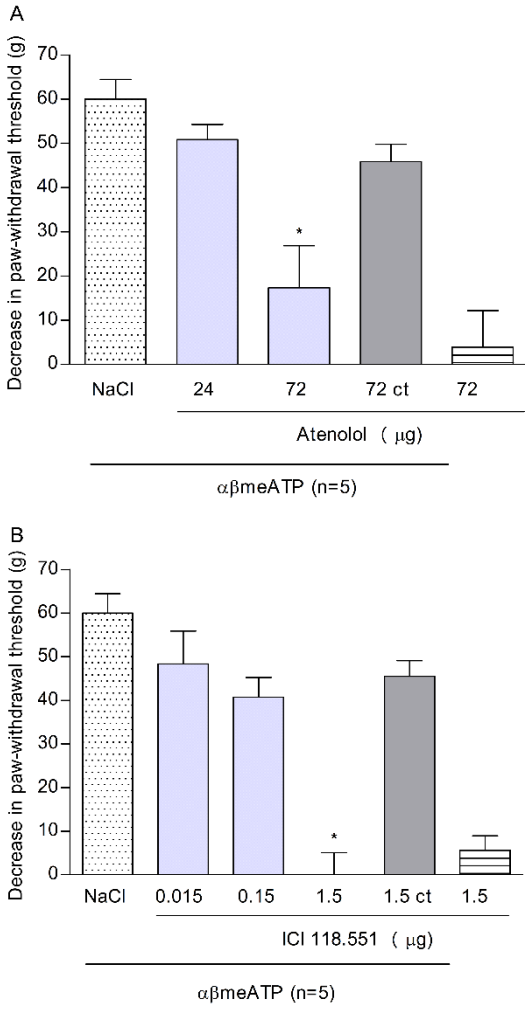


Figura 5

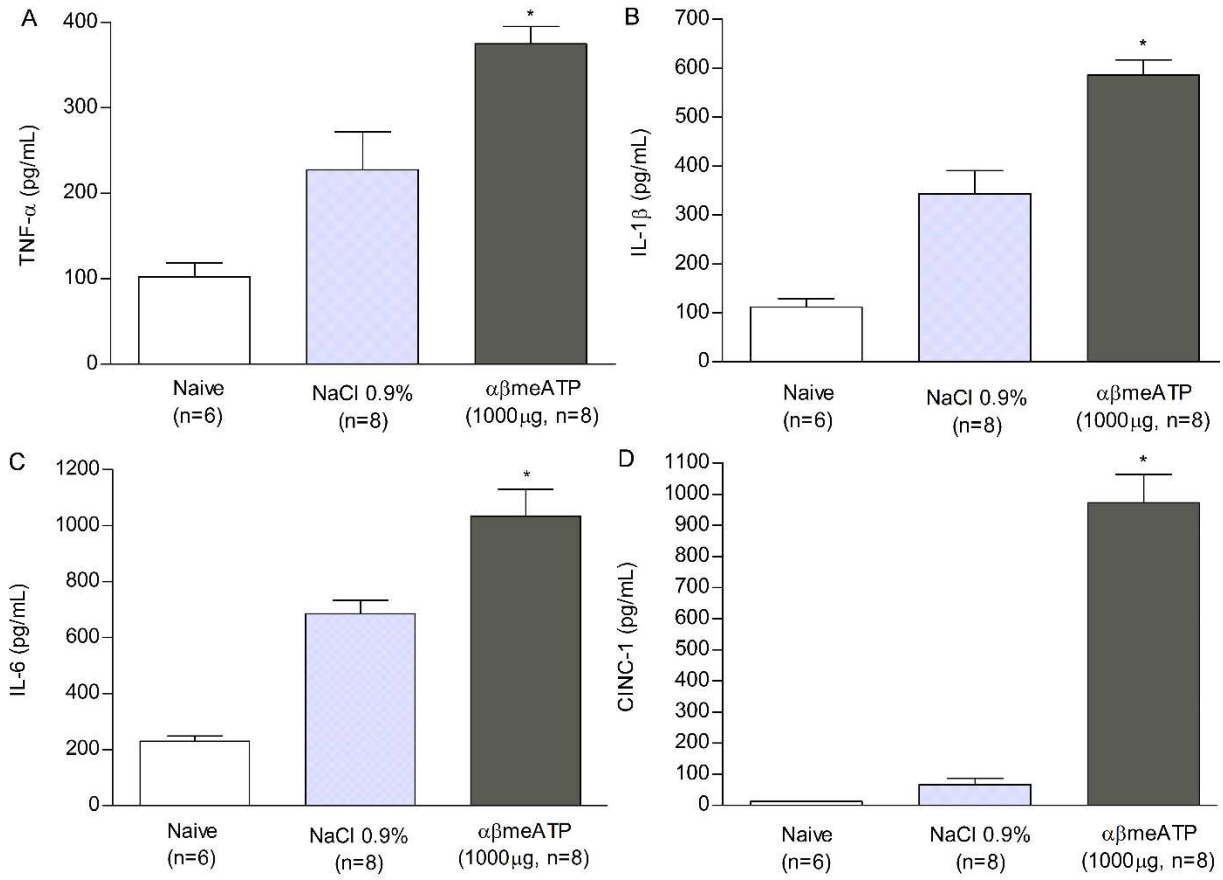
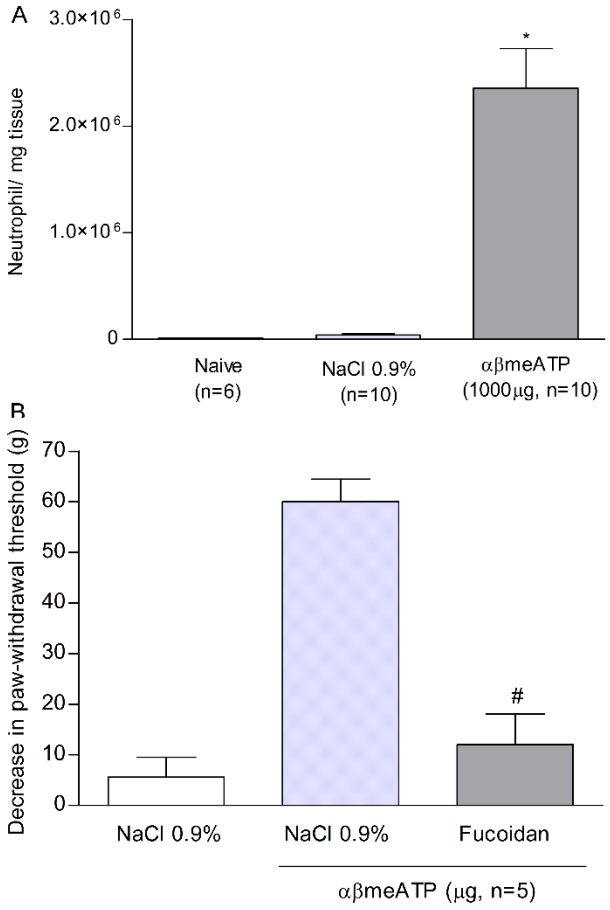

A
TNF-α (pg/mL)
400
300
200
100
0
*
Naive (n=6)
NaCl 0.9% (n=8)
αβmeATP (1000μg, n=8)
B
IL-1β (pg/mL)
700
600
500
400
300
200
100
0
*
Naive (n=6)
NaCl 0.9% (n=8)
αβmeATP (1000μg, n=8)
C
IL-6 (pg/mL)
1200
1000
800
600
400
200
0
*
Naive (n=6)
NaCl 0.9% (n=8)
αβmeATP (1000μg, n=8)
D
CINC-1 (pg/mL)
1100
1000
900
800
700
600
500
400
300
200
100
0
*
Naive (n=6)
NaCl 0.9% (n=8)
αβmeATP (1000μg, n=8)

Figura 6


A
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
Neutrophil/ mg tissue
*
Naive
(n=6)
NaCl 0.9%
(n=10)
αβmeATP
(1000μg, n=10)
B
Decrease in paw-withdrawal threshold (g)
70
60
50
40
30
20
10
0
#
NaCl 0.9%
NaCl 0.9%
Fucoidan
αβmeATP (μg, n=5)

Discussão

Os achados deste estudo nós temos demonstrado, no primeiro momento, que a ativação do receptor P2X3 no músculo gastrocnêmio de ratos pelo agonista não seletivo de receptor P2X3, α,β-meATP, induz hiperalgesia mecânica muscular mediada por bradicinina, prostaglandinas, aminas simpatomiméticas e migração de neutrófilos. Também a ativação dos receptores P2X3 nos músculo de ratos induzem aumento de citocinas pro inflamatórias.

Vimos também que o antagonista seletivo dos receptores P2X3 e P2X2/3 A317491, bloqueou a hiperalgesia muscular induzida pelo agonista não seletivo de receptor P2X3, α,β-meATP no músculo gastrocnêmio demonstrando o papel periférico dos receptores P2X3 na hiperalgesia muscular. Embora o α,β-meATP seja um agonista não seletivo de receptores P2X1, P2X3 e P2X2/3, o envolvimento do receptor P2X1 parece ser improvável, porque a hiperalgesia mecânica muscular induzida pelo α,β-meATP foi completamente revertida pelo A-317491. Além disso, estudos demonstram que o IP5I, um potente antagonista seletivo do receptor P2X1, é ineficiente para reduzir a dor inflamatória. (Honore, Kage *et al.*, 2002). Nossos dados de que a ativação de receptores P2X3 pelo α,β-meATP induz hiperalgesia mecânica, estão apoiados em outros estudos em dor muscular (Shinoda, Ozaki *et al.*, 2005) e no tecido subcutâneo (Prado, Araldi *et al.*). A hiperalgesia mecânica induzida pelo α,β-meATP foi bloqueada pelo antagonista seletivo de receptor de bradicinina B1 or B2, sugerindo que a hiperalgesia mecânica induzida pela ativação do receptor P2X3 nos músculo gastrocnêmio de ratos é mediada, pelo menos em parte, pela bradicinina. Nossos achados estão de acordo com estudos que demonstram a dor muscular crônica (Gerdle, Hilgenfeldt *et al.*, 2008) contração isométrica sustentada (Boix, Roe *et al.*, 2005) do músculo trapézio de humanos é mediado por bradicinina e kalidina, e que a administração de bradicinina em humanos saudáveis induz dor muscular (Babenko, Graven-Nielsen *et al.*, 1999). Considerando que a bradicinina é um mediador inflamatório que está aumentado nas primeiras fases da hiperalgesia inflamatória (Ferreira, Lorenzetti *et al.*, 1993), Ferreira et al., 1993b), é plausível sugerir que a bradicinina é um dos primeiros mediadores inflamatórios aumentados pelo α,β-meATP na dor muscular.

Tem sido demonstrado que a bradicinina induz hiperalgesia por duas vias distintas que em última análise resulta na produção local de prostaglandina e na libertação local de aminas

simpáticas (Ferreira *et al*., 1993a, Ferreira *et al*., 1993b), que sensibilizam diretamente as fibras aferentes primárias (Gold, Shuster *et al.*, 1996; Rush e Waxman, 2004). Também tem sido demonstrado que os níveis de PGE_2 l estão aumentados na dor muscular em pacientes com fibromialgia (Hedenberg-Magnusson, Ernberg *et al.*, 2001) e na dor muscular (Tegeder, Zimmermann *et al.*, 2002). Considerando o as achados deste presente estudo que o inibidor de ciclooxigenase tal como os antagonistas de adrenoceptores β_1- e β_2- Atenolol e ICI 118,551, respectivamente, reduzem a hiperalgesia mecânica muscular induzida pelo α,β-meATP, é plausível sugerir que o α,β-meATP induz o aumento de bradicinina, a qual induz aumento local e produção de prostaglandinas e aumento local de aminas simpatomiméticas.

Tem sido proposto que a bradicinina desencadeia a síntese de prostaglandinas e aminas simpáticas através da libertação das citocinas pró-inflamatórias TNF-α, IL-1β, IL-6 e CINC-1 (Ferreira *et al.,* 1993a, Ferreira *et al.,* 1993b) para induzir a hiperalgesia inflamatória. Nossos achados demonstram que o α,β-meATP também induz aumento de citocinas, é plausível hipotetizar que o α,β-meATP induz aumento de bradicinina a qual, por sua vez, provocou aumento de TNF-α, IL-1β, IL-6 e CINC-1. É interessante salientar que sucessivas injeções de IL-1β no músculo masseter de ratos aumenta a expressão de receptores de P2X3 nos gânglios do trigêmeo. Além disso, as sucessivas injeções IL-1β atenuaram a hiperalgesia muscular induzida pela contração excessiva do músculo masseter e o aumento da expressão de receptores P2X3 no gânglio trigeminal (Noma *et al*., 2013).

Foi recentemente demonstrado que as citocinas pro-inflamatórias TNF-α, IL-1β, IL-6 and CINC-1 induzem migração de neutrófilos e que a hiperalgesia mecânica induzida por cada uma delas é impedida pelo inibidor de migração de neutrófilos fucoidan (Cunha *et al*., 2008). Portanto, as nossas descobertas de que a migração do neutrófilo induzida pelo α, β-meATP no músculo gastrocnêmio e que a inibição da migração de neutrófilos pelo Fucoidan impediu a hiperalgesia mecânica induzida pelo α, β-meATP no músculo, sugerem que a migração de neutrófilos induzida pelo α, β-meATP provavelmente resulta da sua capacidade de induzir a aumento de TNF-α, IL-1β, IL-6 e CINC-1. Portanto nossos achados mostram que a hiperalgesia mecânica induzida pelo α,β-meATP induz migração de neutrófilos no tecido muscular e que a migração de neutrófilos bloqueada pelo fucoidan preveniu a hiperalgesia muscular induzida α,β-meATP-, sugerindo que a migração de neutrófilos induzido α,β-meATP provavelmente resulta da sua capacidade para induzir a libertação de TNF-α, IL-1β, IL-6 e CINC-1.É interessante

salientar que, no tecido subcutâneo, a hiperalgesia mecânica sensibilizando os receptores P2X3 ativados pela carragenina (Oliveira *et al*., 2009) e bradicinina (Fusaro de Oliveira et al. De 2010) não depende de migração de neutrófilos, sugerindo um mecanismo de tecido dependente. Embora tenhamos demonstrado o envolvimento de alguns dos mecanismos inflamatórios, a hiperalgesia mecânica muscular induzida pelo α, β-meATP, pode excluir o envolvimento de outros mecanismos. Tem sido descrito que a inibição da síntese de óxido nítrico previne a nocicepção muscular induzida pelo α, β-meATP no pescoço de ratos (Ellrich *et al*. De 2010, Ristic *et al*., 2010).

Referências

Abbracchio, M. P. e G. Burnstock. Purinergic signalling: pathophysiological roles. Jpn J Pharmacol, v.78, n.2, Oct, p.113-45. 1998.

Aguggia, M. Neurophysiology of pain. Neurol Sci, v.24 Suppl 2, May, p.S57-60. 2003.

Babenko, V. V., T. Graven-Nielsen, *et al.* Experimental human muscle pain induced by intramuscular injections of bradykinin, serotonin, and substance P. Eur J Pain, v.3, n.2, Jun, p.93-102. 1999.

Barclay, J., S. Patel, *et al.* Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. J Neurosci, v.22, n.18, Sep 15, p.8139-47. 2002.

Besson, J. M. e A. Chaouch. Peripheral and spinal mechanisms of nociception. Physiol Rev, v.67, n.1, Jan, p.67-186. 1987.

Bland-Ward, P. A. e P. P. Humphrey. P2X receptors mediate ATP-induced primary nociceptive neurone activation. J Auton Nerv Syst, v.81, n.1-3, Jul 3, p.146-51. 2000.

Boix, F., C. Roe, *et al.* Kinin peptides in human trapezius muscle during sustained isometric contraction and their relation to pain. J Appl Physiol, v.98, n.2, Feb, p.534-40. 2005.

Bonica, J. J. Evolution and current status of pain programs. J Pain Symptom Manage, v.5, n.6, Dec, p.368-74. 1990.

Burnstock, G. Purinergic nerves. Pharmacol Rev, v.24, n.3, Sep, p.509-81. 1972.

Chen, C. C., A. N. Akopian, *et al.* A P2X purinoceptor expressed by a subset of sensory neurons. Nature, v.377, n.6548, Oct 5, p.428-31. 1995.

Christidis, N., S. Kopp, *et al.* The effect on mechanical pain threshold over human muscles by oral administration of granisetron and diclofenac-sodium. Pain, v.113, n.3, Feb, p.265-70. 2005.

Cockayne, D. A., S. G. Hamilton, *et al.* Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. Nature, v.407, n.6807, Oct 26, p.1011-5. 2000.

Cunha, F. Q., S. Poole, *et al.* The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. Br J Pharmacol, v.107, n.3, Nov, p.660-4. 1992.

Dessem, D., R. Ambalavanar*, et al.* Eccentric muscle contraction and stretching evoke mechanical hyperalgesia and modulate CGRP and P2X(3) expression in a functionally relevant manner. Pain, v.149, n.2, May, p.284-95.

Ferreira, S. H., B. B. Lorenzetti*, et al.* Bradykinin release of TNF-alpha plays a key role in the development of inflammatory hyperalgesia. Agents Actions, v.38 Spec No, p.C7-9. 1993.

Gerdle, B., U. Hilgenfeldt*, et al.* Bradykinin and kallidin levels in the trapezius muscle in patients with work-related trapezius myalgia, in patients with whiplash associated pain, and in healthy controls - A microdialysis study of women. Pain, v.139, n.3, Oct 31, p.578-87. 2008.

Gold, M. S., M. J. Shuster*, et al.* Role of a Ca(2+)-dependent slow afterhyperpolarization in prostaglandin E2-induced sensitization of cultured rat sensory neurons. Neurosci Lett, v.205, n.3, Mar 1, p.161-4. 1996.

Hamilton, S. G. ATP and pain. Pain Pract, v.2, n.4, Dec, p.289-94. 2002.

Hamilton, S. G., A. Wade*, et al.* The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat. Br J Pharmacol, v.126, n.1, Jan, p.326-32. 1999.

Hanna, R. L. e M. P. Kaufman. Activation of thin-fiber muscle afferents by a P2X agonist in cats. J Appl Physiol, v.96, n.3, Mar, p.1166-9. 2004.

Hedenberg-Magnusson, B., M. Ernberg*, et al.* Pain mediation by prostaglandin E2 and leukotriene B4 in the human masseter muscle. Acta Odontol Scand, v.59, n.6, Dec, p.348-55. 2001.

Holton, P. The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. J Physiol, v.145, n.3, Mar 12, p.494-504. 1959.

Honore, P., K. Kage*, et al.* Analgesic profile of intrathecal P2X(3) antisense oligonucleotide treatment in chronic inflammatory and neuropathic pain states in rats. Pain, v.99, n.1-2, Sep, p.11-9. 2002.

Huang, J., X. Zhang*, et al.* Inflammatory pain: the cellular basis of heat hyperalgesia. Curr Neuropharmacol, v.4, n.3, Jul, p.197-206. 2006.

Jarvis, M. F., E. C. Burgard*, et al.* A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. Proc Natl Acad Sci U S A, v.99, n.26, Dec 24, p.17179-84. 2002.

Julius, D. e A. I. Basbaum. Molecular mechanisms of nociception. Nature, v.413, n.6852, Sep 13, p.203-10. 2001.

Kennedy, C. e P. Leff. Painful connection for ATP. Nature, v.377, n.6548, Oct 5, p.385-6. 1995.

Khakh, B. S. e R. A. North. P2X receptors as cell-surface ATP sensors in health and disease. Nature, v.442, n.7102, Aug 3, p.527-32. 2006.

Krismer, M. e M. Van Tulder. Strategies for prevention and management of musculoskeletal conditions. Low back pain (non-specific). Best Pract Res Clin Rheumatol, v.21, n.1, Feb, p.77-91. 2007.

Laskin, D. L. e K. J. Pendino. Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol, v.35, p.655-77. 1995.

Lewis, C., S. Neidhart*, et al.* Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. Nature, v.377, n.6548, Oct 5, p.432-5. 1995.

Loram, L. C., A. Fuller*, et al.* Cytokine profiles during carrageenan-induced inflammatory hyperalgesia in rat muscle and hind paw. J Pain, v.8, n.2, Feb, p.127-36. 2007.

Makowska, A., C. Panfil*, et al.* ATP induces sustained facilitation of craniofacial nociception through P2X receptors on neck muscle nociceptors in mice. Cephalalgia, v.26, n.6, Jun, p.697-706. 2006.

Mccleskey, E. W. e M. S. Gold. Ion channels of nociception. Annu Rev Physiol, v.61, p.835-56. 1999.

Mcgaraughty, S., P. Honore*, et al.* Endogenous opioid mechanisms partially mediate P2X3/P2X2/3-related antinociception in rat models of inflammatory and chemogenic pain but not neuropathic pain. Br J Pharmacol, v.146, n.2, Sep, p.180-8. 2005.

Mcgaraughty, S., C. T. Wismer*, et al.* Effects of A-317491, a novel and selective P2X3/P2X2/3 receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. Br J Pharmacol, v.140, n.8, Dec, p.1381-8. 2003.

Mense, S. Algesic agents exciting muscle nociceptors. Exp Brain Res, v.196, n.1, Jun, p.89-100. 2009.

Millan, M. J. The induction of pain: an integrative review. Prog Neurobiol, v.57, n.1, Jan, p.1-164. 1999.

Mork, H., M. Ashina*, et al.* Experimental muscle pain and tenderness following infusion of endogenous substances in humans. Eur J Pain, v.7, n.2, p.145-53. 2003.

Noback, M. A., P. Terpstra*, et al.* A 22 kb DNA sequence in the cspB-glpPFKD region at 75 degrees on the Bacillus subtilis chromosome. Microbiology, v.142 (Pt 11), Nov, p.3021-6. 1996.

Noma, N., M. Shinoda*, et al.* Interaction of IL-1beta and P2X(3) receptor in pathologic masseter muscle pain. J Dent Res, v.92, n.5, May, p.456-60.

Oliveira, M. C., C. A. Parada*, et al.* Evidence for the involvement of endogenous ATP and P2X receptors in TMJ pain. Eur J Pain, v.9, n.1, Feb, p.87-93. 2005.

Oliveira, M. C., A. Pelegrini-Da-Silva*, et al.* Peripheral mechanisms underlying the essential role of P2X3,2/3 receptors in the development of inflammatory hyperalgesia. Pain, v.141, n.1-2, Jan, p.127-34. 2009.

Prado, F. C., D. Araldi*, et al.* Neuronal P2X3 receptor activation is essential to the hyperalgesia induced by prostaglandins and sympathomimetic amines released during inflammation. Neuropharmacology, v.67, Apr, p.252-8.

Reinohl, J., U. Hoheisel*, et al.* Adenosine triphosphate as a stimulant for nociceptive and non-nociceptive muscle group IV receptors in the rat. Neurosci Lett, v.338, n.1, Feb 20, p.25-8. 2003.

Riedel, W. e G. Neeck. Nociception, pain, and antinociception: current concepts. Z Rheumatol, v.60, n.6, Dec, p.404-15. 2001.

Rote, N. S., E. Vogt*, et al.* The role of placental trophoblast in the pathophysiology of the antiphospholipid antibody syndrome. Am J Reprod Immunol, v.39, n.2, Feb, p.125-36. 1998.

Rush, A. M. e S. G. Waxman. PGE2 increases the tetrodotoxin-resistant Nav1.9 sodium current in mouse DRG neurons via G-proteins. Brain Res, v.1023, n.2, Oct 15, p.264-71. 2004.

Schafers, M., L. S. Sorkin*, et al.* Intramuscular injection of tumor necrosis factor-alpha induces muscle hyperalgesia in rats. Pain, v.104, n.3, Aug, p.579-88. 2003.

Sharp, C. J., A. J. Reeve*, et al.* Investigation into the role of P2X(3)/P2X(2/3) receptors in neuropathic pain following chronic constriction injury in the rat: an electrophysiological study. Br J Pharmacol, v.148, n.6, Jul, p.845-52. 2006.

Sherwood, E. R. e T. Toliver-Kinsky. Mechanisms of the inflammatory response. Best Pract Res Clin Anaesthesiol, v.18, n.3, Sep, p.385-405. 2004.

Shinoda, M., N. Ozaki*, et al.* Changes in P2X3 receptor expression in the trigeminal ganglion following monoarthritis of the temporomandibular joint in rats. Pain, v.116, n.1-2, Jul, p.42-51. 2005.

Souslova, V., P. Cesare*, et al.* Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. Nature, v.407, n.6807, Oct 26, p.1015-7. 2000.

Tegeder, L., J. Zimmermann*, et al.* Release of algesic substances in human experimental muscle pain. Inflamm Res, v.51, n.8, Aug, p.393-402. 2002.

Tsuda, M., S. Koizumi*, et al.* Mechanical allodynia caused by intraplantar injection of P2X receptor agonist in rats: involvement of heteromeric P2X2/3 receptor signaling in capsaicin-insensitive primary afferent neurons. J Neurosci, v.20, n.15, Aug 1, p.RC90. 2000.

Van Furth, R., P. H. Nibbering*, et al.* The characterization, origin, and kinetics of skin macrophages during inflammation. J Invest Dermatol, v.85, n.5, Nov, p.398-402. 1985.

Verri, W. A., Jr., T. M. Cunha*, et al.* Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? Pharmacol Ther, v.112, n.1, Oct, p.116-38. 2006.

Waldron, J. B. e J. Sawynok. Peripheral P2X receptors and nociception: interactions with biogenic amine systems. Pain, v.110, n.1-2, Jul, p.79-89. 2004.

Wang, C., G. W. Li*, et al.* Prostaglandin E2 potentiation of P2X3 receptor mediated currents in dorsal root ganglion neurons. Mol Pain, v.3, p.22. 2007.

Wu, G., G. T. Whiteside*, et al.* A-317491, a selective P2X3/P2X2/3 receptor antagonist, reverses inflammatory mechanical hyperalgesia through action at peripheral receptors in rats. Eur J Pharmacol, v.504, n.1-2, Nov 3, p.45-53. 2004.