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Maria Cláudia Gonçalves de Oliveira

FISIOTERAPEUTA

"AVALIAÇÃO DA PRESENÇA DE RECEPTORES PURINÉRGICOS P2X *FUNCIONAIS* NA REGIÃO DA ATM DE RATOS E DO PAPEL DO ATP ENDÓGENO NA SENSIBILIZAÇÃO DOS NOCICEPTORES DA ATM"

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

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(Autor desconhecido)

SUMÁRIO

RESUMO

Muitos estudos evidenciam o papel do ATP no processo de desencadeamento da dor por agir em receptores P2X expressos nos terminais nervosos aferentes nociceptivos. Para investigar se esses receptores exercem uma função na dor da ATM, estudamos a presença de receptores P2X funcionais na ATM de ratos, examinando as conseqüências comportamentais da administração de um agonista seletivo para o receptor P2X α,β-metileno ATP (α,β-me ATP) na região da ATM em ratos conscientes. O envolvimento do ATP endógeno no desenvolvimento da hiperalgesia na ATM foi também determinado pela avaliação do efeito do antagonista de receptores P2 pyridoxal-phosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) na hiperalgesia química induzida pela carragenina na ATM. Administração de α,β-me ATP na região da ATM de ratos produziu significativas respostas nociceptivas que foram significativamente reduzidas pela co-administração do derivado quaternário de lidocaína hidrofílica, QX 314, a 2% ou do antagonista de receptor P2 PPADS. Co-administração de PPADS com carragenina na ATM significativamente reduziu a hiperalgesia química induzida pela carragenina. Os resultados indicam que receptores P2X funcionais estão presentes na ATM e sugerem que o ATP endógeno participe dos mecanismos da dor inflamatória na ATM possivelmente por agir primariamente nesses receptores.

Palavras chave: receptores P2X, ATM, inflamação, dor, α,β-meATP

ABSTRACT

Evidence is accumulating which supports a role for ATP in the initiation of pain by acting on P2X receptors expressed on nociceptive afferent nerve terminals. To investigate whether these receptors play a role in TMJ pain, we studied the presence of functional P2X receptors in rat TMJ by examining the behavioral consequences of the application of the selective P2X receptor agonist α,β-methylene ATP (α,β-meATP) to the TMJ region in the conscious rat. The involvement of endogenous ATP in the development of TMJ hyperalgesia was also determined by evaluating the effect of the general P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on carrageenan-induced chemical TMJ hyperalgesia. Administration of α,β-meATP into the TMJ region of rats produced significant nociceptive responses that were significantly reduced by the co-application of lidocaine N-ethyl bromide quaternary salt, QX-314, (2%) or of the P2 receptor antagonist, PPADS. Co-administration of PPADS with carrageenan into the TMJ significantly reduced carrageenan-induced chemical hyperalgesia. The results indicate that functional P2X receptors are present in the TMJ and suggest that endogenous ATP may play a role in TMJ inflammatory pain mechanisms possibly by acting primarily in these receptors.

Key words: P2X receptors, TMJ, inflammation, pain, α,β-meATP

1. INTRODUÇÃO

A dor é definida pela Associação Internacional para Estudo da Dor (IASP) como sendo uma "experiência sensorial e emocional desagradável associada a um dano tecidual real ou potencial ou descrita nesses termos". É uma experiência multidimensional na qual estão envolvidos vários componentes: motivacional, emocional, sensório-discriminativo, afetivo e cognitivo (Mersky, 1986). Além disso, a dor é um mecanismo de demarcação de limites para o organismo e de aviso quanto à ocorrência de estímulos que possam ser lesivos (Lent, 2001). Uma grande parte dos episódios dolorosos são desencadeados por lesão tecidual, sendo uma reação natural do organismo ao trauma proteger-se através da inflamação e, ao mesmo tempo, alertar o cérebro do perigo iminente através da sensação dolorosa, que impede a ocorrência de uma destruição tecidual ainda maior (Cooper, 1990). No entanto, embora a dor seja essencial para a preservação da vida, em muitas circunstâncias ela perde o seu caráter protetor e passa a constituir a própria doença (Coderre *et al*., 1993).

Dentre as dores somáticas, a dor proveniente da região orofacial corresponde a mais de 25% dos casos (Sessle, 1987; Loe, 1993). Dados epidemiológicos demonstram que de uma amostra de 894 pacientes brasileiros portadores de distúrbios temporomandibulares, 87,9% apresentavam dor orofacial (Luz *et al*., 1997) e, entre as condições dolorosas da região orofacial, a dor proveniente da ATM é a mais freqüente (Luz *et al*., 1997; Irving *et al*., 1999).

A captação e transmissão das informações sensoriais dolorosas provenientes da região orofacial são realizadas por receptores sensoriais nociceptivos e por fibras nervosas aferentes primárias trigeminais, cujos corpos celulares estão localizados no gânglio trigeminal (Machado, 1993).

Segundo Sessle (1986), em um trabalho de revisão, muitas terminações nervosas livres atuam como nociceptores, ou seja, receptores ou órgãos sensoriais sensíveis a estímulos nocivos. A ativação dos nociceptores, por sua vez, pode levar à excitação das fibras nervosas aferentes, transmitindo ao Sistema Nervoso Central informações sensoriais e discriminativas sobre as características espaciais e temporais de um estímulo nocivo. Eles estão presentes em vários tecidos orofaciais: pele, tecido subcutâneo, tecido adiposo, camada adventícia dos vasos sanguíneos, mucosa oral, polpa dental, periósteo, fáscia, músculos, cápsula e ligamentos da ATM (Sessle, 1995). Essas terminações nervosas livres encontradas nos tecidos orofaciais estão associadas a fibras nervosas de pequeno diâmetro e baixa velocidade de condução, representadas pelas fibras mielinizadas A delta e pelas fibras amielinizadas C. Estas duas classes de fibras nervosas aferentes nociceptivas conduzem a informação nervosa dos nociceptores da região orofacial ao complexo nuclear sensorial trigeminal do tronco cerebral. Esse complexo é subdividido em núcleo sensorial principal e núcleo do trato espinhal, o qual é composto pelos subnúcleos oral, interpolar e caudal, sendo o último a principal região receptora das informações nociceptivas da região orofacial (Sessle, 1996). Além do subnúcleo caudal, o subnúcleo interpolar e o oral também estão relacionados à expressão da informação nociceptiva através da ligação entre os interneurônios dos subnúcleos que são capazes de modular as respostas dolorosas entre os mesmos.

Uma vez captado o sinal doloroso, sua interpretação depende da interação entre o complexo neuroanatômico periférico e central. Neste complexo, um conjunto de substâncias neuroquímicas é essencial para a sua funcionalidade. Ao ocorrer uma lesão tecidual, imediatamente uma série de respostas é desencadeada pelo organismo por intermédio da liberação de substâncias tais como: ácido lático, bradicinina, prostaglandinas, aminas simpatomiméticas, substância P, íons potássio, íons hidrogênio, serotonina, histamina, fator de crescimento neural, peptídeo relacionado ao gene da calcitonina, citocinas, óxido nítrico e a molécula de ATP (Dray, 1995; Swift *et al*., 1998).

Todas as células apresentam no citoplasma concentrações milimolares de ATP (Burnstock, 1996), uma das moléculas responsáveis pela geração da energia celular, podendo ser liberada no meio extracelular de duas maneiras: a primeira ocorre por processo passivo, quando há uma lise repentina de células causada por traumas teciduais (Bleehen e Keele, 1977; Maehara *et al*., 1987; Ryan *et al*., 1991) e a segunda, por processo ativo, no qual o ATP é liberado em vesículas (Burnstock, 1995).

Foi proposto o envolvimento da molécula de ATP no mecanismo de desenvolvimento dos sinais dolorosos, verificando-se que administrações de ATP diretamente sobre bolhas cutâneas (Bleehen e Keele, 1977), intradérmicas (Coutts *et al*., 1981) e por iontoforese (Hamilton *et al*., 2000) em humanos produziram sensações dolorosas persistentes e dose-dependentes, sugerindo que o ATP pode ser um ativador de nociceptores e um mediador endógeno de dor.

Com o desenvolvimento das técnicas de cultura celular, uma série de estudos foi realizada com o objetivo de verificar os efeitos do ATP nos neurônios sensoriais aferentes. Estes estudos demonstraram que mais de 90% dos neurônios despolarizam na presença de ATP (Ding *et al*., 2000). A cultura de neurônios sensoriais nociceptivos (aferência da polpa dental) e não nociceptivos de ratos demonstraram que o ATP induz potenciais de ação nas fibras aferentes primárias nociceptivas quando expostas a baixas concentrações de ATP (Cook *et al*., 1997) ou ao citoplasma de células lesadas (Cook e McCleskey, 2002).

Atualmente, tem sido proposto que o ATP pode ser liberado como neurotransmissor com a noradrenalina a partir de terminações simpáticas, podendo agir em terminações nervosas sensoriais nociceptivas durante processos patológicos e contribuindo com o desenvolvimento da dor (Burnstock, 1996).

O ATP no espaço extracelular exerce suas funções através 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 (para revisão veja Abbracchio e Burnstock, 1998). Entre 1992-1996, diversos estudos demonstraram uma 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 — ligando-dependentes (ionotrópicos) — e receptores P2Y — acoplados à proteína G (metabotrópicos).

Os receptores P2X medeiam rápida e não seletiva passagem de cátions (Na^+, K^+, Ca^{2+}) através da membrana celular, resultando em um aumento intracelular de Ca^{2+} e em uma despolarização da membrana (Bean *et al*., 1992; DuByak e El- Moatassim, 1993). O fluxo extracelular direto de Ca^{2+} através dos canais constitui uma significante fonte para o aumento intracelular de Ca^{2+}. No entanto, a despolarização da membrana leva a secundária ativação de canais de Ca^{2+} voltagem-dependentes, contribuindo ainda mais para o aumento intracelular de Ca^{2+}. Como este mecanismo de transdução não depende da produção e difusão de segundos mensageiros no citoplasma ou na membrana celular, o tempo de resposta é muito rápido e, conseqüentemente, possui um importante papel na rápida sinalização neuronal (Ralevic e Burnstock, 1998).

Atualmente, sete subtipos de receptores P2X de mamíferos (P2X1-P2X7) foram identificados por clonagem molecular (Buell *et al*., 1996). Eles foram encontrados em diversos tecidos como sistema nervoso central, sistema nervoso periférico, células sanguíneas, células de músculos lisos e nervos sensoriais (Ralevic e Burnstock, 1998). É importante ressaltar que os receptores de ATP/ADP estão presentes na maioria dos tipos celulares que devem ser rapidamente ativados e/ou recrutados para minimizar perda de sangue ou invasão de patógenos nos locais de lesão tecidual. Estes incluem as plaquetas, neutrófilos, monócitos, macrófagos, células vasculares endoteliais e células vasculares da musculatura lisa (DuByak e El- Moatassim, 1993).

Recentemente, estudos têm demonstrado o envolvimento desses receptores na transmissão da informação nociceptiva através das fibras aferentes sensoriais primárias. Essa idéia vem se consolidando cientificamente após evidências de que o RNAm dos receptores P2X é expresso nos gânglios trigeminais e nos gânglios das raízes dorsais (Chen *et al.,* 1995; Lewis *et al*., 1995; Collo *et al*., 1996; Llewellyn-Smith e Burnstock, 1998; Barden e Bennet, 2000) e, em particular, pelas evidências de que os receptores P2X funcionais são expressos em algumas fibras aferentes de ratos (Bland-Ward e Humphrey, 1997; Dowd *et al*., 1998; Hamilton *et al*., 1999; Tsuda *et al.,* 2000), incluindo a polpa dental (Cook *et al*., 1997). Tais estudos verificaram que a administração de ATP ou de seu análogo α,β-metilenoATP (α,β-meATP) na pata de ratos produz respostas comportamentais nociceptivas que são abolidas por anestesia local, pela administração local de antagonistas do receptor P2X e por dessensibilização ou eliminação das fibras aferentes C, indicando que essas respostas nociceptivas são mediadas por estimulação aferente primárias das fibras C ou Aδ. Hilliges *et al*. (2002) verificou que a administração intracutânea de ATP em humanos ativa fibras aferentes nociceptivas tipo C.

No entanto, não se sabe se os receptores P2X estão presentes nos terminais periféricos das fibras aferentes da ATM. Considerando a alta incidência das condições dolorosas da ATM e a alta freqüência de tratamentos farmacológicos insatisfatórios, estudos mais específicos dos mecanismos periféricos — particularmente dos receptores — envolvidos no processo de desenvolvimento e manutenção da dor na ATM são necessários.

Embora o mecanismo de desenvolvimento das condições dolorosas da ATM não seja completamente conhecido, tem sido demonstrado que mediadores inflamatórios como a prostaglandina E_2, a serotonina e as citocinas pró-inflamatórias (TNF-α, IL-1β) estão presentes em altas concentrações no líquido sinovial de pacientes com disfunções temporomandibulares (para revisão veja Kopp, 2001). A sensibilização e estimulação dos nociceptores aferentes primários são denominadores comuns em todos os tipos de hiperalgesia inflamatória. Nessas circunstâncias, um estímulo, que em tecidos normais

provoca pequeno ou nenhum efeito, agora ativa nociceptores para induzir respostas comportamentais exacerbadas em animais e intensa dor em humanos (Ferreira, 1972,1981; Hedenberg-Magnusson *et al*., 2002).

O aumento das respostas comportamentais nociceptivas mediadas por receptores P2X tem sido demonstrado também na presença de inflamação ou de mediadores inflamatórios, sugerindo que, sob situações de lesão tecidual, o ATP deixa o espaço intracelular para contribuir com o desenvolvimento da hiperalgesia inflamatória. Esta hipótese é sustentada pela demonstração de que a administração subplantar de α,β-me ATP em ratos, um agonista seletivo para receptores purinérgicos P2X, aumenta as respostas comportamentais nociceptivas quando co-administrado com algógenos, mediadores de hiperalgesia e agentes inflamatórios como a 5-hidroxitriptamina (Bland-Ward e Humphrey, 2000), PGE_2 (Hamilton *et al*., 1999) e formalina (Sawynok e Reid, 1997), respectivamente.

Assim, o objetivo deste trabalho era investigar se a ativação de receptores P2X na região da ATM induz nocicepção e avaliar o envolvimento do ATP endógeno no desenvolvimento da hiperalgesia na ATM através da avaliação do efeito do antagonista de receptor P2 pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) na hiperalgesia induzida pela carragenina na ATM.

2. CAPÍTULO

O presente artigo foi submetido ao periódico “European Journal of Pain” e se encontra sob avaliação (Anexo 7).

Evidence for the involvement of endogenous ATP and P2X receptors in TMJ pain.

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Abstract

Evidence is accumulating which supports a role for ATP in the initiation of pain by acting on P2X receptors expressed on nociceptive afferent nerve terminals. To investigate whether these receptors play a role in temporomandibular (TMJ) pain, we studied the presence of functional P2X receptors in rat TMJ by examining the nociceptive behavioral response to the application of the selective P2X receptor agonist α,β-methylene ATP (α,β-meATP) into the TMJ region of rat. The involvement of endogenous ATP in the development of TMJ inflammatory hyperalgesia was also determined by evaluating the effect of the general P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on carrageenan-induced TMJ inflammatory hyperalgesia. Administration of α,β-meATP into the TMJ region of rats produced significant nociceptive responses that were significantly reduced by the co-application of lidocaine N-ethyl bromide quaternary salt, QX-314, (2%) or of the P2 receptor antagonist PPADS. Co-administration of PPADS with carrageenan into the TMJ significantly reduced inflammatory hyperalgesia. The results indicate that functional P2X receptors are present in the TMJ and suggest that endogenous ATP may play a role in TMJ inflammatory pain mechanisms possibly by acting primarily in these receptors.

Key words: P2X receptors, TMJ, inflammation, pain, α,β-meATP

Introduction

P2X receptors are a family of ligand-gated ion channels activated by extracellular ATP that have been reported to play a role in nociception. Evidence for that has been accumulating by the demonstration that mRNA for six of the seven subtypes of P2X receptors are expressed in trigeminal and dorsal root ganglia (Chen *et al*., 1995; Lewis *et al.*, 1995; Collo *et al*., 1996; Llewellyn-Smith and Burnstock 1998, Barden and Bennett, 2000) and, in particular, by the findings that functional P2X receptors are expressed on some sensory afferent nerves in the rat (Bland-Ward and Humphrey, 1997; Dowd *et al*., 1998; Hamilton *et al*., 1999; Tsuda *et al.*, 2000) including the tooth pulp (Cook *et al*., 1997). However, it is not known whether functional P2X receptors are present on the peripheral terminals of primary afferent neurons in the rat temporomandibular joint (TMJ). Considering the high incidence of TMJ pain conditions and also the high rate of unsuccessful pharmacological approaches, a better understanding of the receptors involved in the initiation and maintenance of TMJ pain is necessary. Although the mechanism underlying the TMJ pain conditions is not completely known, it has been shown that inflammatory mediators like prostaglandin E_2, serotonin, pro-inflammatory cytokines (TNF-α, IL-1β) are present at high levels in the synovial fluid of patient with temporomandibular disorders (for review see Kopp, 2001). Sensitization and stimulation of the primary afferent nociceptor are common denominators of all types of inflammatory hyperalgesia. In this circumstance, stimuli, which in a normal tissue have little or no effect, now activate the nociceptors to induce overt behavioral responses in experimental animals and overt pain in humans (Ferreira, 1972,1981; Hedenberg-Magnusson *et al*., 2002).

Enhancement of the P2X receptor mediated nociceptive response has been also reported in the presence of inflammation or inflammatory mediators suggesting that, under situations of tissue injury, ATP may leave the intracellular space to contribute to the development of inflammatory hyperalgesia. This is supported by the demonstration that subplantar injection of α,β-methylene ATP (α,β-meATP) enhances nociceptive behaviors in conscious rats when co-administered with algogens, hyperalgesic mediators and inflammatory agents such as 5-hydroxytryptamine (Bland-Ward and Humphrey, 2000), PGE_2 (Hamilton *et al*., 1999) and formalin (Sawynok and Reid, 1997), respectively.

The aim of this study was to investigate whether activation of P2X receptors located within the TMJ region induces nociception. We also evaluated the involvement of endogenous ATP in the development of TMJ hyperalgesia by evaluating the effect of the P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on carrageenan-induced TMJ hyperalgesia.

Material and methods

Animals:

This study was carried out on male Wistar rats (150 - 250g). The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (± 23ºC) and handled for at least one week prior to the experiments. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas and conformed to IASP guidelines for the study of pain in animals (Zimmermann, 1983).

General Procedures:

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland *et al*., 1991). Each animal was manipulated for 7 days to be habituated to the experimental manipulation. After this period, the animal was placed in a test chamber (30x30x30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress (Abbot *et al*., 1986). The animal was removed from the test chamber and lightly anaesthetized by inhalation of halothane to allow the TMJ injection. Each animal was used once.

TMJ Injections:

TMJ injections were performed via a 30-gauge needle introduced into the left TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50µl).

Measurement of nociceptive responses:

Animals immediately recovered from the anesthesia after TMJ injection. When more than one TMJ injection was given, the rat was returned to the test chamber following the last TMJ injection for an observation period of 30 or 45 min, depending on the experimental group. The recording time was divided into 15 blocks of 3 minutes and a pain score was determined for each block by measuring the number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hindpaw and/or flinching the head in an intermittent and reflexive way characterized by high frequency shakes of the head as previously described (Roveroni *et al*., 2001; Gameiro *et al*., 2003). Rats did not have access to food or water during the test.

Carrageenan-induced chemical hyperalgesia:

Administration of carrageenan has been widely used as model of inflammatory hyperalgesia in cutaneous (Vinegar *et al*., 1987; Cunha *et al*., 1991) and articular (knee joint) tissue (Tonussi and Ferreira, 1992, 1999). In the present study, a low dose of 5-hydroxytryptamine (5-HT) that induces minimal nociceptive behavior was used to detect carrageenan-induced sensitization in TMJ region (Fig. 3).

Drugs:

α,β-methyleneATP lithium salt (α,β-meATP: 500 and 1000 μg, Hamilton *et al*., 1999); 5-hydroxytryptamine (75, 225 and 450 μg); pyridoxal-phosphate-6-azophenyl-2'4'-disulphate (PPADS: 300 μg, Sawynok and Reid, 1997); lidocaine N-ethyl bromide quaternary salt (2% QX-314, Roveroni *et al*., 2001) were obtained from Sigma (MO, USA) and

carrageenan (100 μg, Zanin and Ferreira, 1978) from Sigma (SP, Brazil). All drugs were dissolved in 0.9% NaCl. Volume per injection was 25μl.

Statistical analysis:

Data with homogeneity of variance were analyzed using the T test or One-Way Analysis of Variance (ANOVA) and multiple post-hoc comparisons were performed using Tukey test. A probability level of less than 0.05 was considered to indicate statistical significance. Data are presented in figures and text as means ± S.E.M.

Results

P2X receptors in the TMJ

Presence of functional P2X receptors in the rat TMJ was evaluated by the ability of the selective P2X receptor agonist α,β-meATP to induce behavioral nociceptive responses when administered into the TMJ region of rats. Figure 1 shows that the TMJ injection of α,β-meATP (500 or 1000 μg) induced a significantly greater behavioral response than that induced by its vehicle ($p<0.05$, Tukey test). This response was blocked by the co-administration of 2% QX 314 ($p<0.05$, Tukey test), confirming its nociceptive character. Co-administration of the P2 receptor antagonist PPADS (300 μg) completely blocked α,β-meATP-induced nociception ($p<0.05$, Tukey test) but had no effect by itself ($p<0.05$, Tukey test), suggesting that the nociceptive response induced by intra articular α,β-meATP is a specific response mediated through the PPADS-sensitive P2X receptors located at the TMJ region (Figure 2). Because the effect of α,β-meATP was more evident during the first 30 min post injection, the period of observations was set to 30 min in further experiments.

ATP mediation of carrageenan-induced TMJ chemical hyperalgesia

Carrageenan-induced TMJ hyperalgesia was assessed by the behavioral nociceptive response induced by the application of a low dose of 5-hydroxytryptamine (5-HT; 75 μg) into the rat TMJ previously challenged with carrageenan. This dose of 5-HT was determined by evaluating the behavioral response induced by the application of increasing doses of 5-HT into the rat TMJ (Figure 3A). The behavioral response induced by application of 5-HT 1 hour post the TMJ injection of carrageenan was significantly greater than that induced by its application 3 or 6 hour later ($p<0.05$, Tukey test; Figure 3B);

therefore, a 1 hour interval between injections was used in further experiments. The behavioral response induced by application of 5-HT into the TMJ previously challenged with carrageenan was significantly greater than that induced by the TMJ injection of each of these drugs alone ($p<0.05$, Tukey test; Figure 3C). Co-administration of 2% QX314 with carrageenan significantly reduced ($p<0.05$, t test) the behavioral response induced by a subsequent injection of 5-HT, confirming the nociceptive character of the behavioral response.

Carrageenan-induced chemical TMJ hyperalgesia was significantly reduced ($p<0.05$, Tukey test) by the co-application of the P2 receptor antagonist PPADS with carrageenan. When applied alone, PPADS did not affect the behavioral response induced by a subsequent TMJ injection of 5-HT (Figure 4B). Taken together, these findings suggest that endogenous ATP plays a role in the development of TMJ inflammatory pain.

Discussion

Nociceptive behavioral responses were induced by application of the selective P2X receptor agonist α,β-meATP into the TMJ region of rats as indicated by the blockade of these responses by the co-application of the local anesthetic QX 314. Given that QX 314, lidocaine **N**-ethyl bromide quaternary salt, an analog of lidocaine, is unable to cross the blood brain barrier and that α,β-meATP-induced TMJ nociception was also blocked by the co-administration of the P2 receptor antagonist PPADS, we suggest that functional P2X receptors are present into the TMJ region of rats. The current study, as well as the study of Dowd *et al*. (1998), in which PPADS antagonized α,β-meATP and ATP-induced excitation of nociceptive afferent fibers in the knee joints of rats, indicate that activation of P2X receptors can induce nociception in the articular tissue.

Although the precise mechanism by which α,β-meATP induces TMJ nociception remains to be elucidated, α,β-meATP may directly activates functional P2X receptors located in the TMJ nociceptive primary afferents. Since P2X subunits are present in the cell body of trigeminal nociceptive neurons (Collo *et al*., 1996, Llewellyn-Smith and Burnstock, 1998), they may also occur in their peripheral terminals as supported by the immunohistochemical localization of P2X receptors on the tooth pulp afferents (Cook *et al*., 1997). Since P2X receptors mediate Na^+ and Ca^{2+} influx (for review see Dubyak and El-Moatassim, 1993), activation of P2X receptor in the primary afferent nociceptors induce a short-lasting depolarization and consequently a short-lasting nociceptive behavior. However, our finding that the nociceptive response persisted for approximately 30 min following the TMJ administration of α,β-meATP suggests that α,β-meATP may also indirectly activates the

nociceptor possibly via the release of other mediators from resident cells such as mast cells (Cockcroft and Gomperts, 1979; Jaffar *et al.*, 1990; Osipchuk and Cahalan, 1992; Lee *et al*., 2001) and platelets (Vial *et al*., 1997; Oury *et al*., 2002; Kunapuli *et al*., 2003).

Recently, we have shown that administration of the classical local algesic agent formalin into the rat TMJ region induces a stereotyped and quantifiable nociceptive behavior, mediated by local release of serotonin (Roveroni *et al*., 2001; Parada *et al*., 2001; Doak and Sawynok, 1997). Similar to formalin, administration of 5-HT in the rat TMJ induced a dose dependent nociceptive behavior. In order to assess carrageenan-induced sensitization of the TMJ nociceptors, a low dose of 5-HT (75 μg) that produced minimal nociceptive behavior in normal TMJ was applied to the ipsilateral TMJ 1 hour post carrageenan injection at the same local (Fig 3). Under these conditions 5-HT induced behavioral nociceptive response was significantly greater than that induced by each of these drugs alone, and was used as a quantitative measurement of carrageenan-induced TMJ hyperalgesia.

Co-application of the general P2 receptor antagonist PPADS with carrageenan significantly reduced the carrageenan-induced TMJ hyperalgesia. These findings and the findings of others (Cook and McCleskey, 2002; Dell'Antonio *et al*., 2002) suggest that under situations of tissue injury, ATP may leave the intracellular space to contribute to the development of inflammatory hyperalgesia. Although PPADS is a general P2 receptor antagonist (Ralevic and Burnstock, 1998), our findings suggest that PPADS-sensitive P2X receptors located in the TMJ may be the most likely target for extracellular ATP released from the cytoplasm of damaged cells.

In general, inflammatory hyperalgesia is mediated by the release of arachidonic acid products, such as prostaglandins (Ferreira *et al*., 1988; Cunha *et al*., 1992; Khasar *et al*., 1999) and by sympathomimetic amines such as norephinefrine released at the site of injury (Nakamura and Ferreira, 1987). Both anti-inflammatory drugs (Ferreira, 1972; Bianchi and Broggini, 2002; Rioja *et al*., 2002) and adrenergic antagonists (Nakamura and Ferreira, 1987; Raja, 1995; Khasar *et al*., 1999) are known to reduce inflammatory pain. However, the relative contribution of each inflammatory mediator to inflammatory pain depends on the characteristics of the pathological stimulus and on the tissue involved. In the cutaneous (Vinegar *et al.*, 1987; Cunha *et al*., 1991) and in the articular tissue such as knee joint (Tonussi and Ferreira, 1992, 1999) and temporomandibular joint (*unpublished observations*) carrageenan-induced hyperalgesia is a model of inflammatory pain in which prostaglandins and sympathomimetic amines are the major mediators. In the current study we are showing that endogenous ATP plays an important role in the development of inflammatory hyperalgesia in the TMJ, as indicated by the significant attenuation of carrageenan-induced TMJ hyperalgesia by PPADS. Because ATP released from cytosol of damaged cells provides a rapid nociceptive signal from injured tissue (Cook and McCleskey, 2002), it is possible that the release of inflammatory mediators such as prostaglandins and sympathomimetic amines in the TMJ depends on previous release of endogenous ATP. Consistent with this idea it has been previously reported that ATP induces prostaglandin synthesis (Needleman *et al*., 1974) and stimulates sympathetic transmitter release via P2X receptors (Boehm, 1999). In addition to the indirect effect of ATP on the development of inflammatory hyperalgesia, ATP may directly sensitize primary afferent nociceptors by activating P2X receptors which in turn results in Ca^{2+}

influx. It has been demonstrated that PGE_2-induced hyperalgesia is a Ca^{2+} dependent phenomenon, and that local administration of calcium ionophore produces hyperalgesia (Ferreira and Nakamura, 1979; Parada *et al*., 2003).

In summary, we provided evidence that activation of P2X receptors in the rat TMJ induces nociception and that blockage of PPADS-sensitive P2X receptors decreases carrageenan-induced inflammatory hyperalgesia in rat TMJ. Taken together, these findings point out P2X receptors as potential targets for the development of new analgesic drugs to control TMJ pain.

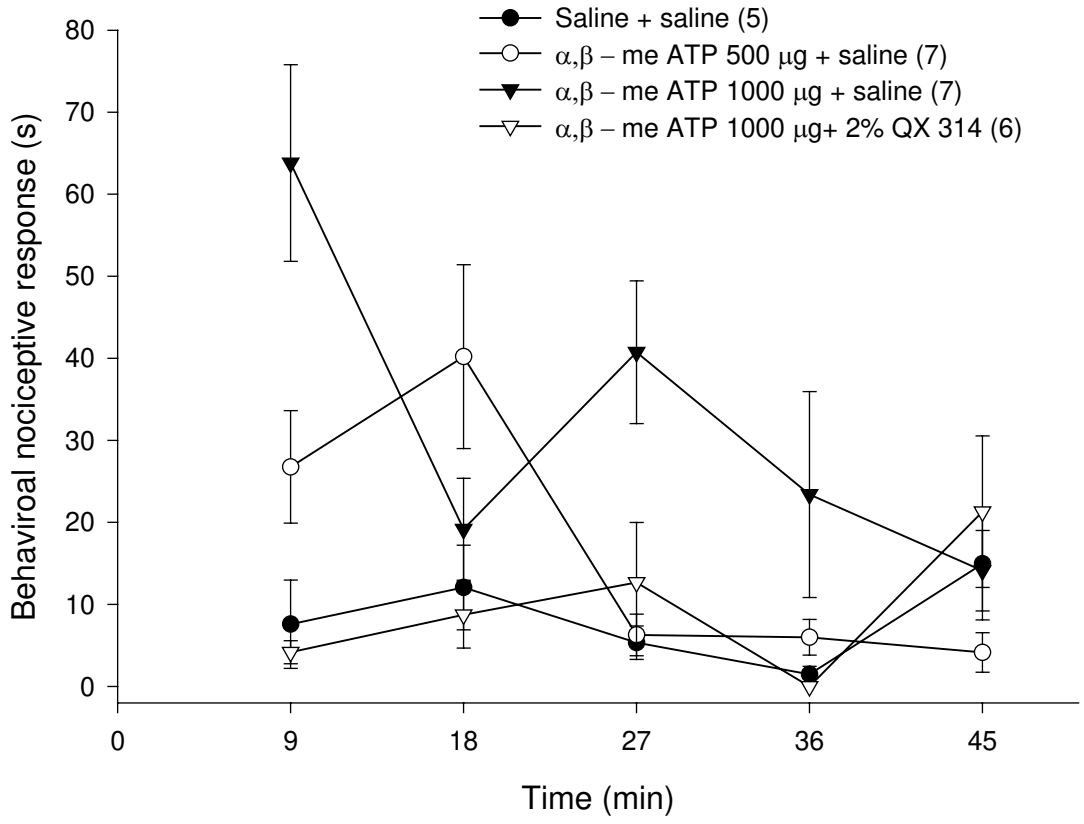

Figure 1, Oliveira et al

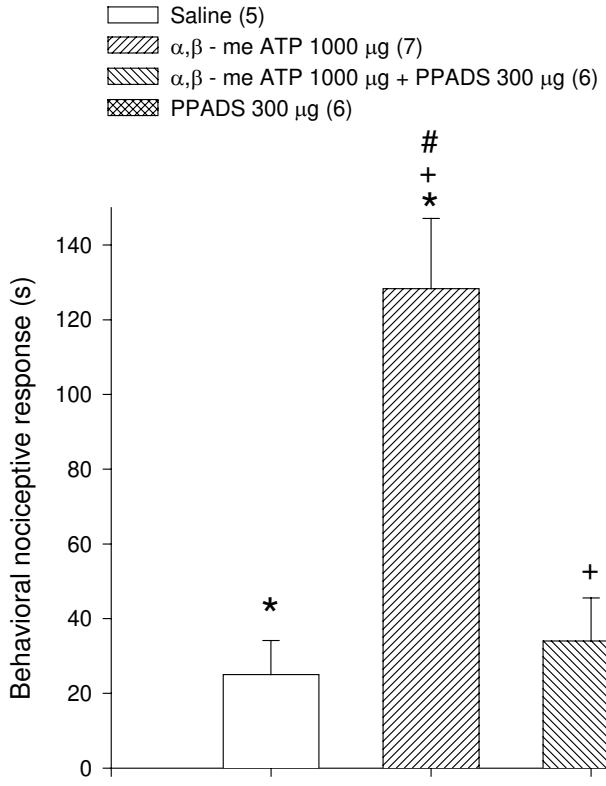

Figure 2, Oliveira et al

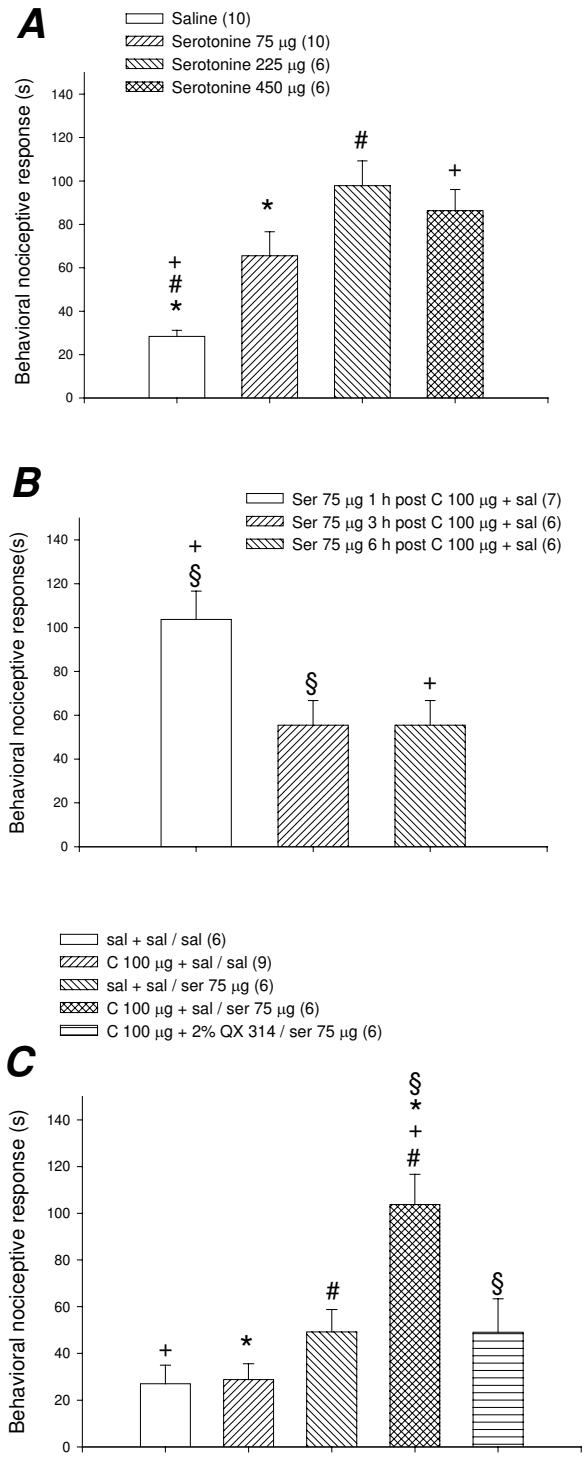

Figure 3, Oliveira et al

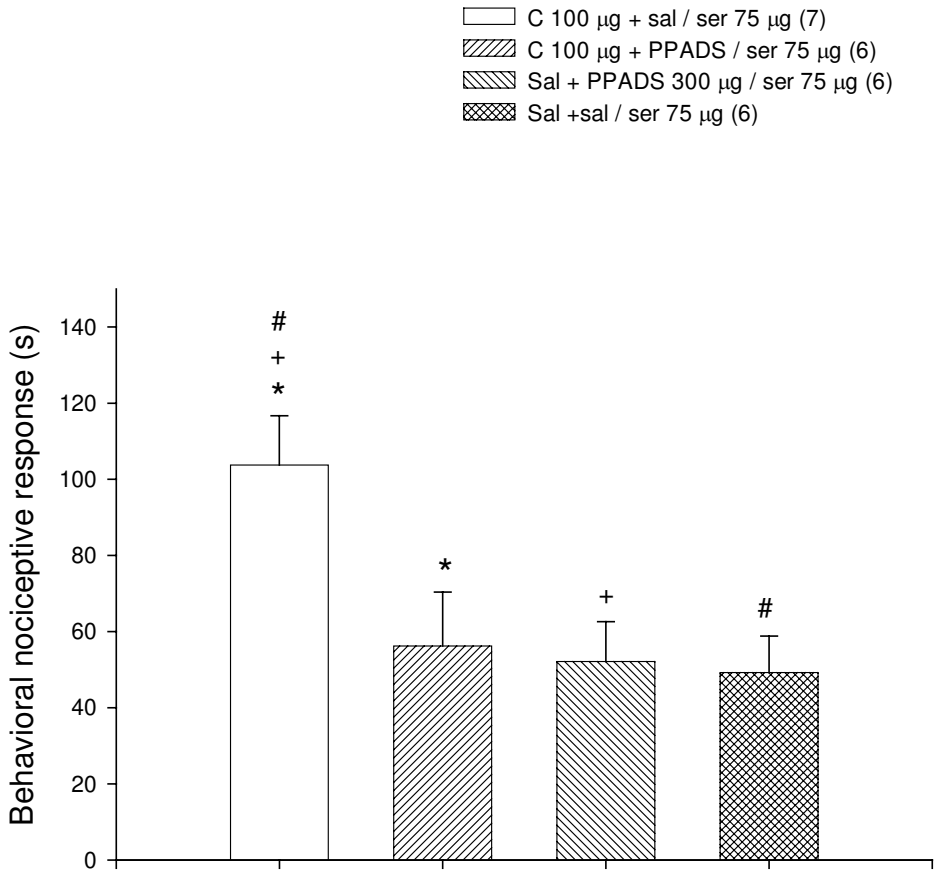

Figure 4, Oliveira et al

Figures:

Figure 1 –

Time course of behavioral response induced by application of a selective P2X receptor agonist into the rat TMJ. The selective P2X receptor agonist, α,β-meATP induced a dose related behavioral response. This response was significantly greater than that induced by its vehicle ($p< 0.05$, Tukey test) and was blocked by the co-administration of 2% QX 314. The sum of the behavioral responses measured for 45 min was used for statistical analyze. In this and subsequent figures, data are presented as mean ± s.e.m. and group sample sizes are shown in parentheses.

Figure 2 –

The effect of co-application of a P2 receptor antagonist in the rat TMJ on α,β-meATP-induced nociception. Co-application of the P2 receptor antagonist PPADS significantly reduced α,β-meATP-induced nociception ($p<0.05$, Tukey test) but had no effect by itself. In this and in subsequent figures significant differences between groups ($p<0.05$) are indicated by matching pair(s) of symbols above the bars; each bar represents the sum of the behavioral responses measured for 30 min (see methods for more details).

Figure 3 –

Caracterization of carrageenan-induced TMJ hyperalgesia

A- Dose related increase of behavioral response induced by 5-HT application in the rat TMJ

B- Time course of behavioral response induced by application of 5-HT in the rat TMJ pretreated with carrageenan. Note that the behavioral response induced by application of 5-HT 1 hour post the TMJ injection of carrageenan was significantly greater ($p<0.05$, Tukey test) than that induced by its application 3 or 6 hour later.

C- The behavioral response induced by application of 5-HT into the TMJ previously challenged with carrageenan was significantly greater than that induced by the TMJ injection of each of these drugs alone. Co-administration of 2% QX314 with carrageenan blocked the behavioral response induced by a subsequent injection of 5-HT. In this and in the subsequent figure, 5-HT was applied in the TMJ 1 hour post carrageenan application. Abbreviations: C, carrageenan; sal, saline; ser, serotonine.

Figure 4 –

The effect of a P2 receptor antagonist on carrageenan-induced TMJ hyperalgesia.

Co-application of the P2 receptor antagonist PPADS blocked α,β-meATP-induced TMJ hyperalgesia as indicated by the reversal of the nociceptive response induced by the application of 5-HT in the TMJ pretreated with carrageenan. PPADS had no effect on the behavioral response induced by 5-HT. Abbreviations: C, carrageenan; sal, saline; ser, serotonine.

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CONCLUSÃO

Concluímos que a ativação de receptores P2X na ATM de ratos induz nocicepção e que o bloqueio de receptores P2X-sensíveis ao PPADS diminui a hiperalgesia inflamatória induzida pela carragenina na ATM de ratos. Esses dados indicam que os receptores P2X são possíveis alvos para o desenvolvimento de novas drogas analgésicas para o controle da dor na ATM.

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ANEXO 1

TABELA 1. Avaliação da presença de receptores P2X *funcionais* na ATM de ratos. A avaliação comportamental foi realizada pelo período de 45 min.

GRUPOS	Resposta Comportamental (s)
Salina (n=5)	41,43 ±15,7 *
α,β-meATP 500μg + Salina (n=7)	134,2 ± 28,8 *
α,β-meATP 1000μg + Salina (n=7)	161,24 ± 22,5 * #
α,β-meATP 1000μg + QX314 (2%) (n=6)	46,9 ± 14,9 #

Nesta tabela, assim como nas subseqüentes, símbolos iguais indicam diferença significativa ($p \leq 0,05$; teste Tukey) entre grupos. Dados expressos como média ± E.P.M.

ANEXO 2

TABELA 2. Efeito da co-administração do antagonista do receptor P2X PPADS (300μg) com o α,β-meATP (1000μg) na região da ATM de ratos. Nesta tabela, assim como nas subseqüentes, a avaliação comportamental foi realizada pelo período de 30 min.

Grupos	Resposta Comportamental (s)
Salina (n=5)	25,00 ± 9,13 *
α,β-meATP 1000μg + Salina (n=7)	128,31 ± 18,78 * + #
α,β-meATP 1000μg + PPADS 300μg (n=6)	34,02 ± 11,52 +
PPADS 300μg + Salina (n=6)	31,84 ± 6,21 #

ANEXO 3

TABELA 3a. Efeito da administração de diferentes concentrações de serotonina na região da ATM de ratos.

Grupos	Resposta Comportamental (s)
Salina (n=10)	28,37 ± 2,76* $^{\#+}$
Serotonina 75 μg (n=10)	65,55 ± 11,03*
Serotonina 225 μg (n=6)	97,79 ± 11,47 $^{\#}$
Serotonina 450 μg (n=6)	86,33 ± 9,72 $^{+}$

TABELA 3b. Evolução temporal da hiperalgesia induzida pela carragenina.

Grupos	Resposta Comportamental (s)
C 100μg + sal/ 1h após ser 75μg (n=7)	103,75 ± 12,92 $^{\S+}$
C 100μg + sal/ 3h após ser 75μg (n=6)	55,49 ± 11,22 §
C 100μg + sal/ 6h após ser 75μg (n=6)	58,15 ± 9,58 $^{+}$

C = carragenina, sal = salina, ser = serotonina.

TABELA 3c. Avaliação do papel do ATP endógeno na hiperalgesia induzida pela administração de carragenina na ATM de ratos.

Grupos	Resposta Comportamental (s)
sal + sal/ 1h após sal (n=6)	26,96 ± 7,99 $^{+}$
C100μg + sal/ 1h após sal (n=9)	28,83 ± 6,76 *
sal + sal/ 1h após ser 75μg (n=6)	49,23 ± 9,58 $^{\#}$
C100μg + sal/ 1h após ser 75μg (n=7)	103,75 ± 12,92 $^{+}$ * $^{\# \S}$
C100μg + QX314 (2%)/ 1h após ser 75μg (n=6)	49,09 ± 14,23 §

ANEXO 4

TABELA 4. Efeito da co-administração do antagonista do receptor P2 na hiperalgesia induzida pela carragenina na ATM.

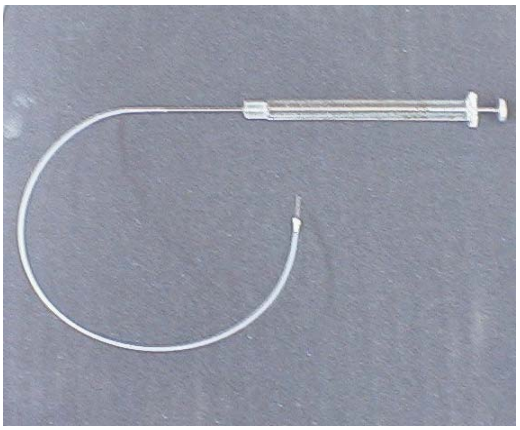
Grupos	Resposta Comportamental (s)
C100µg + sal/ 1h após ser 75µg (n=7)	103,75 ± 12,92 $^{\#\ +}$ *
C100µg + PPADS 300µg/ 1h após ser 75µg (n=6)	56,22 ± 14,16 *
sal + PPADS 300µg/ 1h após ser 75µg (n=6)	52,14 ± 10,47 $^{+}$
sal + sal/ 1h após ser 75µg (n=6)	49,23 ± 9,58 $^{\#}$

ANEXO 5

Câmara de Observação (30 x 30 x 30 cm)

Cronômetro

Contador



Agulha calibre 30 conectada à seringa microlitro Hamilton pela cânula de polietileno P 50

Inserção da agulha na ATM esquerda

ANEXO 6

ANEXO 7

----- Original Message -----
From: EJP
To: Claudia Tambeli
Sent: Friday, January 09, 2004 7:24 AM
Subject: Re: Ms 4/2004

Dear Dr. Tambeli,

Many thanks for your manuscript "Evidence for the involvement of endogenous ATP and P2X receptors in TMJ pain", which has been assigned manuscript number 4/2004.-

Would you be kind enough to fax a covering letter to **+ 34 91 885 48 07**, which should be signed by *all* authors, and include an assertion that the work the article represents has not been submitted for publication elsewhere.

The referees for the manuscript will now be selected and we will let you know their comments as soon as possible. Many thanks for your interest in the European Journal of Pain.

Yours sincerely,
Professor Fernando Cervero
Editor-in-Chief