UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

Luciane Lacerda Franco Rocha Rodrigues
Cirurgiã Dentista

"COMPONENTE SIMPÁTICO PERIFÉRICO DA DOR INFLAMATÓRIA DA ATM "

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

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2006

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecário: Marilene Girello – CRB-8ª. / 6159

R618c

Rodrigues, Luciane Lacerda Franco Rocha.
Componente simpático periférico da dor inflamatória da ATM. / Luciane Lacerda Franco Rocha Rodrigues. -- Piracicaba, SP : [s.n.], 2006.

Orientadores: Cláudia Herrera Tambeli, Maria Cecília Ferraz de Arruda Veiga
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Aminas. 2. Simpatomiméticos. 3. Articulação temporomandibular. 4. Hiperalgesia. 5. Carragenina. 6. Receptores adrenérgicos. I. Tambeli, Cláudia Herrera. II. Veiga, Maria Cecília Ferraz de Arruda. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

(mg/fop)

Título em inglês: Peripheral sympathetic component of the TMJ inflammatory pain
Palavras-chave em inglês (Keywords): 1. Amines. 2. Sympathomimetics. 3. Temporomandibular joint. 4. Hyperalgesia. 5. Carrageenan. 6. Receptors, adrenergic

Área de concentração: Fisiologia Oral

Titulação: Doutor em Odontologia
Banca examinadora: Eduardo Dias de Andrade, Élio Itoshi Shinohara, Renata Cunha Matheus Rodrigues Garcia, Valdir Quintana Gomes Júnior, Cláudia Herrera Tambeli
Data da defesa: 21/02/2006

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 21 de Fevereiro de 2006, considerou a candidata LUCIANE LACERDA FRANCO ROCHA RODRIGUES aprovada.

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DEDICATÓRIA

Dedico este trabalho ao meu pai, Raul, que hoje já não se encontra entre nós, mas durante a sua jornada na Terra foi um grande exemplo de determinação e sabedoria a ser seguido. À minha mãe, Vera, que tanto nos mostrou a importância de continuarmos estudando. Ao meu marido, Hamilton, pela paciência que teve em dividir os nossos momentos de lazer com este trabalho e, ao Guilherme, nosso filho tão esperado e amado, meu muito obrigada pelo apoio recebido para conclusão desta jornada.

AGRADECIMENTOS

À Profa. Dra. Cláudia Herrera Tambeli, minha amiga de tantos anos, meu muito obrigada por ter me orientado e, principalmente pelo apoio e pelo incentivo através do seu exemplo de determinação dentro de seus objetivos, que muito contribuiu para a conclusão deste trabalho.

À Profa. Dra. Maria Cecília Ferraz de Arruda Veiga, obrigada por me acolher nos momentos mais difíceis e pelo constante bom humor.

À Dra. Adriana Pelegrini da Silva, por ter me acolhido tantas vezes em sua residência durante a sua gravidez e pelas sugestões realizadas no artigo publicado.

À doutoranda e amiga, Maria Cláudia Gonçalves de Oliveira Fusaro, pela boa vontade e companheirismo na execução deste trabalho.

Ao Prof. Dr. Carlos Amílcar Parada, o meu muito obrigada pelas valiosas sugestões e colaboração neste trabalho.

À Profa. Dra. Fernanda Klein Marcondes, pelo exemplo de organização e competência.

Às Professoras Doutoras Cecília Gatti Guirado, Célia M. Rizatti Barbosa e Maria Beatriz D. Gavião, por terem aceitado fazer parte da minha banca examinadora na qualificação e pela colaboração nas sugestões apresentadas.

Aos Professores Doutores Eduardo Dias de Andrade, Élio Itoshi Shinohara, Norair Salviano dos Reis e Valdir Quintana Gomes Júnior, pessoas pelas quais tenho profunda admiração, meu muito obrigada por me concederem à honra de fazer parte da minha banca examinadora e pelas valiosas sugestões apresentadas na minha defesa de tese/.

Ao Prof. Dr. Norair Salviano dos Reis, meu muito obrigada por ter se mostrado tão disposto a participar da minha banca, mas que por motivo de saúde não pôde estar presente na minha defesa de tese.

Aos Professores Doutores Franco Arsati, Renata Cunha Matheus Rodrigues Garcia e Ynara Bosco de Oliveira Lima Arsati por terem aceitado fazer parte da minha banca examinadora como suplentes.

À Profa. Renata Cunha Matheus Rodrigues Garcia, por ter substituído o Prof. Dr. Norair Salviano dos Reis e ter se mostrado bastante interessada no assunto e pela colaboração nas sugestões apresentadas.

Ao técnico de laboratório Carlos Alberto Feliciano, pelo auxílio técnico e pela boa vontade sempre presente em nos atender.

À Sra. Maria Elisa dos Santos, secretária do Programa de Pós Graduação em Odontologia e à Sra. Eliete Riguetto, secretária do Depto. de Ciências Fisiológicas, meus sinceros agradecimentos pela dedicação e boa vontade sempre presentes.

À Sra. Érica Alessandra Pinho Sinhoreti pela especial atenção a mim concedida em todas as vezes que fora solicitada.

Aos colegas da Pós Graduação, em especial a Tatiana Cunha, Ana Paula Tanno, Carol Calil, Juliana Clemente, Gustavo Gameiro e Luciano José Pereira pelos bons momentos que passamos juntos.

À minha irmã Indiana e ao meu cunhado Paulo Henrique, por estarem sempre prontos a me ajudar nas traduções e versões do artigo.

À minha irmã Christiane, ao meu cunhado Jorge e filhas, pelo incentivo na conclusão deste trabalho.

Aos meus irmãos, Rudy e Dado, que mesmo à distância sempre torceram pelo meu sucesso profissional.

À minha mãe, Vera, que me ajudou tanto, tomando conta do Guilherme para que eu pudesse concluir mais essa etapa.

Agradeço ao Depto. de Ciências Fisiológicas pela excelente condição de trabalho.

Agradeço ainda a Deus, pela oportunidade de viver!!!

Agradeço a todos, que direta ou indiretamente participaram para que esse trabalho pudesse ser concluído.

SUMÁRIO

LISTA DE ABREVIATURAS

ATM - articulação temporomandibular

DTMs – disfunções temporomandibulares

AINES – antiinflamatórios não esteroidais

COX – enzima ciclooxigenase

TNF α - fator de necrose tumoral alfa

IL – interleucina

IL-1 – interleucina 1

IL-6 – interleucina 6

IL-8 – interleucina 8

IL-1β – interleucina 1 beta

PGE_2 **–** prostaglandina E_2

C – carragenina

5-HT - 5-hidroxytriptamina ou serotonina

INDO – indometacina

G – guanetidina

s.c. – subcutânea

i.p. – intraperitoneal

P – propranolol

A - atenolol

ICI – ICI 118,551

RESUMO

Considerando que a ATM recebe uma rica inervação simpática, o objetivo deste estudo foi investigar o papel das aminas simpatomiméticas na hiperalgesia da ATM induzida pela carragenina, além de validar a natureza inflamatória do modelo de hiperalgesia quimicamente induzida pela carragenina na ATM de ratos. Uma pequena dose de 5-hydroxytriptamina (5-HT; 75μg) que induz resposta comportamental nociceptiva mínima, foi aplicada na região da ATM de ratos 1h após a injeção de carragenina (C; 100μg) para detectar a sensibilização induzida pela carragenina na região da ATM, que foi avaliada pela soma das respostas nociceptivas comportamentais, como coçar a região orofacial e levantar a cabeça. O bloqueio da síntese de prostaglandinas pela indometacina sistêmica (2,5mg/kg) ou local (10μg) antes do início da inflamação pela carragenina diminuiu significativamente a hiperalgesia da ATM. A depleção das aminas simpatomiméticas pela guanetidina (30mg/kg por três dias consecutivos antes da injeção de carragenina na ATM) ou a co-aplicação de antagonistas dos adrenoceptores β (propranolol nas doses de 0,25 e 2,25μg), assim como os antagonistas dos adrenoceptores β_2 (ICI 118,551 nas doses de 0,05 e 0,1 μg) com carragenina (C; 100 μg) reduziram significativamente a hiperalgesia na ATM. A co-aplicação de antagonistas dos adrenoceptores β_1 (atenolol nas doses de 6, 18, 54 ou 162μg) não afetou as respostas comportamentais induzidas pela carragenina (C; 100μg). Indometacina local, propranolol e ICI 118,551 não tiveram efeito quando injetados na ATM contra lateral, o que indica uma participação periférica das prostaglandinas e das aminas simpatomiméticas nesta hiperalgesia. Estes resultados sugerem que as aminas simpatomiméticas são liberadas no local da injúria onde as mesmas contribuem para a hiperalgesia inflamatória na ATM através dos adrenoceptores β_2, indicando que os mesmos são possíveis alvos para o desenvolvimento de novas drogas analgésicas no controle da dor da ATM.

Palavras-chave: Aminas simpatomiméticas, ATM, hiperalgesia química, carragenina, adrenoceptores β.

ABSTRACT

The aim of this study was to further validate our carrageenan-induced temporomandibular joint (TMJ) inflammatory hyperalgesia model in rats by showing that administration of indomethacin before the initiation of inflammation would diminish the TMJ hyperalgesia. By using this model, it was investigated whether norepinephrine and local β adrenoceptors contribute to the development of inflammatory TMJ hyperalgesia. Carrageenan-induced TMJ hyperalgesia was assessed by measuring the behavioral nociceptive responses, such as rubbing the orofacial region and flinching the head, induced by the injection of a low dose of 5-hydroxytryptamine into the TMJ sensitized one hour before by a TMJ injection of carrageenan. Blockade of prostaglandin synthesis by indomethacin prior to initiation of inflammation by carrageenan significantly attenuated the TMJ hyperalgesia. The guanethidine depletion of norepinephrine or the blockade of β_2 but not the blockade of the β_1 adrenoceptor by the selective adrenoceptor antagonists ICI 118.551 and atenolol, respectively, significantly reduced carrageenan-induced TMJ hyperalgesia. In this study, we further validate our carrageenan-induced TMJ hyperalgesia model to study the mechanisms involved in inflammatory TMJ hyperalgesia and to test the analgesic effect of different types of peripheral analgesics. By using this model, we show that norepinephrine is released at the site of injury where it contributes to the development of the inflammatory TMJ hyperalgesia. It is proposed that the contribution of norepinephrine to the development of the inflammatory TMJ hyperalgesia is mediated by the activation of β_2-adrenoceptors. Perspective: The findings that local sympathomimetic amines contribute to the inflammatory TMJ hyperalgesia by acting on β_2-adrenoceptors may be relevant to clinical TMJ inflammatory pain states less sensitive to non-steroidal anti-inflammatory drugs.

Key words: TMJ, carrageenan, chemical hyperalgesia, sympathomimetic amines, β adrenoceptors.

1. INTRODUÇÃO GERAL

O estudo da dor na articulação temporomandibular (ATM) é de grande relevância científica já que esta acomete mais de 12% da população (Von Korff et al., 1988; Dworkin et al., 1990; Carlson and Le Resche, 1995) e é muitas vezes refratária aos tratamentos existentes. Assim sendo, o conhecimento da fisiopatologia dessa dor torna-se fundamental não só para a determinação de tratamentos mais apropriados, como também para o desenvolvimento de novos medicamentos.

A dor é definida pela "Associação Internacional para o Estudo da Dor" (IASP) como "uma experiência sensitiva e emocional desagradável associada à lesão tecidual real ou potencial, ou ainda, descrita nestes termos". Portanto, em humanos, a dor é uma percepção subjetiva com uma dimensão psicológica. Já, a ativação dos nociceptores por uma injúria tecidual e a transmissão desses sinais ao sistema nervoso central (SNC) constitui o processo de nocicepção. Desta forma, para animais experimentais, a utilização do termo nocicepção é mais adequada, reservando-se o termo dor aos seres humanos, nos quais é possível a discriminação do componente emocional (Noback *et al*. 1996).

A informação nociceptiva é convertida em potenciais de ação pelos nociceptores (transdução) e transmitida pelos nervos espinhais e cranianos à medula espinhal e tronco encefálico, respectivamente. Os nociceptores são terminações nervosas livres de neurônios pseudo-unipolares, cujos corpos celulares encontram-se nos gânglios das raízes dorsais e nos gânglios trigeminais (Kandel *et al*., 2000). Os nociceptores possuem limiares de ativação específicos que os distinguem de outras fibras sensoriais, uma vez que só são excitáveis por estímulos intensos, como calor nocivo, pressão intensa ou substâncias químicas irritantes, mas não por estímulos não nocivos como luz e toque (Julius e

Basbaum, 2001). Os axônios desses neurônios pseudo-unipolares, denominados fibras aferentes primárias, conduzem a informação nociceptiva ao SNC. Essas fibras são classificadas em: 1) fibras A-δ, as quais são finas e mielinizadas, com velocidade de condução de 5 a 30m/s e; 2) fibras C, mais finas e não mielinizadas, com velocidade de condução de cerca de 1m/s. As fibras C são chamadas de polimodais por responderem a estímulos nocivos de origem diversa, como térmica, mecânica ou química. Já as fibras A-δ, respondem apenas a estímulos térmicos ou mecânicos intensos (Julius e Basbaum, 2001). Em relação ao processo nociceptivo, esses neurônios de primeira ordem possuem três funções: 1) detecção do estímulo nocivo ou potencialmente injuriante (transdução), 2) passagem dessa informação sensorial da periferia para a medula espinhal (condução) e transferência sináptica dessa mesma informação aos neurônios localizados em lâminas específicas do corno dorsal da medula espinhal (transmissão) (Kidd e Urban, 2001). Na via de condução da informação dolorosa da região orofacial, ou seja, na via trigeminal os impulsos são carregados por neurônios nociceptivos primários, a partir da face e de grande parte da boca, atingindo ao tronco encefálico via nervo trigêmeo. Os estímulos são retransmitidos por meio do núcleo espinhal trigeminal, chamado subnúcleo caudal. Esses sinais ascendem para os centros superiores, incluindo o tálamo e o córtex cerebral. A sinapse entre o neurônio periférico e os neurônios no subnúcleo caudal é um importante local para processamento do sinal. Nesse nível, a informação é modulada por mediadores químicos liberados de terminais de vários grupos de neurônios: os primários aferentes, dos núcleos trigeminais e neurônios de centros superiores.

Processos inflamatórios na ATM podem resultar em hiperalgesia, ou seja, em sensibilização periférica dos nociceptores, (Alstergren e Kopp, 2000; Kopp, 2001; Oliveira

et al., 2005) caracterizada por um aumento na excitabilidade da membrana neuronal, devido a liberação de mediadores inflamatórios no local da lesão (Alstergren e Kopp, 2000; Kopp, 2001; Suzuki et al., 2003).

A sensibilização dos nociceptores nos tecidos decorre de vários mecanismos.

1) Redução do limiar de geração de potenciais – os nociceptores são sensibilizados por substâncias algiogênicas, provenientes dos vasos sanguíneos, liberadas no ambiente tecidual por mastócitos, leucócitos e células traumatizadas. Entre estas substâncias destacam-se a bradicinina, a acetilcolina, a histamina, a serotonina, o leucotrieno, a tromboxana, o fator de ativação plaquetário, os radicais ácidos, os íons potássio, as citocinas e principalmente as prostaglandinas.
2) Atividade do sistema neurovegetativo simpático: em processos inflamatórios, o sistema simpático libera noradrenalina e prostaglandinas nos tecidos, que contribuem para sensibilizar os nociceptores.

Grande parte dos episódios dolorosos é desencadeada por lesão tecidual. Por exemplo, um traumatismo na ATM promove destruição das células da cápsula articular e desencadeia um processo inflamatório no tecido conjuntivo subjacente. O fluído sinovial apresenta, então, altos níveis de mediadores inflamatórios como PGE_2 (Kopp, 2001). Em estados hiperalgésicos, o limiar nociceptivo é diminuído e um estímulo não nocivo, como o movimento mandibular pode causar dor (Alstergren e Kopp, 2000), enquanto que, um estímulo nocivo pode causar dor aumentada (De Laat et al., 1998).

A dor de origem inflamatória é mediada principalmente pelas prostaglandinas (Ferreira *et al*. 1988; Cunha *et al*. 1991; 1992; Khasar *et al*. 1999; Alstergren e Kopp, 2000), mas também pode ter contribuição de aminas simpatomiméticas (Nakamura e Ferreira, 1987).

Nakamura e Ferreira (1987) demonstraram que a hiperalgesia induzida experimentalmente pela administração de carragenina na pele é reduzida não só por inibidores da ciclooxigenase (COX), como a indometacina, como também por bloqueadores de receptores β adrenérgicos, principalmente os β_1. Esta liberação de prostaglandinas e aminas simpatomiméticas pela carragenina parece não ocorrer diretamente, mas através da liberação de uma cascata de mediadores. Ferreira (1980) mostrou que macrófagos e mastócitos sinalizam para outras células a existência de injúria tissular, via liberação de citocinas, como o fator de necrose tumoral (TNF α) e interleucinas (IL)s. As citocinas parecem constituir uma ligação da injúria celular ou do reconhecimento de agentes estranhos com a liberação de mediadores inflamatórios, que por fim são os responsáveis pelos sinais e sintomas locais e sistêmicos da inflamação.

Na hiperalgesia promovida pela carragenina na pata de ratos ocorre a liberação de TNF-α , estimulando a liberação de IL-1 e IL-6, que por sua vez, estimulam a liberação local de prostaglandinas. O TNF-α também induz a liberação da IL-8, que ativa o componente simpático (Cunha *et al*., 1992), constituindo-se assim, o processo em duas vias finais paralelas de mediadores inflamatórios a partir da carragenina, uma mediada pela ação da ciclooxigenase e outra simpática.

Embora os mecanismos envolvidos na dor da ATM não sejam completamente conhecidos, foi demonstrado que a PGE_2, serotonina e citocinas pró inflamatórias (TNF-α, IL-1β) estão presentes em grandes concentrações no fluído sinovial de pacientes com desordens temporomandibulares (Kopp, 2001). Assim sendo, o conhecimento dos principais mediadores que contribuem para hiperalgesia é de suma importância para o

desenvolvimento de novos medicamentos para tratar a dor presente nas disfunções temporomandibulares (DTMs). (Alstergren e Kopp, 2000).

Na clínica, o efeito analgésico dos antiinflamatórios não esteroidais (AINES), que bloqueiam a atividade da enzima ciclooxigenase, tem comprovado o papel das prostaglandinas, principalmente a PGE_2 na hipersensibilização dos nociceptores (Ferreira, 1980). No entanto, a participação do componente simpático em algumas condições dolorosas parece ser especialmente importante em situações onde a dor é refratária ao uso dos AINES (Nakamura e Ferreira, 1987; Duarte et al., 1988). Muitas patologias associadas à dor na ATM não são eficientemente reduzidas pelo uso de AINES (Ferreira, 1980), sugerindo o envolvimento de outros mediadores neste processo.

Como a ATM recebe rica inervação simpática (Yoshino *et al*., 1988; Widenfalk e Wiberg, 1990; Kido *et al*., 2001), é possível que as aminas simpatomiméticas participem do desenvolvimento da hiperalgesia na ATM. No entanto, esta participação ainda precisa ser investigada.

Oliveira *et al*. (2005) ao estudar a importância de receptores purinérgicos na hiperalgesia na ATM necessitava de um modelo de hiperalgesia local. Na tentativa de desenvolvê-lo, foi injetado 100μg de carragenina na ATM, sensibilizando-a. Uma hora após, aplicou-se 75μg de serotonina, para assim avaliar a resposta comportamental nociceptiva, que se apresenta no animal sensibilizado, mas é mínima no animal controle (injetado previamente com salina). A função da serotonina, então, é promover um estímulo químico para permitir a mensuração do comportamento animal (Oliveira *et al*., 2005). Assim, este modelo (publicado em Oliveira *et al*., 2005; ver anexo seis) permite-nos estudar

os mecanismos fisiopatológicos envolvidos na hiperalgesia da ATM, abrindo perspectivas para tratamentos alternativos da dor no local.

2. PROPOSIÇÃO GERAL

Os objetivos deste trabalho foram:

Validar o modelo de hiperalgesia química induzida pela carragenina na ATM, reforçando a natureza inflamatória, ou seja, comprovar a participação das prostaglandinas neste processo hiperalgésico. Como alguns pacientes com dor inflamatória na ATM não respondem ao efeito analgésico dos AINEs, foi proposto também estudar o papel do sistema simpático na hiperalgesia da ATM, através da utilização desse modelo experimental.

A resposta hiperalgésica da ATM de ratos foi avaliada frente ao tratamento intraarticular com: (1) indometacina, inibidor não seletivo da COX e (2) guanetidina (depletor dos estoques intracelulares da norepinefrina) além de antagonistas β adrenérgicos não específicos, propranolol, e específicos para β_1, atenolol e para β_2, ICI 118,551.

3. CAPÍTULO

O presente artigo foi submetido a publicação no periódico “The Journal of Pain” e encontra-se sob avaliação.

Peripheral sympathetic component of the temporomandibular joint inflammatory pain in rats

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Key words: TMJ, carrageenan, chemical hyperalgesia, sympathomimetic amines,

β adrenoceptors.

Abstract

The aim of this study was to further validate our carrageenan-induced temporomandibular joint (TMJ) inflammatory hyperalgesia model in rats by showing that administration of indomethacin before the initiation of inflammation would diminish the TMJ hyperalgesia. By using this model, it was investigated whether norepinephrine and local β adrenoceptors contribute to the development of inflammatory TMJ hyperalgesia. Carrageenan-induced TMJ hyperalgesia was assessed by measuring the behavioral nociceptive responses, such as rubbing the orofacial region and flinching the head, induced by the injection of a low dose of 5-hydroxytryptamine into the TMJ sensitized one hour before by a TMJ injection of carrageenan. Blockade of prostaglandin synthesis by indomethacin prior to initiation of inflammation by carrageenan significantly attenuated the TMJ hyperalgesia. The guanethidine depletion of norepinephrine or the blockade of β_2 but not the blockade of the β_1 adrenoceptor by the selective adrenoceptor antagonists ICI 118.55 and atenolol, respectively, significantly reduced carrageenan-induced TMJ hyperalgesia. In this study, we further validate our carrageenan-induced TMJ hyperalgesia model to study the mechanisms involved in inflammatory TMJ hyperalgesia and to test the analgesic effect of different types of peripheral analgesics. By using this model, we show that norepinephrine is released at the site of injury where it contributes to the development of the inflammatory TMJ hyperalgesia. It is proposed that the contribution of norepinephrine to the development of the inflammatory TMJ hyperalgesia is mediated by the activation of β_2-adrenoceptors.

Perspective: The findings that local sympathomimetic amines contribute to the inflammatory TMJ hyperalgesia by acting on β_2-adrenoceptors may be relevant to clinical TMJ inflammatory pain states less sensitive to non-steroidal anti-inflammatory drugs.

Introduction

Temporomandibular joint disorders, especially those associated with acute trauma, internal derangement, or arthritis are commonly associated with acute or chronic inflammation.[1,43] They represent a group of chronic painful conditions involving the muscles of mastication and the temporomandibular joint (TMJ) with a prevalence in the general population up to 12%.[9,22,49]

Inflammatory TMJ conditions can result in TMJ hyperalgesia produced by peripheral sensitization of TMJ nociceptors [1,29,35,36,38] and by central sensitization of the nociceptive neurons of the trigeminal brainstem sensory nuclear complex.[21,30,41] Peripheral sensitization as well as central sensitization are characterized by an increase in the neuronal membrane excitability by inflammatory mediators released at the site of injury[1,29,43] and by neuropeptide and excitatory amino acid released at the trigeminal brainstem sensory nuclear complex[3,4,7,52], respectively. Some of the inflammatory mediators released at the site of injury including $PGE_{2,}$ are present at high levels in the synovial fluid of patients with temporomandibular disorders.[29] During hyperalgesic states, nociceptive threshold is lowered and a non noxious stimulus such as jaw movement can induce pain[1] as well as noxious stimulus can induce increased pain.[18] Non-steroidal anti-inflammatory drugs (NSAIDS) are frequently used to manage inflammatory pain.[19,32,44] The analgesic action of these drugs results from the blockade of prostaglandins synthesis, thus preventing the peripheral sensitization of nociceptors. [23, 24] Considering that many patients are intolerant to prolonged treatment with NSAIDS and that not all patients with TMJ inflammatory pain respond to its effects[44] a better understanding of the neurochemicals and receptors involved in these conditions is necessary.

It is well known that inflammatory pain has a sympathetic component[31, 33] that may predominate in pain less sensitive to NSAID and that TMJ receives a rich sympathetic innervation.[28, 50, 51] However, whether sympathomimetic amines play a role in the development of TMJ hyperalgesia remains to be investigated. Our recently described model of carrageenan-induced hyperalgesia in the rat TMJ[36] may contribute to that. In this model a low dose of 5-hydroxytryptamine (5-HT) that induces minimal nociceptive behavior is injected into the TMJ sensitized one hour before by a TMJ injection of carrageenan. The chemical stimulation induced by the TMJ injection of 5-HT is used to detect carrageenan-induced hyperalgesia in the TMJ region. In order to distinguish this type of inflammatory hyperalgesia from that detected by mechanical stimulation, the experimental model was termed carrageenan-induced chemical hyperalgesia in the rat TMJ. The aim of this study was to further validate this model by showing that administration of indomethacin before the initiation of inflammation would diminish the TMJ hyperalgesia. By using this model, it was investigated whether norepinephrine and local β adrenoceptors contribute to the development of inflammatory TMJ hyperalgesia by depleting intracellular stores of norepinephrine or by blocking articular β adrenoceptors.

Material and Methods

Animals

This study was carried out on 154 male Wistar rats (200-300g). The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00A.M.) with food and water available ad libitum. They were maintained in a temperature-controlled room (±23°C) 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 the pain in animals.[53]

General Procedures

Each animal was placed in a test chamber (30×30×30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period. Each animal was used once and immediately sacrificed after used. Testing sessions took place during light phase (between 09:00AM and 5:00PM) in a quiet room maintained at 23°C. [39]

TMJ injection

Animals were briefly anesthetized by inhalation of halothane and the posteroinferior border of the zygomatic arch was palpated. The needle was inserted immediately inferior to this point and advanced in an anterior direction until reach the posterolateral aspect of the condyle. TMJ injections were performed via a 30-gauge needle introduced to the left TMJ at the moment of the injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50µL). Volume per injection was 15µL. Each animal regained consciousness approximately 30 seconds after discontinuing the anesthetic and was returned to the test chamber.

After the conclusion of each experiment, animals were anesthetized with an intraperitoneal injection of a mixture of urethane (1g/kg) and α-chloralose (50mg/kg). The Evans blue dye (0.1%, 5mg/Kg) was then administered systemically in order to visualize the inflammation-induced plasma extravasation of Evans Blue dye bound to plasma protein upon *post-mortem* examination of the injected TMJs. [25] The correct site of injection was indicated by the observation that the plasma extravasation induced by the TMJ injections was restricted to the TMJ region.

Measurement of behavioral nociceptive responses

Two TMJ injections were given at one hour interval and each rat returned to the test chamber following the last TMJ injection for an observation period of 30 min. Rats immediately recovered from the anesthesia after each TMJ injection. The recording time was divided into 10 blocks of 3 min 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 and hindpaw and/or flinching the head in an intermittent and reflexive way characterized by high frequency shakes of the head as previously described. [12, 36, 40] Rats did not have access to food or water during the test.

Carrageenan-induced chemical hyperalgesia in the TMJ

Carrageenan-induced TMJ hyperalgesia was assessed by measuring these behavioral nociceptive responses induced by application of a low dose of 5-hydroxytryptamine (5-HT; 75µg) into the TMJ challenged 1 hour prior by the injection carrageenan (100µg).[36]

Drugs and doses

The following drugs were administered: Carrageenan 100µg[36]; 5-hydroxytryptamine 75µg[36]; Indomethacin 10µg[17], 2.5mg/kg[26]; Propranolol hydrochloride ((*RS*)-1-[(1-

Methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol hydrochloride), β adrenoceptor antagonist 0.25µg and 2.25µg[16]; Atenolol ((*RS*)-4-[2-Hydroxy-3-[(1methylethyl)amino] ropoxy]benzeneacetamide), β1 adrenoceptor antagonist 6µg, 18µg, 54µg or 162µg[16]; ICI 118,551 hydrochloride ((±)-1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), β2 adrenoceptor antagonist 0.05µg and 0.1µg[37]; Guanethidine, sympathetic neuron-blocking agent 30 mg/kg for 3 days, s.c.[11]

Indomethacin was dissolved in Tris (0.2 M Tris; pH 8.3)[5] and all other drugs were dissolved in saline (0.9% NaCl). Carrageenan was obtained from Sigma, SP, Brazil; ICI 118,551 hydrochloride was obtained from Tocris, USA, and all other drugs and reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Experimental design

Each rat was given a TMJ injection of a specific combination of two drugs followed, one hour later, by a TMJ injection of either 5-HT or its vehicle control (saline = 0.9% NaCl). Rats were randomly assigned to separate groups of animals.

The TMJ injection of 5-HT or saline one hour after the TMJ injection of carrageenan plus saline (n=7 and 9, respectively) or saline plus saline (n=6 in both groups) was used to characterize carrageenan-induced chemical TMJ inflammatory hyperalgesia.

To further validate this model of TMJ inflammatory hyperalgesia, indomethacin was injected either systemically or locally. Systemic indomethacin was injected via an intraperitonel injection performed 30 minutes before the TMJ injection of carrageenan plus saline followed, one hour later, by the TMJ injection of 5-HT (n=6). Local indomethacin was co-injected with carrageenan into the TMJ and was followed, one hour later, by the TMJ injection of 5-HT (n=6). In another experimental group, Tris was co-injected with

saline into the TMJ and was followed, one hour later, by the TMJ injection of saline (n=4). To control for the possibility that local indomethacin could enter the circulation after injected into the TMJ to act at a site distant from the site of administration, indomethacin was injected into the right TMJ, and carrageenan plus saline was injected into the left TMJ (contralateral), which was also injected with 5-HT one hour later (n=6). Furthermore, to control for the possibility that indomethacin could affect the behavioral response induced by the TMJ injection of 5-HT, systemic indomethacin was injected 30 minutes before the TMJ injection of saline plus saline followed, 1 hour later, by the TMJ injection of 5-HT (n=7). For the same reason, in another experimental group, local indomethacin plus saline was injected into the TMJ and was followed, 1 hour later, by the TMJ injection of 5-HT (n=6).

To test whether sympathomimetic amines such as norepinephrine play a role in carrageenan-induced chemical TMJ inflammatory hyperalgesia, we depleted the peripheral stores of norepinephrine by systemically applying guanethidine (30 mg/Kg, sc) for 3 days[11] before the TMJ injection of carrageenan plus saline followed, one hour later, by the TMJ injection of 5-HT (n=7). Guanethidine treatment was also applied before the TMJ injection of saline plus saline followed, one hour later, by the TMJ injection of 5-HT (n=6) to control for the possibility that guanethidine could affect the behavior response induced by 5-HT.

To evaluate the contribution of β adrenergic-receptors to the TMJ hyperalgesia induced by carrageenan, the β adrenergic receptor antagonist propranolol (0.25 or 2.25μg, n=6 in both cases) was co-applied with carrageenan into the TMJ and one hour later, 5-HT was injected into the ipsilateral TMJ. To further evaluate the contribution of β adrenergic-receptors to the TMJ hyperalgesia induced by carrageenan, we next asked which subtypes of β adrenergic-receptors contribute to this TMJ hyperalgesia. To answer this question, the

β_1 adrenoceptor antagonist atenolol (6µg, n=7; 18µg, n=6; 54µg, n=6; or 162 µg, n=6) or the β_2 adrenoceptor antagonist ICI 118,551 (0.05 or 0.1µg, n=6 in both cases) was co-applied with carrageenan into the TMJ and one hour later, 5-HT was injected into the ipsilateral TMJ. In order to confirm the peripheral action of the β adrenoceptor antagonists that had any effect, the highest dose of either propranolol or ICI 118.551 (n=6 in both cases) was injected into the right TMJ and carrageenan plus saline was injected into the left TMJ (contralateral), which was also injected with 5-HT one hour later. To control for the possibility that these β adrenoceptor antagonists could affect the behavioral response induced by the TMJ injection of 5-HT into the TMJ previously injected with saline plus saline, either propranolol (0.25 or 2.25µg, n=6 in both cases) or ICI 118.551 (n=5) was co-applied with saline into the TMJ and was followed, 1 hour later, by the TMJ injection of 5-HT.

Statistical Analysis

The sum of the behavioral responses measured for 30 minutes was used for statistical analyzes. 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 ± SEM.

Results

Carrageenan-induced chemical TMJ inflammatory hyperalgesia

To access carrageenan-induced chemical TMJ inflammatory hyperalgesia in rats, we applied a low dose of 5-hydroxytryptamine (5-HT) into the TMJ challenged by the injection of carrageenan 1 hour prior, as previously described.[36] Under this condition, the behavioral nociceptive response measured was significantly greater ($p<0.05$, Tukey test) than that induced by each of these drugs combined with saline, and was used as a quantitative measurement of carrageenan-induced chemical TMJ hyperalgesia (Fig.1).

Indomethacin

Systemic indomethacin (2.5mg/kg; i.p.) significantly reduced the behavioral nociceptive response induced by the injection of 5-HT into the TMJ pretreated with carrageenan plus saline (Fig. 2A; $p<0.05$, t test). This finding indicates that prostaglandins contribute to the TMJ hyperalgesia induced by carrageenan. Similar results were obtained with the co-application of indomethacin (10µg) with carrageenan into the TMJ followed, one hour later, by the injection of 5-HT into the ipsilateral TMJ (Fig. 2B; $p<0.05$, Tukey test). Injection of the vehicle control Tris plus saline followed, one hour later, by the injection of saline into the ipsilateral TMJ did not affect the rat's behavior response, because this response was similar to that induced by the TMJ injection of the saline combination followed, one hour later, by the injection of saline into the ipsilateral TMJ. Indomethacin had no effect when injected into the contralateral TMJ, confirming its local action. Neither systemically nor locally applied indomethacin significantly affected the behavioral response induced by the injection of 5-HT into the TMJ pretreated with saline

plus saline ($p>0.05$, t test), further confirming the contribution of prostaglandins to the TMJ hyperalgesia induced by carrageenan.

Guanethidine

Depletion of peripheral stores of norepinephrine by guanethidine significantly reduced the nociceptive response induced by the injection of 5-HT into the TMJ pretreated with carrageenan plus saline (Fig. 3; $p<0.05$, t test). This finding suggests that norepinephrine contributes to the TMJ hyperalgesia induced by carrageenan. Guanethidine pretreatment (30 mg/Kg, sc) did not significantly affect the behavioral response induced by the injection of 5-HT into the TMJ pretreated with saline plus saline ($p>0.05$, t test), further confirming the contribution of norepinephrine to the TMJ hyperalgesia induced by carrageenan.

β-adrenoceptor antagonists

Co-application of propranolol (0.25 or 2.25µg) with carrageenan into the TMJ significantly reduced the nociceptive response induced by the injection of 5-HT into the ipsilateral TMJ one hour later (Fig. 4A; $p<0.05$, Tukey test). The lack of effect of the injection of the highest dose of propranolol into the contralateral TMJ, confirms its local action.

Co- application of ICI 118.551 (0.05 or 0.1µg) (Fig. 4C; $p<0.05$, Tukey test) but not the co-application of atenolol (6, 18, 54 or 162µg) (Fig. 4B; $p>0.05$, Tukey test) with carrageenan into the TMJ significantly reduced the nociceptive response induced by the injection of 5-HT into the ipsilateral TMJ one hour later. The lack of effect of the injection of the highest dose of ICI 118.551 into the contralateral TMJ, confirms its local action. Neither propranolol nor ICI 118.551 at highest doses significantly affected the behavioral

response induced by the injection of 5-HT into the TMJ pretreated with saline plus saline ($p>0.05$, t test), further confirming the contribution of β-adrenoceptors to the TMJ hyperalgesia induced by carrageenan.

Discussion

In this study we further validate our carrageenan-induced chemical TMJ inflammatory hyperalgesia model in rats[36] by showing that blockade of prostaglandin synthesis by indomethacin prior to initiation of inflammation by carrageenan significantly attenuates TMJ hyperalgesia. By using this model, we show that sympathomimetic amines contribute to the development of TMJ hyperalgesia, probably by acting at β_2- adrenoceptors located in the TMJ region. This is based on the findings that depletion of intracellular stores of norepinephrine or blockade of articular β_2-adrenoceptors prior to initiation of inflammation by carrageenan significantly attenuated the TMJ hyperalgesia.

Validation of the carrageenan-induced TMJ hyperalgesia model by indomethacin

It is well known that the analgesic action of NSAIDs such as indomethacin results from blockade of the synthesis of cyclooxigenase products such as prostaglandins.[10,13,45] Also well known, is that prostaglandins are common mediators of inflammatory processes and induce nociceptor sensitization.[6,8,46] Therefore, the finding that indomethacin significantly attenuated the hyperalgesia induced by carrageenan in the TMJ indicates that prostaglandin contributes to this articular hyperalgesic condition. This finding is consistent with those previously demonstrated in other tissues, such as the cutaneous[16,48] and the articular tissue (knee joint)[46,47], and further validates the carrageenan-induced TMJ hyperalgesia model to study the mechanisms involved in TMJ inflammatory pain and to test the analgesic effect of different types of peripheral analgesics. Given that NSAIDs may also posses a central analgesic effect by attenuating prostaglandin production in the spinal cord, [42] the effect of systemic indomethacin might be due, at least in part, to a central effect. However, the findings that local application of indomethacin similarly attenuated

carrageenan-induced TMJ hyperalgesia, an effect that was not observed when indomethacin was injected into the contralateral TMJ, confirms that prostaglandin is released at the inflamed TMJ.

Role of norepinephrine in carrageenan-induced TMJ hyperalgesia

In addition to prostaglandins, sympathomimetic amines such as norepinephrine are also released at the site of inflammation[20,33] where they contribute to the inflammatory hyperalgesia.[2,27] Therefore, our finding that depletion of intracellular stores of norepinephrine by guanethidine significantly attenuated the TMJ hyperalgesia induced by carrageenan indicates that norepinephrine contributes to the hyperalgesic state in the TMJ, as previously demonstrated in other tissues.[14,15,16,33,46] This finding is consistent with the dense innervation of the temporomandibular joint by sympathetic fibers arising from cells of the superior cervical ganglion[28,50,51] from where norepinephrine may be released. This sympathetic component of TMJ inflammatory pain may predominate in some patients that suffer from this painful condition and may explain why some of these patients are unresponsive to the analgesic effect of NSAIDS.

Mechanism of action of norepinephrine in carrageenan-induced TMJ hyperalgesia

Our data suggest that the mechanism by which norepinephrine contributes to carrageenan-induced TMJ hyperalgesia is mediated by the activation of β_2- adrenoceptors; specifically, TMJ injection of the selective β_2- adrenoceptor antagonist ICI 118.551 but not of the β_1-adrenoceptor antagonist atenolol significantly attenuated the TMJ hyperalgesia. This suggestion, which is consistent with the finding that norepinephrine-induced mechanical hyperalgesia is blocked by the β adrenoceptor antagonist propranolol[33] does not exclude the involvement of other mechanisms as well as the possibility of other related-

monoamines to act on β_2-adrenoceptors to contribute to carrageenan-induced TMJ hyperalgesia.

Site of action of norepinephrine in carrageenan-induced TMJ hyperalgesia

The contribution of norepinephrine to carrageenan-induced TMJ hyperalgesia via β_2-adrenoceptor activation probably does not result from a direct action of this amine on the primary afferent nociceptive terminals. Although no previous studies appear to have examined the presence of β-adrenoceptor RNA or binding sites within nociceptive trigeminal neurons, the β-adrenoceptor RNA was not detected in the dorsal root ganglion.[34] In fact, it has been suggested that in hyperalgesic states, the site of action of norepinephrine is not on the primary afferents but rather is presynaptic on the sympathetic post-ganglionic terminals.[31]

Comparison with other types of hyperalgesia

The inflammatory TMJ hyperalgesia observed in the present study resembles other types of inflammatory hyperalgesia in that, like them, it is mediated by prostaglandins and sympathomimetic amines such as norepinephrine. However, while carrageenan-induced chemical TMJ hyperalgesia is mediated by local β_2-adrenoceptors activation (current study), carrageenan-induced mechanical hyperalgesia in the subcutaneous tissue is mediated by local β_1-adrenoceptors activation.[16] Another difference is that carrageenan-induced chemical TMJ hyperalgesia reaches its hyperalgesic peak 1 hour after the carrageenan injection [36] whereas carrageenan-induced mechanical hyperalgesia in the subcutaneous tissue it does only 3 hours after the carrageenan injection.[46] Therefore, the patophysiological characteristics of the hyperalgesic state may differ depending on the local of the inflammation or on the hyperalgesic stimulus.

In summary, this study further validates our carrageenan-induced TMJ hyperalgesia model to study the mechanisms involved in inflammatory TMJ pain and to test the analgesic effect of different types of peripheral analgesics. In addition, we conclude that sympathomimetic amines such as norepinephrine are released at the site of injury where they contribute to the inflammatory TMJ hyperalgesia by acting on β_2-adrenoceptors. The relationship between our findings and the clinical TMJ inflammatory pain is not yet clear. However, the involvement of sympathomimetic amines in TMJ hyperalgesia may be relevant to clinical TMJ inflammatory pain states less sensitive to NSAIDs.

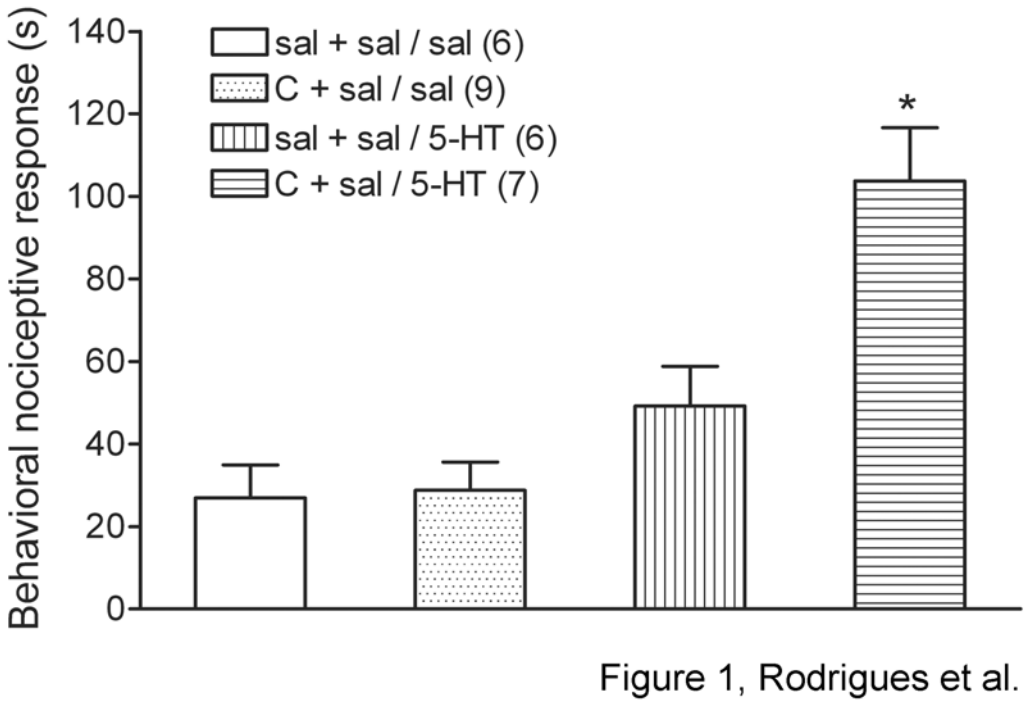

Fig. 1- Characterization of carrageenan-induced TMJ hyperalgesia.

The behavioral response induced by application of 5-HT into the TMJ previously challenged with carrageenan (C, 100µg) was significantly greater than that induced by the TMJ injection of each of these drugs combined with saline. The symbol (*) indicates a response significantly greater than that induced by other treatments ($p<0.05$, Tukey test). In this and in subsequent figures, 5-HT was injected into the TMJ 1h after the TMJ injection of carrageenan. *Abbreviations:* C, carrageenan and sal, saline (0.9% NaCl).

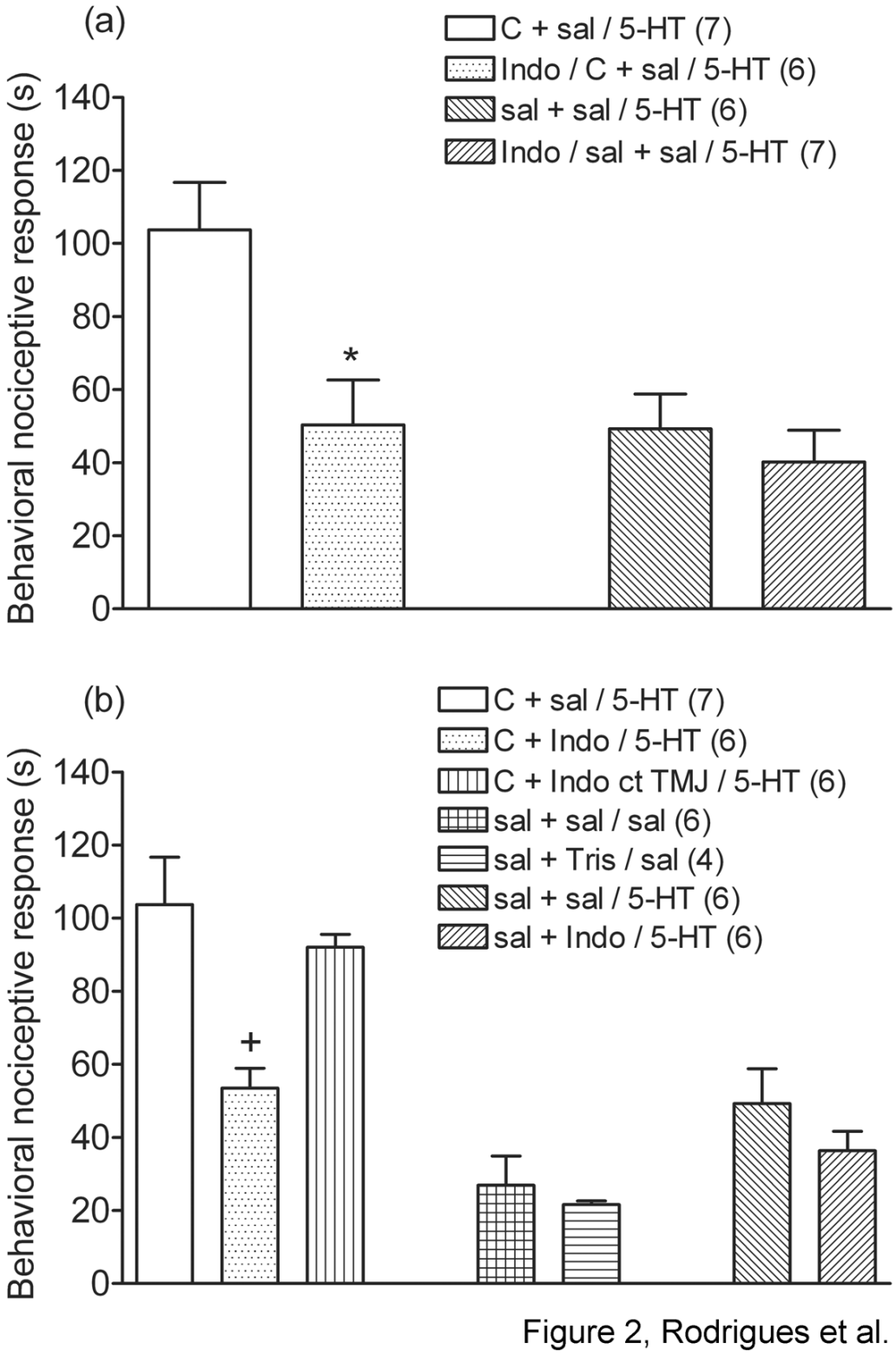

Figure 2, Rodrigues et al.

Fig. 2- Effect of indomethacin on carrageenan-induced TMJ hyperalgesia.

(a) Application of systemic indomethacin (Indo, 2.5mg/kg; i.p.) 30 min before the injection of carrageenan plus saline (C, 100μg) into the TMJ significantly reduced the hyperalgesic effect of carrageenan as indicated by the significant reduction of the behavioral nociceptive response induced by application of 5-HT (75μg) into the TMJ pretreated with carrageenan plus saline ($p<0.05$, Tukey test). The symbol (*) indicates a response significantly lower ($p<0.05$, Tukey test) than that induced by the treatment on the left (C 100 μg + sal / 5-HT 75 μg).

(b) Co-application of indomethacin (Indo, 10μg) with carrageenan plus saline (C, 100μg) into the TMJ significantly reduced carrageenan-induced TMJ hyperalgesia as indicated by the reduction of the nociceptive response induced by application of 5-HT into the TMJ pretreated with carrageenan ($p<0.05$, Tukey test). Co-application of Tris (the vehicle of indomethacin) plus saline followed, one hour later, by the injection of saline into the ipsilateral TMJ, produced similar results to that induced by the TMJ injection of saline plus saline followed, one hour later, by the injection of saline into the ipsilateral TMJ. Indomethacin (Indo, 10μg) had no effect when injected into the contralateral TMJ. The symbol ($^{+}$) indicates a response significantly lower ($p<0.05$, Tukey test) than that induced by the treatment on the left (C 100 μg + sal / 5-HT 75 μg). Neither systemic nor local indomethacin significantly affected the behavioral response induced by the injection of 5-HT into the TMJ previously injected with saline plus saline ($p>0.05$, Tukey test).

Abbreviations: C, carrageenan; Indo, indomethacin; sal, saline (0.9% NaCl); ct, contralateral.

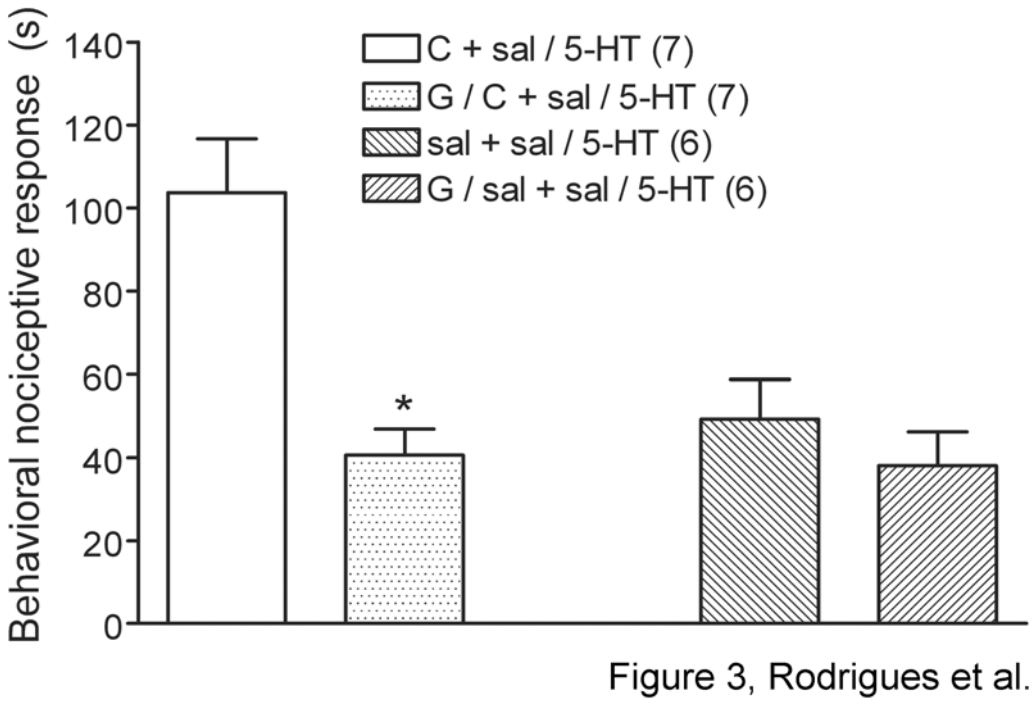

Fig. 3- The effect of sympathomimetic amines depletion by guanethidine on carrageenan-induced TMJ hyperalgesia.

Guanethidine (G, s.c., 30mg/kg for 3 days before the TMJ injection of carrageenan) significantly reduced carrageenan-induced TMJ hyperalgesia as indicated by the reduction of the nociceptive response induced by application of 5-HT into the TMJ pretreated with carrageenan plus saline ($p<0.05$, Tukey test). Guanethidine did not significantly affect the behavioral response induced by the injection of 5-HT into the TMJ previously injected with saline plus saline ($p>0.05$, Tukey test). The symbol (*) indicates a response significantly lower ($p<0.05$, Tukey test) than that induced by treatment on the left (C 100 μg + sal / 5-HT 75 μg).

Abbreviations: C, carrageenan; G, guanethidine and sal, saline (0.9% NaCl).

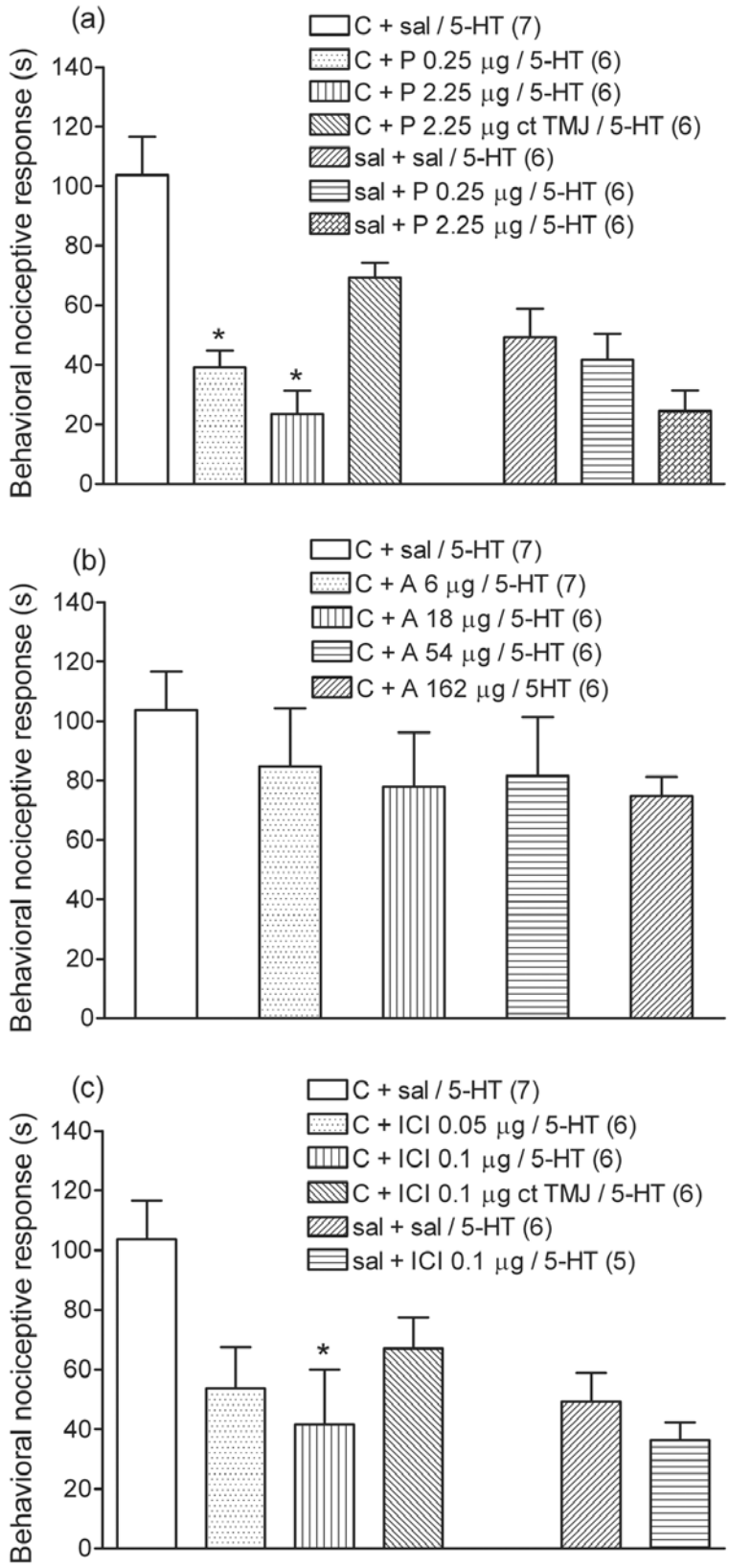

Figure 4, Rodrigues et al.

Fig. 4- The effect of β adrenoceptor antagonists on carrageenan-induced TMJ hyperalgesia. (a) Co-application of propranolol (P, 0.25μg or 2.25μg) with carrageenan (C, 100μg) into the TMJ significantly reduced, in a dose related fashion, carrageenan-induced TMJ hyperalgesia as indicated by the reduction of the nociceptive response induced by application of 5-HT in the TMJ pretreated with carrageenan ($p<0.05$, Tukey test). Propranolol (P, 2.25μg) had no effect when injected into the contralateral TMJ. The symbol (*) indicates responses significantly lower ($p<0.05$, Tukey test) than that induced by the treatment on the left (C 100 μg + sal / 5-HT 75 μg).

(b) Co-application of the β_1 adrenoceptor antagonist, atenolol (A, 6, 18, 54 or 162μg), with carrageenan (C, 100μg) had no effect on carrageenan-induced TMJ hyperalgesia ($p>0.05$, Tukey test).

(c) Co-application of the β_2 adrenoceptor antagonist, ICI (0.05 or 0.1μg), with carrageenan (C, 100μg) significantly reduced, in a dose related fashion, carrageenan-induced TMJ hyperalgesia as indicated by the reduction of the nociceptive response induced by application of 5-HT into the TMJ pretreated with carrageenan plus saline ($p<0.05$, Tukey test). The symbol (*) indicates responses significantly lower ($p<0.05$, Tukey test) than that induced by the first treatment on the left (C 100 μg + sal / 5-HT 75 μg). Neither Propranolol nor ICI 118.551 significantly affected the behavioral response induced by the injection of 5-HT into the TMJ previously injected with saline plus saline ($p>0.05$, Tukey test). *Abbreviations:* C, carrageenan; P, propranolol; A, atenolol; ICI, ICI 118.551; sal, saline (0.9% NaCl) and ct, contralateral.

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4. CONCLUSÃO GERAL

Este trabalho validou o modelo de hiperalgesia química induzida pela administração de carragenina na ATM de ratos, demonstrando para isso que a administração sistêmica (i.p.) ou intraarticular de indometacina antes do início da inflamação reduz a hiperalgesia inflamatória na ATM de ratos. Além disso, demonstrou que a norepinefrina e adrenoceptores β2 localizados na região da ATM participam dessa resposta, enquanto que os β1 não participam, já que tanto o pré-tratamento com guanetidina, como o uso local de antagonistas dos adrenoceptores β não específicos (propranolol) e antagonistas dos adrenoceptores β_2 (ICI 118,551) na ATM reduziram significativamente a hiperalgesia inflamatória, o que não foi observado com a injeção local do antagonista do adrenoceptor β_1, atenolol. Diante do exposto, pode-se concluir que a hiperalgesia química induzida pela administração de carragenina na ATM de ratos é mediada por prostaglandinas e pelas aminas simpatomiméticas, como por exemplo, a norepinefrina, via adrenoceptores β_2, e que esses receptores podem constituir alvos em potencial para o desenvolvimento de novas drogas analgésicas no controle da dor inflamatória da ATM.

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* De acordo com a norma da UNICAMP/FOP, baseada no modelo Vancouver.

Abreviatura do periódico em conformidade com o Medline.

ANEXO 1

TABELA 1- Efeito da hiperalgesia induzida pela administração de carragenina na ATM de ratos. Nesta tabela, assim como nas subseqüentes, a avaliação comportamental foi realizada no período de 30 minutos.

GRUPOS	RESPOSTA COMPORTAMENTAL
Sal + sal/ 1h após sal (6)	26,96 ± 7,99 $^{+}$
C 100μg + sal/ 1h após sal (9)	28,83 ± 6,76 $^{\#}$
Sal + sal/ 1h após 5-HT 75μg (6)	49,23 ± 9,58 *
C 100μg + sal/ 1h após 5-HT 75μg (7)	103,75 ± 12,92 $^{+\#*}$

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

ANEXO 2

TABELA 2A - Efeito da administração sistêmica de indometacina (2,5 mg/kg; i.p.; 30 min antes da injeção de carragenina (C; 100 μg) na hiperalgesia induzida pela carragenina na ATM de ratos.

GRUPOS	RESPOSTA COMPORTAMENTAL
C 100 μg + sal/ 1h após 5-HT 75μg (7)	103,75 ± 12,92 $^{+\#*}$
I 2.5 mg/kg/ C100μg + sal/5 -HT 75μg (6)	57,62 ± 18,34 $^{+}$
Sal + sal/ 1h após 5-HT 75 μg (6)	49,23 ± 9,58 $^{\#}$
I 2,5 mg/kg/ sal + sal/ 5-HT 75μg (7)	40,24 ± 7,85 *

TABELA 2B - Efeito da co-administração de indometacina (10 μg) com carragenina (100 μg) na hiperalgesia induzida pela carragenina na ATM de ratos.

GRUPOS	RESPOSTA COMPORTAMENTAL
C 100 μg + sal/ 1h após 5-HT 75μg (7)	103,75 ± 12,92 $^{+\#*\delta\varphi\psi}$
C 100 μg + I 10μg/ 1h após 5-HT 75μg(6)	52,62 ± 17,23 $^{+}$
C 100 μg + I 10μgct ATM/ 5-HT 75μg (6)	92,10 ± 3,17 $^{\#}$
Sal + Sal/ 1h após sal (6)	26,96 ± 7,99 *
Sal + Tris/ 1h após sal (4)	21,58 ± 0,94 $^{\delta}$
Sal + sal/ 1h após 5-HT 75 μg (6)	49,23 ± 9,58 $^{\varphi}$
Sal + I 10μg/ 1h após 5-HT 75 μg (6)	36,38 ± 13,82 $^{\psi}$

ANEXO 3

TABELA 3 – Efeito do pré tratamento de 3 dias com guanetidina (30mg/kg; s.c.), depletor das aminas simpatomiéticas periféricas, na hiperalgesia induzida pela carragenina na ATM.

GRUPOS	RESPOSTA COMPORTAMENTAL
C 100 μg + sal/ 1h após 5-HT 75μg (6)	103,75 ± 12,92 $^{+ \# *}$
G/ C 100 μg + sal/ 1h após 5-HT 75μg (6)	40,61 ± 5,80 $^{+}$
Sal + sal/ 1h após 5-HT 75 μg (6)	49,23 ± 9,58 $^{\#}$
G/ Sal + sal/ 1h após 5-HT 75 μg (6)	38,05 ± 7,47 *

ANEXO 4

TABELA 4A - Efeito da co-administração do antagonista do receptor adrenérgico β, propranolol (0,25 e 2,25μg) com carragenina (C; 100μg) na hiperalgesia induzida pela carragenina na ATM de ratos.

GRUPOS	RESPOSTA COMPORTAMENTAL
C 100 μg + sal/ 1h após 5-HT 75 μg (7)	103,75 ± 12,92 $^{+ \# * \delta \varphi \psi}$
C 100 μg + P 0,25 μg/ 5-HT 75 μg (6)	39,12 ± 5,08 $^{+}$
C 100 μg + P 2,25 μg/ 5-HT 75 μg (6)	23,50 ± 7,12 $^{\#}$
C 100 μg + P 2,25 μg ct / 5-HT 75 μg (6)	69,34 ± 4,58 *
Sal + sal/ 1h após 5-HT 75 μg (6)	49,23 ± 9,58 $^{\delta}$
Sal + P 0,25 μg/ 5-HT 75 μg (6)	41,77 ± 7,76 $^{\varphi}$
Sal + P 2,25 μg/ 5-HT 75 μg (6)	24,52 ± 6,26 $^{\psi}$

TABELA 4B – Efeito da co-administração do antagonista adrenérgico β_1, atenolol (6, 18 , 54 e 162μg) com carragenina (C; 100μg) na hiperalgesia induzida pela carragenina na ATM de ratos.

GRUPOS	RESPOSTA COMPORTAMENTAL
C 100 μg + sal/ 1h após 5-HT 75 μg (7)	103,75 ± 12,92
C 100 μg + A 6μg/ 5-HT 75 μg (7)	84,76 ±18,14
C 100 μg + A 18μg/ 5-HT 75 μg (6)	77,84 ± 16,71
C 100 μg + A 54μg/ 5-HT 75 μg (6)	81,56 ± 17,98
C 100 μg + A 162μg/ 5-HT 75 μg (6)	74,82 ± 5,89

TABELA 4C – Efeito da co-administração do antagonista adrenérgico β_2, ICI 118,551 (0,05 e 0,1μg) com carragenina (C; 100μg) na hiperalgesia induzida pela carragenina na ATM de ratos.

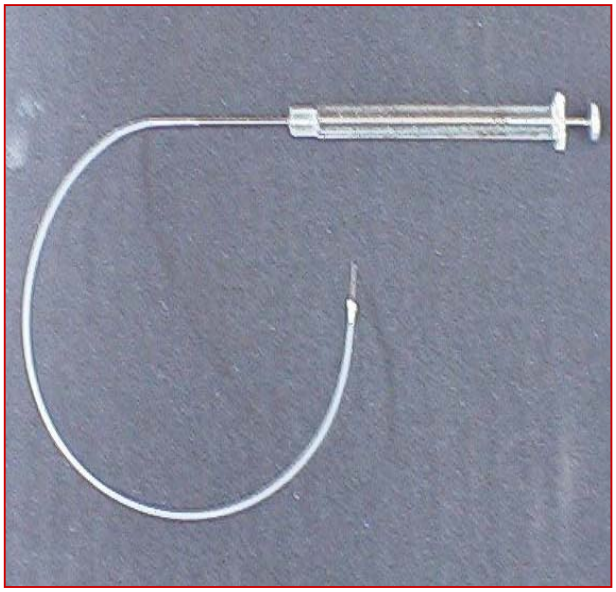
GRUPOS	RESPOSTA COMPORTAMENTAL
C 100 μg + sal/ 1h após 5-HT 75 μg (7)	103,75 ± 12,92 $^{+ \# * \delta \varphi}$
C 100 μg + ICI 0,05μg/ 5-HT 75 μg (6)	53,60 ± 12,60 $^{+}$
C 100 μg + ICI 0,1μg/ 5-HT 75 μg (6)	41,60 ± 16,78 $^{\#}$
C 100 μg + ICI 0,1 μg ct/ 5-HT 75 μg (6)	67,05 ± 9,42 *
Sal + sal/ 1h após 5-HT 75 μg (6)	49,23 ± 9,58 $^{\delta}$
Sal + ICI 0,1μg/ 5-HT 75 μg (5)	36,24 ± 5,40 $^{\varphi}$

ANEXO 5

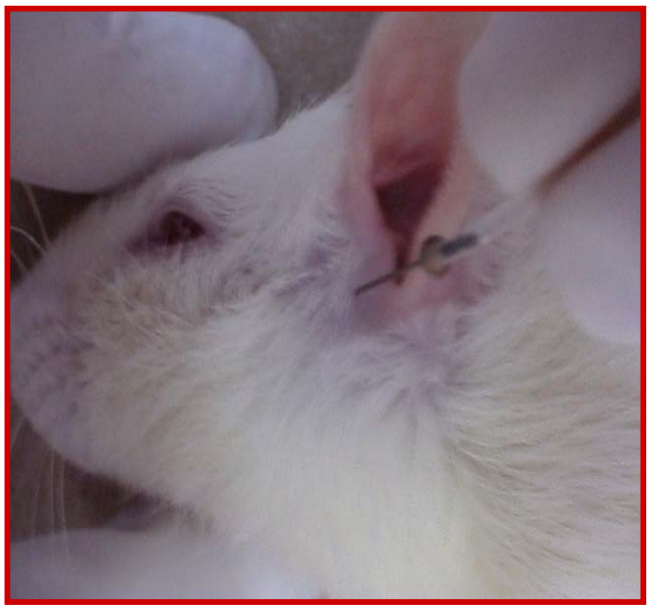
Câmara de observação (30x30x30cm)

Cronômetro

Contador



Agulha conectada a seringa microlitro Hamilton pela cânula de polietileno P50



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

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Received 9 January 2004; accepted 27 April 2004
Available online 21 July 2004

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. Application 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-application 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.

Keywords: P2X receptors; TMJ; Inflammation; Pain; α,β-meATP

1. 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

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doi:10.1016/j.ejpain.2004.04.006

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 and pro-inflammatory cytokines (TNF-α, IL-1β) are present at high levels in the synovial fluid of patients 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 (Sawynok and Reid, 1997; Hamilton et al., 1999; Bland-Ward and Humphrey, 2000). These findings taken together with a number of recent reports using gene knockout methods (Cockayne et al., 2000; Souslova et al., 2000), antisense oligonucleotide technologies (Barclay et al., 2002; Honore et al., 2002) and selective P2X antagonists (Dell'Antonio et al., 2002, Jarvis et al., 2002) suggest that under situations of tissue injury, ATP may leave the intracellular space to contribute to the development of inflammatory hyperalgesia.

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.

2. Materials and methods

2.1. Animals

This study was carried out on male Wistar rats (150–250 g). The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available ad libitum. They were maintained in a temperature-controlled room (±23 °C) 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).

2.2. General procedures

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23 °C (Rosland, 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 (30 × 30 × 30 cm mirrored-wood chamber with a glass at the front side) for a 15-min habituation period to minimize stress (Abbott 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.

2.3. 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 (50 μl).

2.4. 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 min. The recording time was divided into 10 blocks of 3 min 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.

2.5. 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).

2.6. 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; 100 and 300 μg, Sawynok and Reid, 1997) and 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 25 μl.

2.7. Statistical analysis

The sum of the behavioral responses measured for 30 min was used for statistical analyzes. 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 ± SEM.

3. Results

3.1. 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 applied into the TMJ region of rats. Fig. 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-application of 2% QX 314 ($p < 0.05$, Tukey test), confirming its nociceptive character. Co-application 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 (Fig. 1).

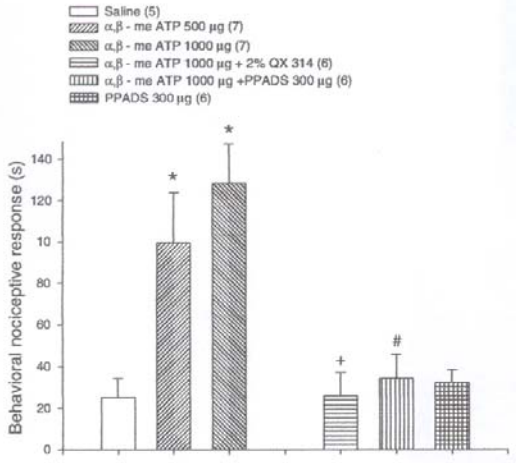

Fig. 1. Behavioral response induced by application of the selective P2X receptor agonist α,β-meATP into the rat TMJ. The selective P2X receptor agonist, α,β-meATP induced a dose-related behavioral response; the symbol (*) indicates that this response was significantly greater than that induced by its vehicle ($p < 0.05$, Tukey test). Co-application of 2% Qx 314 or of the P2 receptor antagonist PPADS significantly reduced α,β-meATP-induced nociception; PPADS had no effect by itself. The symbol ($^{+}$ or $^{\#}$) indicates a response significantly lower than that induced by α,β-me ATP 1000 μg ($p < 0.05$, Tukey test). In this and subsequent figures, data are presented as mean ± SEM, group sample sizes are shown in parentheses and each bar represents the sum of the behavioral responses measured for 30 min (see Section 2 for more details).

3.2. 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 (Fig. 2(a)). The behavioral response induced by application of 5-HT 1 h post the TMJ injection of carrageenan was significantly greater than that induced by its application either 3 or 6 h later ($p < 0.05$, Tukey test; Fig. 2(b)); therefore, a 1 h 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; Fig. 2(c)). Co-application of 2% QX 314 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) in a dose-dependent fashion by the co-application of the P2 receptor antagonist PPADS (100 or 300 μg) with carrageenan (Fig. 3). When applied alone, PPADS did not affect the behavioral response induced by a subsequent TMJ injection of 5-HT (Fig. 3). Taken together, these findings suggest that endogenous ATP plays a role in the development of TMJ inflammatory pain.

4. 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

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 (Roveroni et al., 2001), mediated by local release of serotonin (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 h post the carrageenan injection (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. Although other inflammatory agents such as mustard oil and formalin are commonly used in the TMJ pain studies, these agents are known to induce primarily overt pain rather than sensitization of nociceptors. It has been demonstrated that mustard oil directly stimulate the nociceptors (Woolf and Wall, 1986; Jordt et al., 2004) and that the mechanism underlying formalin-induced nociception involves the release of serotonine and histamine which, in turn, stimulate the nociceptors by activation 5-HT_{1A}, 5-$HT_{4/3}$ and H_1 receptors, respectively (Parada et al., 2001). In general, inflammatory hyperalgesia is mediated by the release of arachidonic acid products, such as prostaglandins (Ferreira et al., 1988; Cunha et al., 1992) and by sympathomimetic amines such as norephinefrine released at the site of injury (Nakamura and Ferreira, 1987; Khasar et al., 1999). 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 co-application of the general P2 receptor antagonist PPADS with carrageenan significantly reduced the carrageenan-induced TMJ hyperalgesia. These findings taken together with a number of reports using gene knockout methods (Cockayne et al., 2000; Souslova et al., 2000), antisense oligonucleotide technologies (Barclay et al., 2002; Honore et al., 2002) and selective P2X antagonists (Dell'Antonio et al., 2002; Jarvis 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. 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.

Acknowledgements

We thank Carlos Alberto Feliciano for excellent technical assistance.

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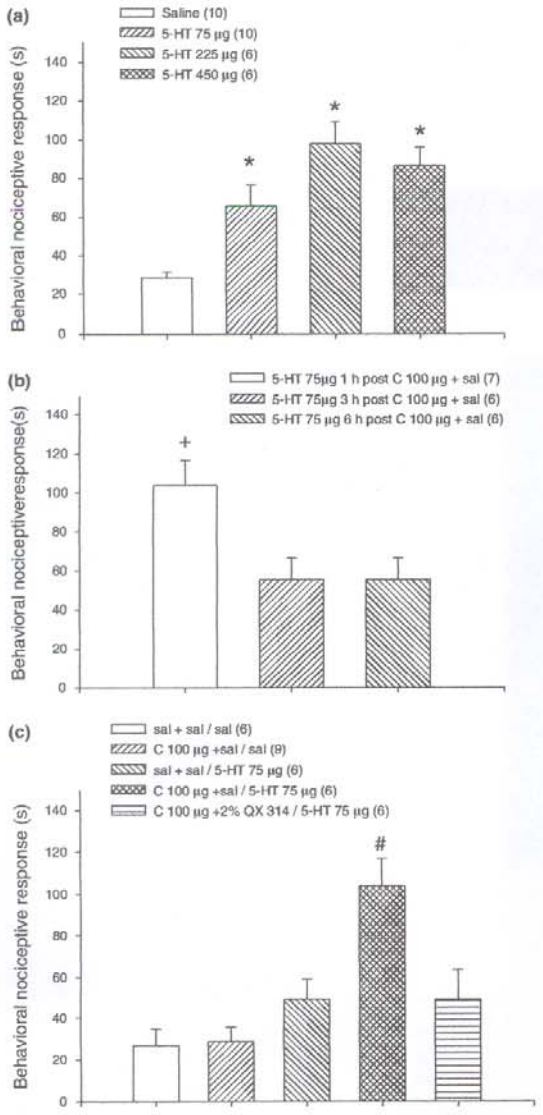

Fig. 2. Characterization of carrageenan-induced TMJ hyperalgesia. (a) Application of 5-HT into the rat TMJ induced a dose-related behavioral response; the symbol (*) indicates that this response was significantly greater than that induced by saline ($p < 0.05$, Tukey test). (b) Time course of behavioral response induced by application of 5-HT in the rat TMJ pretreated with carrageenan. The symbol (+) indicates that the response induced by application of 5-HT 1-h post the TMJ injection of carrageenan was significantly greater than that induced by its application either 3 or 6 h later ($p < 0.05$, Tukey test). (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% QX 314 with carrageenan blocked the behavioral response induced by a subsequent injection of 5-HT. The symbol (#) indicates a response significantly greater than that induced by other treatments. In this and in the subsequent figure, 5-HT was applied in the TMJ 1 h post the carrageenan application. *Abbreviations*: C, carrageenan and sal, saline.

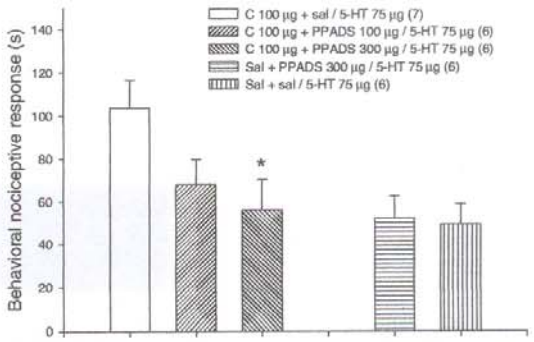

Fig. 3. The effect of a P2 receptor antagonist on carrageenan-induced TMJ hyperalgesia. Co-application of the P2 receptor antagonist PPADS blocked in a dose-related fashion carrageenan-induced TMJ hyperalgesia as indicated by the reversal of the nociceptive response induced by application of 5-HT in the TMJ pretreated with carrageenan. PPADS had no effect on the behavioral response induced by 5-HT. The symbol (*) indicates a response significantly lower ($p < 0.05$, Tukey test) than that induced by treatments on the left (first two bars). *Abbreviations*: C, carrageenan and sal, saline.

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 activate 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). Although activation of peripheral P2X receptors induces a short-lasting depolarization mediated by $Na^+$and Ca^{2+} influx (for review see Dubyak and El-moatassim, 1993) and consequently, a short-lasting nociceptive behavior, the nociceptive response induced by activation of P2X receptors in the TMJ persisted for approximately 30 min following the TMJ application of α,β-meATP. This finding suggests that α,β-meATP may also indirectly activate the nociceptor possibly via the release of other mediators from resident cells such as mast cells (Cockcroft and Gomperts, 1979; Jaffar and Pearce, 1990; Osipchuk and Cahalan, 1992; Lee et al., 2001) and platelets (Vial et al., 1997; Oury et al., 2002; Kunapuli et al., 2003).

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