UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

GILSON GONÇALVES DOS SANTOS

Mecanismo de ação analgésica da dipirona:

Envolvimento dos receptores canabinóides CB1 e CB2 no tecido periférico.

Mechanism of the analgesic action of dipyrone:

Involvement of CB1 and CB2 cannabinoid receptors in the peripheral tissue.

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Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do titulo de Doutor em Biologia Funcional e Molecular na área de Fisiologia.

Dissertation presented to the Institute of Biology of University of Campinas as a partial fulfillment of the requirement for the Ph.D. degree in Functional e molecular biology.

Orientador: Profº Dr. Carlos Amilcar Parada

Campinas, 2018

Agência(s) de fomento e nº(s) de processo(s): CAPES

Ficha catalográfica Universidade
Estadual de Campinas
Biblioteca do Instituto de Biologia Mara
Janaina de Oliveira - CRB 8/6972

D741m

Dos Santos, Gilson Goncalves, 1986-
Mechanism of the analgesic action of dipyrone : involvement of CB1 and CB2 cannabinoid receptors in the peripheral tissue / Gilson Goncalves dos Santos. – Campinas, SP : [s.n.], 2018.

Orientador: Carlos Amilcar Parada.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Dipirona. 2. Hiperalgesia. 3. Receptores canabinóides. 4. Receptores opióides. I. Parada, Carlos Amilcar, 1960-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Mecanismo de ação analgésica da dipirona : envolvimento dos receptores canabinóides CB1 e CB2 no tecido periférico

Palavras-chave em inglês:

Dipyrone
Hyperalgesia
Receptors, Cannabinoid
Receptors, Opioid

Área de concentração: Fisiologia
Titulação: Doutor em Biologia Funcional e Molecular

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Data de defesa: 19-06-2018
Programa de Pós-Graduação: Biologia Funcional e Molecular

Campinas, 02 de julho de 2018.

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Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

DEDICATÓRIA

Aos meus pais, Inês e Ivanilde, por todo amor, dedicação incansável e por serem precursores da minha vida, educação e maneira de ver o mundo.

Aos meus irmãos, fraternos e os que adquiri ao longo da minha jornada, que de um modo ou outro contribuíram para o meu crescimento.

Aos meus amigos da Casa F4 que me incomodaram muito, que estavam ao meu lado, que me escutavam nas preparações de seminários e desta defesa.

Ao professor Carlos Amilcar Parada, que foi orientador e amigo! Que viu em mim um potêncial a ser trabalhado e que não perguntou na primeira abordagem “de qual universidade pública você veio” mas perguntou “com o que você gosta de trabalhar”!

AGRADECIMENTOS

A meu orientador ***Prof. Dr. Carlos Amilcar Parada***, por ser uma pessoa acessível, humilde, pelo apoio, confiança e pela orientação;

Aos ***Amigos de Laboratório (LeD)*** que desde o estágio em 2012 me receberam bem, me apoiaram e me suportaram estes 6 anos (a reciproca também é verdadeira) !! Pelos momentos de cumplicidade e divertidos, que me ajudaram a amenizar as dificuldades diárias;

Ao ***Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)*** pela concessão da bolsa de mestrado e pelo apoio financeiro nesta pesquisa;

Aos ***professores do Departamento Biologia Funcional e Molecular***, pelas aulas ministradas e pelo exemplo e dedicação à pesquisa;

Aos **membros da banca examinadora desta tese**.

RESUMO

Contexto e objetivo: A dipirona é um analgésico utilizado no controle da dor moderada. É rapidamente metabolizada em dois metabolitos bioativos: 4–metil-aminoantipirina (4-MAA), por hidrólise, o qual é metabolizado em 4-aminoantipirina (4-AA), por reação enzimática. O objetivo deste estudo foi verificar a participação dos receptores canabinoides CB1 e CB2 e sua relação com os receptores TRPV1 e opióides do tecido periférico no efeito analgésico da dipirona, 4-MAA ou 4-AA. **Métodos:** PGE_2 (100 ng), carragenina (100 μg) ou capsaicina (0.12 μg) foram administradas na pata de ratos Wistar. A hiperalgesia mecânica induzida por carragenina foi avalidada por von-Frey eletrônico e a nocicepção avaliada pela contagem de flinches. Para investigar o envolvimento dos receptores canabinóides CB1, CB2, opióides e TRPV1, seus antagonistas (respectivamente, AM251, AM630, naloxona e capsazepina) foram administrados 30 min antes da dipirona, 4-MAA e 4-AA. Espectrometria de massa foi utilizada para analisar a hidrolise da dipirona para 4-MAA. O efeito do 4-AA no receptor TRPV1 foi analisado por ensaio de Ca^{2+} em neurônios do gânglio da raiz dorsal (DRG) mantidos em cultura. **Resultados:** Os resultados demonstraram que a hiperalgesia induzida por carragenina foi reduzida de maneira dose-dependente pelo tratamento local com dipirona ou 4-MAA. Os dados também demonstraram que a dipirona foi localmente metabolizada em 4-MAA no tecido subcultâneo. Administração local de AM630, Naloxona ou norBNI, mas não de Naltrindole ou CTOP, reverteu totalmente o efeito anti-hiperalgésico da dipirona ou do 4-MAA. A administração local de 4-AA reduziu a nocicepção e o aumento de $[Ca^{2+}]_i$ induzido por capsaicina em neurônios do DRG. O efeito anti-nociceptivo e a redução do $[Ca^{2+}]_i$ promovidos por 4-AA foram revertidos por AM251. PGE_2 aumentou a nocicepção induzida por capsaicina, a qual foi revertida por 4-AA. Em cultura de neurônios sensibilizados por PGE_2 o metabolito 4-AA induziu aumento $[Ca^{2+}]_i$ que foi revertido pela admininistração de capsazepina (antagonista de TRPV1). AM251 reduziu ambos, o $[Ca^{2+}]_i$ induzido por 4-AA e por capsaicina. **Conclusão:** Os resultados demonstram que a dipirona é localmente metabolizada em 4-MAA. Os dados sugerem que este metabólito promove analgesia pela interação entre o sistema canabinóide e opióide em particular pela ativação dos receptores CB2 e Kappa opioide. Por sua vez, o metabólito 4-AA reduziu a atividade das fibras TRPV1+ por dessensibilizarem os receptores TRPV1 via ativação dos receptores canabinóides CB1.

Palavras-chave: Dipirona; 4-metil-amianoantipirina; 4-aminoatipirina, receptores canabinoides, receptores opióides.

ABSTRACT

Background and aim: Dipyrone is an analgesic used to control moderate pain. It is metabolized in two bioactive metabolites: 4-methylaminoantipyrine (4-MAA) by hydrolysis and 4-aminoantipyrine (4-AA) by enzymatic reaction. The aim of this study was to investigate the involvement of peripheral cannabinoid CB1 and CB2 receptors and their relation with TRPV1 and opioid receptors, respectively, in the anti-hyperalgesic effect of Dypirone, 4-MAA or 4-AA. **Methods:** PGE2 (100 ng), carrageenan (100 µg) or capsaicin (0.12) were administered in the hind paw of male Wistar rats. The carrageenan-induced mechanical hyperalgesia was evaluated by electronic von-Frey and capsaicin-induced nociception was evaluated by counting flinches. To analyze the involvement of cannabinoid CB1, CB2, TRPV1 and opioid receptors their antagonist (respectively AM251, AM630, naloxone and capsazepine) were administered 30 min before dipyrone, 4-MAA or 4-AA. Mass spectrometry was used to analyse dipyrone's hydrolysis to 4-MAA. The effect of 4-AA on TRPV1 was analyzed by Calcium-influx assay in Dorsal root ganglian (DRG) keept in culture. **Results:** The results showed that the hyperalgesia induced by the inflammatory agent carrageenan was reduced in a dose dependent-manner by 4-MAA or dipyrone. Dipyrone was locally metabolized to 4-MAA in the subcutaneous tissue of hind paw. Local administration of AM630, Naloxone or norBNI but not of naltrindole or CTOP reduced the analgesic effect of dipyrone or 4-MAA. The local administration of capsaicin induced nociception and [Ca2+]i that was reduced by 4-AA. 4-AA reduces nociception and the calcium influx was reduced by AM251. PGE2 increases the capsaicin-induced nociception that was reversed by 4-AA. 4-AA increased [Ca2+]i in neurons sensitized by PGE2 that was reduced by capsazepine, a TRPV1 antagonist. AM251 reduced the [Ca2+]i induced by 4-AA or capsaicin. **Conclusion:** These results demonstrated that dipyrone is locally hydrolyzed to 4-MAA. Also, these data suggested that this metabolite induced analgesic effect through an interaction of cannabinoid and opioid system. 4-AA reduced the activity of the TRPV1+ (fiber C) by desensitization of TRPV1 through cannabinoid CB1 receptor. These data also suggested that the efficacy analgesic of dipyrone is increased in inflammatory conditions.

Keywords: Dipyrone; 4-methyl-amianoantipirine; 4-aminoatipirine, cannabinoid receptors, opioid receptors.

LISTA DE ABREVIATURAS

4-AA= 4-aminoantipirina

4-MAA= 4-Metil-aminoantipirina

AIEs= Anti-inflamatórios esteroidais

AINEs = Anti-inflamatórios não esteroidais

AMPc= Monofosfato cíclico de adenosina

Ca^{2+} = cálcio

CB1= Receptor Canabinoide 1

CB2= Receptor Canabinoide 2

COX= Ciclooxigenase

GMPc= Monofosfato cíclico de guanosina

h = hora

K^+= Potássio

K_{ATP}= Canais de Potássio sensíveis ao ATP

mRNA = Ácido ribonucléico mensageiro

Na^+=Sódio

NaCL = cloreto de sódio

ng= Nanograma

SNC = sistema nervoso central

SNP=Sistema nervoso periférico

SP = substância P

TRPV1= Receptor Vanilóide de potêncial transiente 1

µg = micrograma

µL=microlitro

SUMÁRIO

1. INTRODUÇÃO

1.1 Historico da dor

Embora o termo “dor” esteja melhor descrito e esclarecido atualmente, na história da humanidade esse termo passou por varias definições. Na pré-história a dor era entendida como sendo causada por “espíritos malignos” e o tratamento era feito por “xamãs”, “feiticeiros” e “curandeiros”, que exorcisavam o doente, além de utilizarem ervas, pedras, trepanações e “magias” para auxiliar na “cura”. O homem primitivo também interpretava a dor como resultado de “possessão”, assim empregava práticas religiosas como “sacrifício”, “exorcismo” o até mesmo a “sangria” para retirada desta “possessão” o que levaria ao alívio da dor (Bonica, 1990). Ainda Platão e, depois, Aristóteles viam a dor e o prazer como emoções. A partir de Descartes a dor deixa de ser uma experiência "espiritual" para ser compreendida como uma experiência física (Hardy, 2002).

Se analisarmos o étimo, a palavra “Dor” deriva do latim “*dolor*”, que significa punição ou pena. Nas civilizações antigas, o controle da dor era baseado em crenças já que nesta época a experiência de dor era derivada do coração, cérebro e preocupações religiosas. Nas epócas pre-aristotélicas, práticas como sangria e exorcismo eram utilizadas como métodos para o alívio da dor. Somente então quando os avanços científicos melhoraram a compreensão sobre a dor, tratamentos como álcool e ópio passaram a ser empregados (Hardy, 2002).

Dor pode ser definida como uma percepção desagradável associada à ativação de vias nociceptivas. Esta definição reconhece a dor como uma experiência, já que envolve as dimensões sensorial-discriminativa e afetivo-emocional (Merskey & Bogduk (Eds), 1994).

Diversos estudos têm demonstrado que a dor é um dos problemas sérios da nossa sociedade e tem levado a um aumento da busca pelo seu controle, nos diversos níveis de assistência à saúde, seja ele primário, secundário ou terciário (Andersson, 1999; Volinn et al 1991; Straker et al. 2012; Coggon et al., 1992). A dor tem efeitos direto no bem-estar e na qualidade de vida resultando em redução da função física e emocional (Niv & Kreitler, 2001). A dor inflamatória, dentre os vários tipos de dor é a mais comum, o que tem motivado o desenvolvimento de estudos que possibilitem a compreensão da dor inflamatória .

A dor inflamatória é decorrente da ação dos mediadores inflamatórios que induzem a sensibilização dos nociceptores. Termos como ‘hiperalgesia’ e ‘alodinia’ são usados para descrever esta sensibilização contudo em contextos diferentes (Julius, 2001; Millan, 1999).

A figura 1, é representativa destas duas condições demonstrando que em uma condição de injúria ocorre um deslocamento da curva para a esquerda.

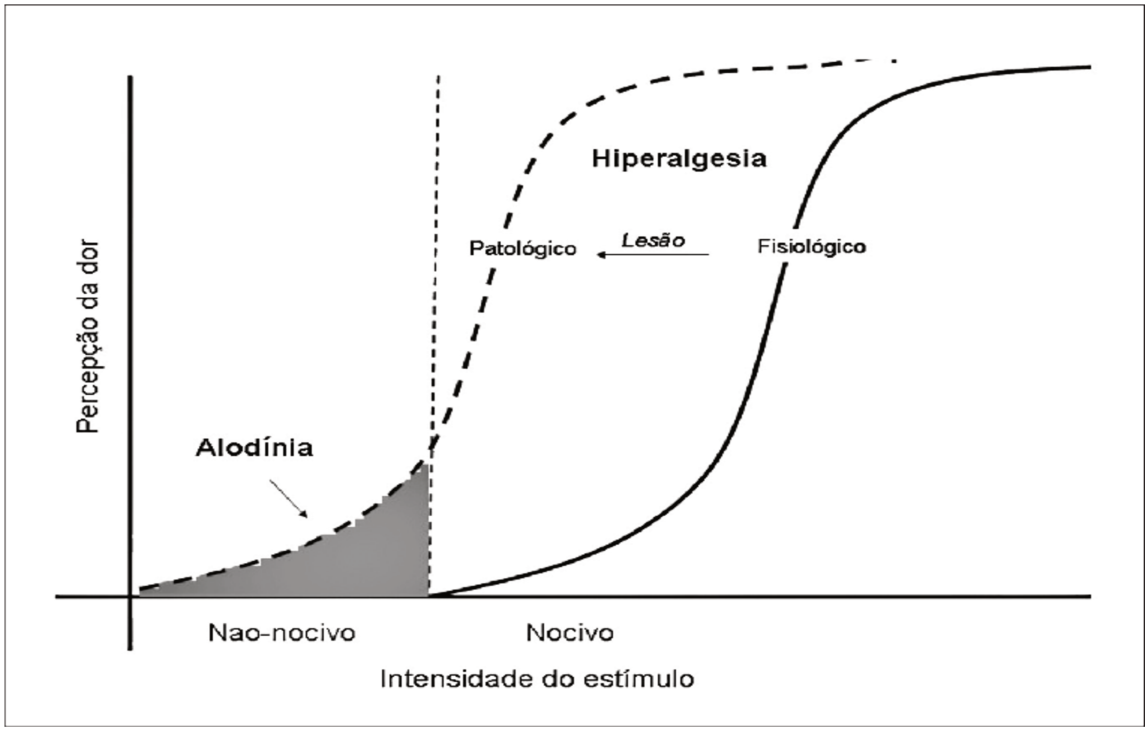

Fig.1. Adaptado de (Wood et al., 2014) A “hiperalgesia” e “alodinia” demonstradas a patir de um desvio para esquerda e proporcionalmente a intensidade do estímulo.

1.2 Hiperalgesia Inflamatória

Como previamente descrito durante um quadro inflamatório ou lesão tecidual mediadores inflamatórios são liberados levando a sensibilização dos nociceptores. A sensibilização dos nociceptores é caracterizada pela diminuição do limiar de excitabilidade neuronal de modo a facilitar a estimulação da via nociceptiva (Riedel & Neeck, 2001). Portanto a hiperalgesia inflamatória ocorre em resposta a um fenômeno neuronal no qual mediadores inflamatórios ativam receptores metabotrópicos e/ou canais iônicos dos nociceptores. Isto leva a uma ativação de vias de sinalização intracelular, deixando essas células mais próximas de seu limiar de excitabilidade (Hartmann et al., 2004).

Peptideos (ex. bradicinina), lipídeos (ex. prostaglandinas), neurotransmissores (ex. serotonina (5-HT) e ATP) e neurotrofinas (ex. NGF) são alguns dos mediadores inflamatórios finais que ativam receptores neuronais e a uma subsequente sensibilização neuronal. A ativação do nociceptor gera então potenciais de ação que são conduzidos da periferia ate o sistema nervoso central, onde então serão processadas e definidas pelo indivíduo como uma sensação desagradavel (Julius, 2001).

Entre os mediadores que levam a sensibilização neuronal, podemos citar os prostanóides que são os produtos finais da via do metabolismo do ácido aracdônico pela ação da enzima ciclo-oxigenase (COX). Os prostanóides atuam como mediadores locais em vários tecidos, em condições fisiológicas e fisiopatológicas (Narumiya et al., 1999). Desde o trabalho de Vane (1971), primeiro a relatar que drogas como a aspirina impedem o desenvolvimento de inflamação através do bloqueio da síntese de prostanóides, tem sido amplamente aceito que prostanóides estão envolvidos na dor, febre, edema, e vários aspectos da inflamação. Entre eles, a prostaglandina E_2 (PGE_2) é considerado como sendo o principal prostanóide pró-inflamatório e desempenha um papel importante no processamento nociceptivo na medula espinhal, assim como na periferia e no gânglio da raiz dorsal (Yaksh et al., 1999; Araldi et al., 2013).

Alguns modelos experimentais são empregados para analisar o envolvimento de receptores especificos na excitabilidade neuronal. Como esquematizado na Figura 2, a carragenina (agente inflamatório) induz a liberação de bradicinina a qual leva a liberação de ATP e ativação de duas vias distintas, a via das aminas simpatéticas e a via das prostaglandinas. Estes três últimos, ATP, aminas e prostaglandinas levarão a ativação de seus respectivos receptores

neuronais, culminando na ativação de vias de sinalização intracelular tendo como resposta final o aumento da excitabilidade neuronal (Levine et al., 1999; Ma et al., 2013).

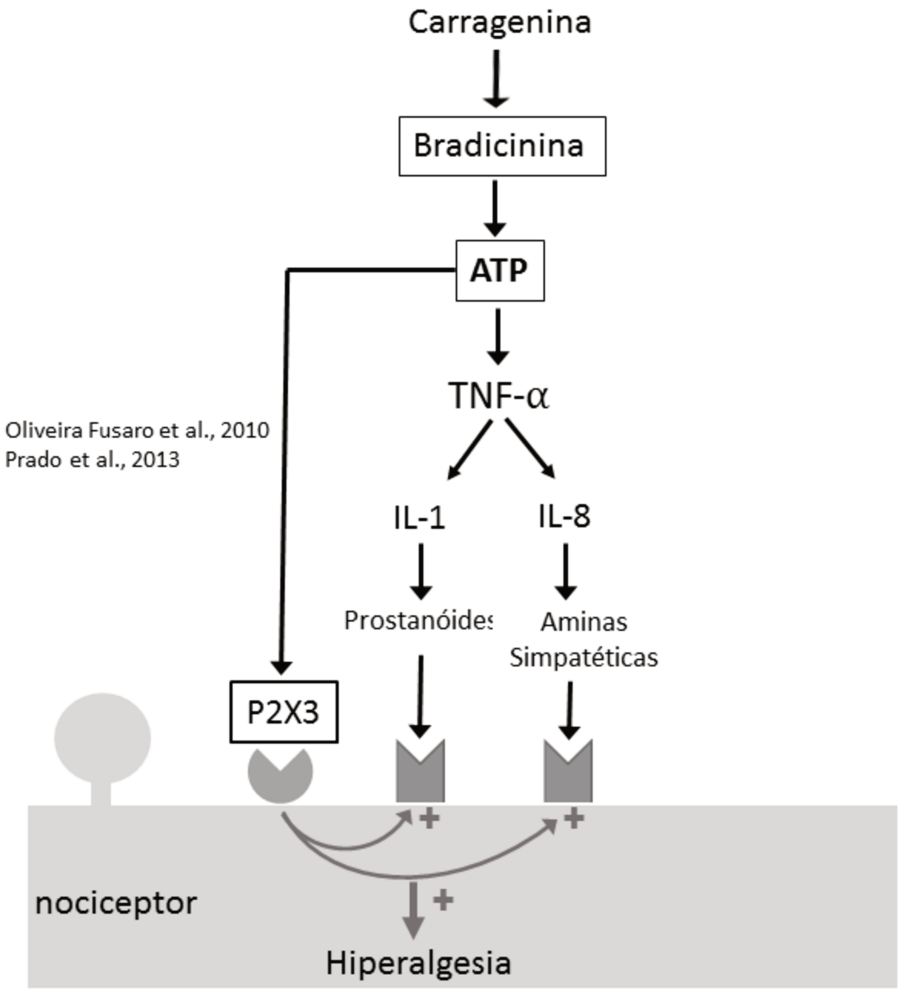

Figura.2. Hiperalgesia inflamatória induzida por carragenina. A administração de carragenina induz liberação bradicina e consequente liberação de de mediadores inflamatorios finais como prostanóides (PGE_2) e amina simpatomiméticas.

Enquanto a carragenina induz a liberação em casacata de mediadores inflamatórios que induzem a sensibilização neuronal, o modelo de nocicepção induzida por capsaicina leva a ativação direta de receptores TRPV1 em fibras nociceptivas do tipo C. A capsaicina é um agonista de TRPV1 e a ativação deste receptor induz aumento da concentração intracelular de cálcio, levando assim a despolarização neuronal. Os eventos moleculares envolvidos (ativação de receptores TRPV1, entrada de cátions, despolarização de fibras C) são bem mais compreendidos do que aqueles envolvidos em outros modelos de dor, onde vários mediadores químicos estão envolvidos (Meisner et al., 2006)

Estes modelos são ferramentas para estudar respostas inflamatórias, a reversão ou prevenção dessas respostas. De fato, o uso destes modelos experimentais tem permitido a compreesão do mecanismo de ação de drogas, como no exemplo da aspirina, que atua impedindo o desenvolvimento da inflamação através do bloqueio da síntese de prostanóides (Vane, 1971).

1.3 Controle Farmacológico da Dor

O tratamento inadequado da dor aguda pode ser um gatilho para o desenvolvimento de dor persistente e crônica, evidenciando assim a importância de um tratamento rápido e efetivo desse processo (Dahl & Møiniche, 2004). Existem várias abordagens terapêuticas famacológicas para o tratamento da dor aguda incluindo o uso de analgésicos como a dipirona, acetaminofeno (paracetamol), antiinflamatórios não esteroidais (AINEs) e opioides (Abbott & Hellemans, 2000; Edwards et al., 2010)

Embora a dor tenha seu papel fisiológico protetivo, quando a mesma se instala torna-se um problema clínico, fazendo-se necessário a intervenção farmacológica para preservar a saúde e qualidade de vida do indivíduo. Ainda, a intervenção farmacológica é importante por que a dor impõe limitações em níveis sociais bem como em níveis econômicos (Phillips & Harper, 2011; Taylor, 2011).

Os anti-inflamatórios não esteroidais (AINEs), tais como ácido acetilsalicílico e ibuprofeno controlam a dor e a inflamação por inibirem a atividade da ciclo-oxigenase (COX). Como bem conhecido a COX é responsável pela formação de mediadores inflamatórios finais, que levam a sensibilização do nociceptor. Portanto, a inibição de COX impede assim a sensibilização neuronal (Mubagwa et al., 2012). Já os anti-inflamatórios esteroidais (AIEs) tem suas principais ações sobre o processo inflamatório por inibir a enzima fosfolipase A2 cuja inibição diminui a concentração de ácido araquidônico para ser processada pela COX. Além desta ação, os AIEs podem inibir a transcrição dos genes de citocinas inflamatórias como Interferon gama, fator de necrose tumoral e a expressão de COX. Por estas ações, os AIEs também estão na lista de drogas imunossupressoras (Balbino et al., 2011; Bateman et al., 2012; Bovill et al., 1997; Koppala et al., 2010).

Outra classe de fármaco importante que induzem analgésia são os opióides, que atuam por uma modulação direta pelo bloqueio de canais iônicos presentes na membrana celular do

nociceptor, levando a hiperpolarização. Ainda, estes receptores, na membrana celular, são acoplados a proteína G. Quando estimulados por um fármaco opióide, ocorre a inibição da enzima adenilato ciclase, reduzindo o nível intracelular de adenosil monofosfato cíclico (AMPc). Com isso há o fechamento de canais de cálcio voltagem dependente nas terminações nervosas pré-sinápticas, reduzindo a liberação de neurotransmissores, porem não os canais de K^+ na membrana pós-sinaptica. Isso causa uma hiperpolarização desse neurônio, bloqueando assim o estimulo doloroso (Jordan & Devi, 1999). Drogas como morfina e a dipirona ainda atuam por ativação da via L-arginina-GMPc-K_{ATP} induzindo a hiperpolarização neuronal (Dos Santos et al., 2014; Duarte & Ferreira, 1992).

Apesar de seus benefícios AINEs, AIEs e ópióides possuem efeitos colaterais indesejados. Por exemplo, os AINEs estão relacionados com o desenvolvimento de graves problemas gástricos e cardíacos devido ao bloqueio das enzimas COX-1 e COX-2, respectivamente (Cunha et al., 2008). Os AIEs, como os corticosteroides, podem causar imunossupressão, aumentando a possibilidade de infecções. Podem ainda, em altas doses, provocar pode provocar transtornos do humor, distúrbios de comportamento e reações psicóticas (Bovill et al., 1997; Koppala et al., 2010). Os opióides, por sua vez, estão diretamente relacionados com efeitos adversos como depressão do sistema imunológico, constipação, tolerância e dependência (Manchikanti & Singh, 2008; Volkow & McLellan, 2016).

Assim a dipirona surge como uma alternativa visto que é um analgésico potente largamente utilizado na prática médica no controle da dor, apresentando poucos efeitos colaterais, baixo custo e alta eficácia. Dessa forma evidencia-se a importância de uma melhor compreensão acerca de seu mecanismo de ação visando fornecer possíveis alvos para o tratamento e controle da dor (Calixto et al., 1998; Levy et al., 1998; Edwards et al., 2010).

1.4 Dipirona e seus metabólitos bioativos

Na Alemanha em 1922, desenvolveu-se um fármaco, descrito inicialmente como anti-inflamatório, conhecido como Dipirona (Metamizol). Devido a sua eficácia e baixa toxicidade, tornou-se um medicamento muito usado mundialmente no controle da dor (Edwards et al., 2010). Anos após o início de sua sua comercialização, a dipirona foi associada a agranulocitose e banida em alguns países ou teve seu uso restrito para a modalidade "uso sob prescrição médica" (Chetley, International, & Drugs, 1999; Edwards et al., 2010; Schönhöfer, Offerhaus, & Herxheimer, 2003).

Embora não exista nenhuma comprovação científica sistematizada de que a dipirona induza agranulocitose, estudos mostram que uma possível incidência de agranulocitose relacionada ao uso de dipirona seria de 56 casos por 30 milhões de exposições, ou seja, 1,5 caso por milhão, o que, em termos de saúde pública, pode ser considerado um risco muito baixo (Editorial – DOL, março 2012; Frederic et al., 2008).

Como demonstrado na figura 3, a dipirona é hidrolisada para 4-metilaminoantipirina (4-MAA), que no fígado é metabolizado em 4-formilaminoantipirina (4-FAA), 4-aminoantipirina (4-AA) e 4-acetilaminoantipirina (4-AAA). Os metabólitos, 4-MAA e 4-AA, são considerados bioativos, uma vez que os mesmos possuem atividade analgésica e são encontrados no plasma após a sua administração (Dos Santos et al., 2014; Pierre et al., 2007; Rogosch et al., 2012).

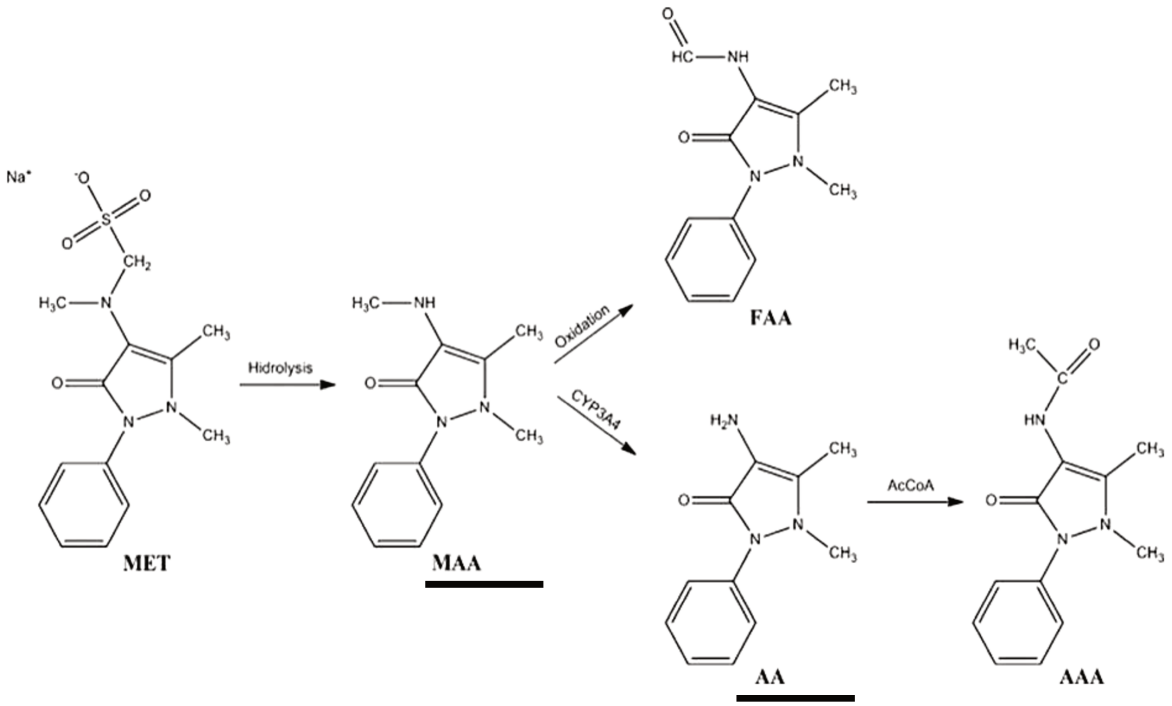

Figura. 3. Metabolização da dipirona (MET) para os metabolitos bioativos MAA e AA (respectivamentes descritos neste trabalho descrito como 4-MAA e 4-AA). Adaptado de Rogosch et al. (2012).

Apesar da dipirona ser comercializada há quase 100 anos, o seu mecanismo de ação ainda não é completamente esclarecido, sendo assim é pláusivel pensar que o fato da dipirona ter sido banida de alguns países ou mesmo ter seu uso restrito, tenham contribuido para tal fato. Embora, inicialmente a dipirona tenha sido comercializada como anti-inflamatório, hoje sabe-se que a mesma possui mecanismos de ação independente da síntese de PGE_2 (Dos Santos et al., 2014; Lorenzetti, & Ferreira, 1992; Ferreira, 1985; Pierre et al., 2007).

Ainda, dados da literatura mostram que a dipirona induz analgesia pela ativação da via L-arginina-NO-K_{ATP} (Alves & Duarte, 2002; Duarte et al., 1992; Lorenzetti & Ferreira, 1985). Dados mais recentes do nosso laboratório atribuem a ativação da mesma via para o metabólito 4-MAA, que é formado pela hidrólise da dipirona através do metabolismo de primeira passagem (Dos Santos et al., 2014). Contudo, esta não parece ser a única via analgésica da dipirona, pois outros trabalhos tem demonstrado envolvimento de receptores canabinóides e opióides (Maione et al., 2015; Rogosch et al., 2012). De fato, resultados do nosso laboratório demonstram que apenas o metabólito 4-AA possui um efeito analgésico dependente do receptor canabinóide CB1 em modelo de hiperalgesia induzida por PGE_2 (Dos Santos et al., 2014). Embora no modelo de hiperalgesia induzida por PGE_2 não tenhamos observado envolvimento o de CB2 no efeito

analgésico da dipirona (Figura 5, Dos Santos et al., 2014) é importante considerar que receptores CB2 são marjoritariamente expressos em células imunes (Piomelli et al ., 2003; Ibrahim et al., 2005), assim modelos experimentais de dor inflamatória que induz migração de células para o sitio da inflamação podem ser mais pertinentes contribuir para demonstrar um possível papel do receptor CB2 na ação analgésica da dipirona.

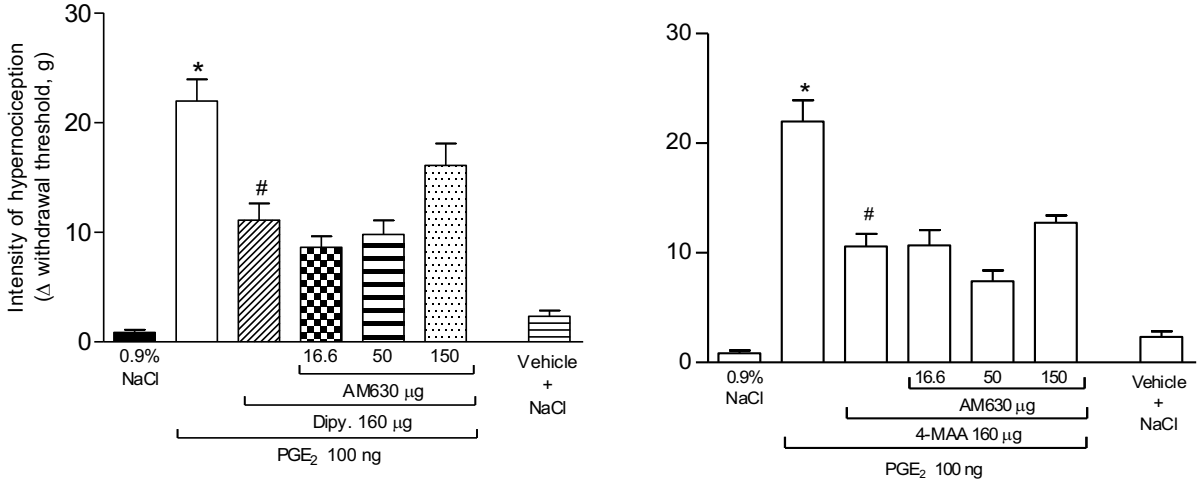

Figura 4. O efeito anti-hiperalgésico da dipirona ou 4-MAA na hiperalgesia induzida por PGE_2 não envolveativação do receptor canabinóide tipo CB2. Antagonista do receptor canabinóide CB2 (AM630), Prostaglandina E2 (PGE_2), Dipy (Dipirona) e 4-metil-aminoantipirina (4-MAA) (extraído de Dos Santos et al., 2014).

1.5 Interação dos receptores canabinoides tipo 1 (CB1) e TRPV1 na modulação da dor

Dados da literatura têm demonstrado que receptores CB1 são amplamente expressos em diversas estruturas encefálicas, medula espinal , gânglios da raiz dorsal e neurônios nociceptivos aferentes primários (Ahluwalia et al., 2000; Engel et al., 2011; Fioravanti et al., 2008). Assim, a modulação direta dos receptores CB1 nos nociceptores tem sido estudada para o controle da dor (Ahluwalia et al., 2000; Howlett et al., 2010; Sacerdote et al., 2000). Estudos tem demonstrado que ativação do receptor CB1 possui um efeito inibitório sobre os receptores TRPV1 (Mahmud et al., 2009; Sántha et al., 2010).

Por outro lado, a ativação de CB1 também apresenta um papel excitatório sobre os canais iônicos TRPV1 (Mahmud et al., 2009; Sántha et al., 2010). De fato a atividade constitutiva do receptor CB1 está relacionada com o processo de dessensitização dos receptores TRPV1,

sobretudo em condições inflamatórias. Contudo, ambas condições contribuem para analgesia, já que a inibição do TRPV1 diminui a despolarização neuronal e o papel excitatorio do receptor CB1 sobre o receptor TRPV1 induz aumento da concentração de cálcio intracelular levando à dessensitização do receptor TRPV1, provavelmente por uma hiperatividade (Chen et al., 2016; Vásquez et al., 2003; Di Marzo et al., 2014; Mackie et al., 1995).

O receptor CB1, é um receptor transmembrana acoplado a proteína Gi, e a sua ativação leva a diminuição da atividade das proteino-kinases (PKA e PKC). Estas proteína kinases PKA ou PKC podem induzir a fosforilação de TRPV1 o que contribui para o aumento de correntes de cálcio induzido por capsaicina. Por outro lado, estas mesmas proteino quinases podem induzir a ativação neuronal da fosfatase dependente de Ca^{2+} (calcinerina) que desfosforilam o receptor. Este efeito da calcinerina estaria envolvido na desensitização do receptor. Assim, outros estudos tem demonstrado o envolvimento da CamMKII na desensitizacao de TRPV1. De fato, o complexo CaM/Ca^{2+} tem sido identificado por ligar ao dominio terminal COOH do receptor TRPV1 uma regiao determinante para desensitizacao do receptor (Numazaki et al., 2003; Gordon et al ., 2004).

1.6 Interação entre o sistema canabinóide e receptores opióides na modulação da dor

Receptores opióides, assim como os receptores canabinóides, são um grupo de receptores acoplados à proteína G, expressos no sistema nervoso central e periférico. Os efeitos analgésicos induzidos pela ativação dos receptores opióides involvem à hiperpolarização neuronal devido à abertura de canais de potássio do tipo K_{ATP} (Duarte et al., 2003). Ainda, estes receptores quando estimulados diminuem a atividade da enzima adenilato ciclase isto leva há uma redução o nível intracelular de adenosil monofosfato cíclico (AMPc) o que leva ao fechamento de canais de cálcio voltagem dependente nas terminações nervosas pré-sinápticas, reduzindo a liberação de neurotransmissores (Jordan & Devi, 1999).

Estudos tem sugerido uma interação entre os sistemas canabinóide e opióide no controle da dor. Receptores canabinoides do tipo 2 (CB2) são receptores acoplados à proteína G e predominantemente expressos em células não neuronais, tais como células do sistema imunológico e queratinócitos (Freund et al., 2003; Ashton & Glass, 2007; Machado et al., 2014).

Estudos comportamentais e moleculares demonstraram que o efeito anti-hiperalgésico induzido pela ativação do receptor CB2 envolve a liberação de opióide endógeno (Ibrahim et al., 2005) e consequentemente a ativação da via L-arginina-NO-cGMP-K_{ATP} nos neurônios nociceptivos periféricos (Negrete et al., 2011), reforçando a hipótese interação entre estes dois sistemas. Ainda, leucócitos também têm sido apontados como células importantes na modulação da dor por serem células imunes que são precursores de dinorfina, a qual também atua em receptores opióides nas terminações periféricas de neurônios aferentes primários (Stein et al., 2010; Stein & Lang, 2009).

Portanto, a interação entre os receptores CB2 e opióides nos tecidos periféricos podem representar uma nova estratégia terapêutica para o controle da dor inflamatória com reduzidos efeitos indesejáveis quando comparados ao uso de anti-inflamatórios ou opioides exógenos.

II – PROPOSIÇÃO

Considerando que: (1) Um provável envolvimento de receptores canabinóides no efeito da dipirona (Rogosch et al., 2012). (2) O efeito anti-hiperalgésico da dipirona (Duarte et al., 2003) ou do 4-MAA (Dos Santos et al., 2014) depende da ativação da via L-arginina-NO-cGMP-K_{ATP}. (3) O efeito anti-hiperalgésico induzido pela ativação do receptor CB2 envolve a liberação de opióide endógeno e também da ativação da via L-arginina-NO-cGMP-K_{ATP} (Negrete et al., 2011). O primeiro objetivo deste estudo foi investigar o envolvimento do receptor CB2 e receptores opióides no efeito analgésico da dipirona e 4-MAA na hiperalgesia inflamatória induzida por carragenina.

Considerando que: (1) O efeito anti-hiperalgésico do metabólito 4-AA depende da ativação de receptor CB1 (Dos Santos et al, 2014). (2) A ativação do receptor CB1 induz um efeito inibitório e ou excitatório sobre os receptores TRPV1 (Cheng et al., 2016). O segundo objetivo deste trabalho foi investigar o envolvimento do receptor TRPV1 no efeito anti-hiperalgésico do 4-AA e se sim, verificar uma possível interação entre os receptores CB1 e TRPV1 neste efeito.

3.1 Objetivos Específicos

I. Investigar o envolvimento do receptor CB2 e dos receptores canabinóides no efeito analgésico da dipirona e 4-MAA na hiperalgesia inflamatória induzida por carragenina. Portanto foi analisado:

- O efeito anti-hiperalgésico da dipirona e 4-MAA sobre a hiperalgesia inflamatória induzida por carragenina.
- O envolvimento do receptor CB2 no efeito analgésico da dipirona e do 4-MAA em modelo de hiperalgesia induzido por carragenina.
- O envolvimento dos receptores opióides no efeito analgésico da dipirona e do 4-MAA em modelo de hiperalgesia induzido por carragenina.

II. Investigar o envolvimento do receptor TRPV1, bem como a interação do receptor canabinoide CB1 com TRPV1 no efeito analgésico do 4-AA. Assim, analisamos:

- O efeito analgésico do 4-AA na nocicepção induzida por capsaicina.
- O envolvimento do receptor cannabinoide CB1 no efeito analgésico do 4-AA.
- O efeito da PGE_2 na nocicepçao induzida por capsaicina

- O efeito do 4-AA em cultura de neurônios do DRG sensibilizados com PGE_2.
- O envolvimento do receptor canabinóide CB1 no efeito analgésico do 4-AA em cultura de neurônios do DRG sensibilizados com PGE_2.

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

III – CAPÍTULO 1

O presente artigo foi submetido no periódico “European Journal of Pharmacology”.

Dipyrone is locally hydrolyzed to 4-methylaminoantipyrine and its analgesic effect depends of CB2 and Kappa opioid receptors activation

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Abstract

Dipyrone is an analgesic pro-drug that has been used in clinical to control moderate pain due to its high analgesic efficacy and low toxicity. Dipyrone is hydrolyzed to 4-methylaminoantipyrine (4-MAA) which is metabolized to 4-aminoantipyrine (4-AA). The aim of this study was to investigate the involvement of peripheral cannabinoid CB2 and opioid receptors activation in the local anti-hyperalgesic effect of dipyrone or 4-MAA. The inflammatory agent, carrageenan was administered in the hindpaw of male Wistar rats, and the mechanical nociceptive threshold was quantified by electronic von Frey test. Dipyrone or 4-MAA was locally administered 2.5 hours after carrageenan. The hindpaw's tissue was harvested after dipyrone injection and its hydrolysis to 4-MAA was analyzed by mass spectrum. The selective CB2 receptor antagonist, AM630, naloxone a non-selective opioid receptor antagonist, nor-BNI a selective κ-opioid receptor, CTOP a selective μ-opioid receptor or naltrindole a selective δ-opioid receptor was administered 30 min before 4-MAA. The results demonstrated that carrageenan-induced mechanical hyperalgesia was inhibited by dipyrone or 4-MAA in a dose-response manner. Dipyrone administered in the hindpaw was completely hydrolyzed to 4-MAA. The anti-hyperalgesic effect of 4-MAA was completely reversed by AM630, naloxone and nor-BNI, but not by CTOP or naltrindole. These data suggest that the local analgesic effect of dipyrone is mediated by its hydrolyzed bioactive form, 4-MAA and, at least in part, depends on CB2 receptor and opioid kappa receptor activations. In conclusion, the analgesic effect of dipyrone may involve a possible interaction between the cannabinoid and opioid system in the peripheral tissue.

Keywords: Dipyrone; 4-methylaminoantipyrine; cannabinoid receptors; opioid receptor.

1. Introduction

Dipyrone is an analgesic drug that has been used in clinical practice in some countries for a more than ten decades due to its high analgesic efficacy and low toxicity. However, its analgesic mechanism of action is not completely understood. As a pro-drug, dipyrone is characterized by fast hydrolysis to 4-methylaminoantipyrine (4-MAA) (Pierre et al., 2007), which is then metabolized to 4-formylaminoantipyrine (4-FAA), 4-aminoantipyrine (4-AA), and 4-acetylaminoantipyrine (4-AAA). After oral administration of dipyrone two metabolites are found in human plasma and cerebrospinal fluid with the same analgesic effect than dipyrone, 4-MAA and 4-AA. For that reason they are called dipyrone bioactive metabolites (Pierre et al., 2007; Rogosch et al., 2012).

Initially, the analgesic effect of dipyrone was associated to its ability in reducing prostaglandins synthesis. However, it has been demonstrated that dipyrone inhibits the hyperalgesia induced by prostaglandin showing that the analgesic effect of dipyrone does not depend on prostaglandins synthesis (Lorenzetti & Ferreira, 1985). Also, like dipyrone, we have recently demonstrated that 4-MAA induces anti-hyperalgesic effect by the activation of L-argenine-NO-cGMP-K_{ATP} pathway (Dos Santos et al., 2014). Moreover, recent data have suggested an involvement of cannabinoids and opioids receptors activation in the analgesic effect of dipyrone (Rogosch et al., 2012; Silva et al., 2016; Vazquez et al., 2005).

Cannabinoids receptors, CB1 and CB2, are G-protein-coupled receptors (GPCRs). CB1 are expressed primarily in the central and peripheral nervous system and CB2 are most expressed in immune cells and keratinocytes (Ahluwalia et al., 2000; Maione et al., 2015; Yang et al., 2013). The anti-hyperalgesic effect of CB1 receptor activation is mediated by acting directly in the nociceptor (Ahluwalia et al., 2000). On the other hand, the anti-hyperalgesic effect of CB2 activation leads to release of endogenous opioids and subsequently neuronal opioids receptors activation (Ahluwalia et al., 2000; Ashton and Glass, 2007).

Likewise, opioids receptors are a group of G protein-coupled receptors expressed in central and peripheral nervous system (Ji et al., 1995). Delta (δ), kappa (κ) and mu (μ) opioid receptors can be activated by endogenous opioids, respectively encephalin, dynorphin and endorphin. Moreover, an interaction between cannabinoid and opioid systems in pain management has been suggested (Machado et al., 2014; Negrete et al., 2011). In fact, behavioral and molecular studies have demonstrated that activation of CB2 receptor induced release of

dynorphin-A and subsequently κ-opioid receptor activation (Machado et al., 2014). Therefore, the aim of this study was to verify the involvement of peripheral cannabinoid CB2 and opioids receptors in dipyrone and 4-MAA's analgesic effect.

2. Materials and methods

2.1 Animals

A total of 180 male Wistar rats weighing 150-250 g, 4-6-weeks-old were obtained from the Multidisciplinary Center for Biological Research (CEMIB - UNICAMP, SP, Brazil). Experimental protocols were approved by Ethics Committee for Animal Research of the State University of Campinas (CEUA, protocol number: 3372-1). The animals were housed in plastic cages with food (commercial chow for rodents) and filtered water available *ad libitum*. Testing sessions took place during the light phase (09:00 AM - 5:00 PM) in a quiet room maintained at 23º C. All experiments were conducted according to the IASP guidelines for the use of laboratory animals (Zimmermann, 1983) and the Brazilian Society of Laboratory Animal Science (SBCAL). All efforts were made to minimize both the stress of rats and the number of animals per group.

2.2 Drugs and doses

The following drugs were used: PGE_2 (100 ng/paw); carrageenan (100 ng/paw); AM630 a selective cannabinoid CB2 receptor antagonist (16.6, 50 and 150 µg/paw, Machado et al., 2014; L C R Silva, Romero, Guzzo, & Duarte, 2012), dipyrone (8, 80, 160 and 320 µg/paw, (Alves and Duarte, 2002; Vivancos et al., 2004); 4-MAA (4-methylaminoantipyrine; 8, 80, 160 and 320 µg/paw (Dos Santos et al., 2014); Naloxone a non-selective opioid receptor antagonist (2, 10 and 20 µg/paw (Napimoga et al., 2008); NorBNI a selective κappa opioid receptor antagonist (5, 10 and 50 µg/paw (Silva et al., 2016); Naltrindole a selective δ opioid receptor (1, 3 and 9 µg/paw (Ferreira et al., 2017); CTOP a selective mu opioid receptor antagonist (8, 20 and 32 µg/paw, (Ferreira et al., 2017). AM630 and Naltrindole were dissolved in propylene glycol and 10% DMSO; the other drugs were dissolved in 0.9% NaCl. All drugs were obtained from Sigma-Aldrich (MO, USA), except 4-MAA that was obtained from TLC-USA.

2.3 Subcutaneous injection

Drugs or vehicle were injected subcutaneously in the rat hindpaw (intraplantar surface) with aid of a BD *Ultra-Fine ® (30 gauge)* needle of insulin. The animals were quickly contained and the volume of 50 µl was injected.

2.4 Nociceptive paw electronic pressure-meter test: von Frey test

Rats were placed 15–30 min before testing in acrylic cages (12 × 20 × 17 cm) with a wire grid floor. During this adaptation period, the paws were tested around 2–3 times. The test consisted of inducing a hindpaw flexion reflex with a hand-held force transducer (Insight, Ribeirao Preto – Brazil) coupled with a 0.5 mm^2 polypropylene tip. Below the grid a titled mirror provided a clear view of the rat's hindpaw. The investigator applyed the tip between the five distal footpads increasing gradually the pressure. The maximal applied force was 80 g. The stimulus was repeated around to six times, but usually, three times until the animal has presented three similar measurements. Animals that did not present a consistent response were discarded. The measurement of the pressure (calibrated in grams) was automatically recorded when the paw was withdrawn. The hyperalgesia is presented as Δ withdraw threshold (intensity of hyperalgesia), in other words results were obtained by subtracting the basal values (before injections) from those obtained after treatment.

2.5 Mass spectrometry

2.5.1 ESI-MS

High performance liquid chromatography (HPLC) -grade methanol was purchased from Burdick & Jackson (Muskegon, MI). Solution of dipyrone was diluted in methanol (1:1000) and and injected with 3.0 µL min-1 of flow rate on Q-ExactiveTM (Thermo Scientific, Germany), mass spectrometer with orbitrap analyzer, via ESI, the experiments was performed in the positive mode.

2.5.2 DESI-MSI

The frozen tissue were sliced using a Leica CM 1900 cryostat microtome (Leica Biosystems, Nussloch, Germany), under -20° C, and each slice were cut at a thickness of 14 µm.

Tissue sections were transferred by thaw mounting onto conventional microscope glass slides without any surface treatment and were stored at −80 °C until the analysis.

The analyses were performed in a Q-Exactive® (Thermo Scientific, Germany) a hybrid Quadrupole-Orbitrap mass spectrometer with a resolution of 75,000 at *m/z* 400 coupled with a source DESI-2D platform of Prosolia® (model OS-3201) for data acquisition. The data was converted in image by Firefly v.1.3.0.0, the treatment were performed in BioMAP software (version 3.8.04). The DESI configuration was optimized, 55° spray angle, 5.0 kV spray voltage, 160 psi N_2 nebulizing gas pressure and a sprayed solvent of methanol (HPLC grade) at a 1.5 μL min^{-1} flow rate, in positive mode. Images were collected from m/z 100−1200 with a step sized of 200 μm, a scan rate of 740 μm s−1, and a pixel size of 200 μm × 200 μm.

2.6 Statistical analysis

To determine if there were differences between groups, one-way ANOVA or unpaired *t*-test was performed, and values of $p<0.05$ were considered statistically significant. The statistical analysis of the results obtained was performed in the GraphPad Prism v7.00 for Windows (GraphPad Software). The results were presented as the mean ± S.E.M of six rats per group. The differences between the groups were compared using one-way ANOVA to obtain the degree of significance, followed by the Tukey multiple comparison test to compare the groups and doses (behavioral experiments).

3 Results

3.1 Local administration of dipyrone or 4-methylaminoantipyrine (4-MAA) in peripheral tissue reduces the carrageenan-induced hyperalgesia.

Local administration of carrageenan (100 μg/paw) in subcutaneous tissue of rat hindpaw induced mechanical hyperalgesia measured 3 hours after its injection. Dipyrone or 4-MAA (all 8; 80; 160 or 320 μg/paw), was administered at the same site 2.5 h after carrageenan. As shown in figure1 (panel A and B), dipyrone or 4-MAA (80, 160 and 320 μg/paw) decreased the mechanical hyperalgesia in a dose-related manner (one-way ANOVA, Tukey test, $P<0.05$). The administration of dipyrone (160 μg/paw) or 4-MAA (160 μg/paw) in the contralateral hindpaw did not change the mechanical withdrawal threshold, ruling out their systemic effect. This dose was used in the following experiments.

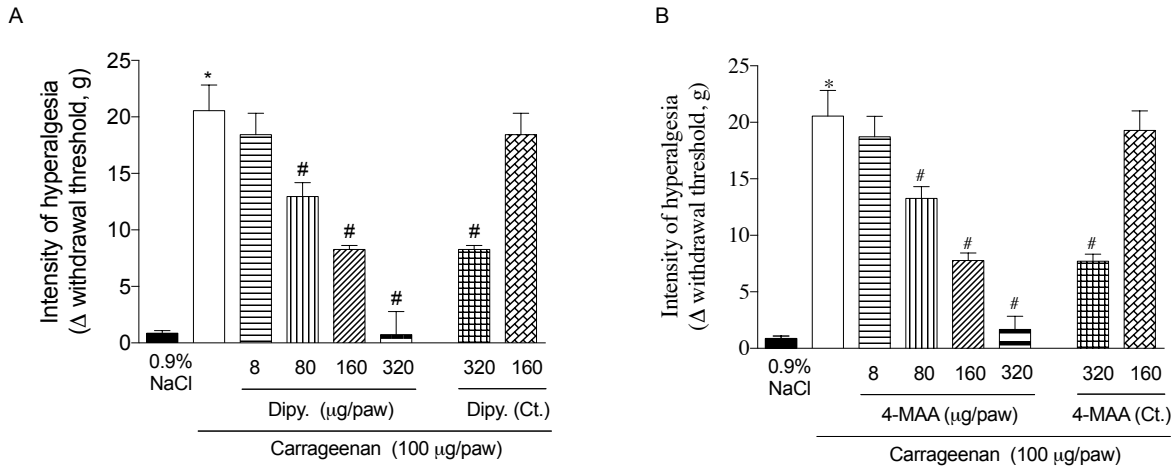

Figure 1. *Local administration of dipyrone or 4-methylaminoantipyrine (4-MAA) in peripheral tissue reduces the Carrageenan-induced hyperalgesia.* Dipyrone (Dipy) or 4-MAA administered in the hindpaw 2.5 h after carrageena inhibited in a dose-dependent manner the mechanical hyperalgesia evaluated 30 min later (respectively Fig. **1A**, **1B**). Dipyrone or 4-MAA (160 μg/paw) but not (320 μg/paw), administered in contra-lateral paw (Ct) did not change the mechanical withdrawal threshold, ruling out its systemic effect. The symbol "*" means different from control group (0.9% NaCl administration; 50 μL), "#" means different from carrageenan group (one-way ANOVA, Tukey test, $P<0.05$).

3.2 Dipyrone is hydrolysate to 4-MAA in the hindpaw.

Because 4-MAA has the same action of dipyrone to the same dose, we performed an assay to analysis whether dipyrone is metabolized to 4-MAA in the peripheral tissue. As shown in figure 3, the mass spectrum showed two noticed peaks, the first at m/z = 218.12843 (1.65 ppm of error)

and the second *m/z* = 240.11021 (2.16 ppm of error). These ions were assigned to the product of hydrolysis of the dipyrone, the 4-MAA $[4\text{-MAA+H}]^+$ and the adduct of the 4-MAA with Na, $[4\text{-MAA+Na}]^+$ respectively. No signal of dipyrone (MW: 333 g/mol) was found in the mass spectrum, neither protonated nor sodiated. This result demonstrated that dipyrone was hydrolyzed to 4-MAA, when solubilized in water.

As shown in figure 2, panel B and C, the distribution of 4-MAA in hindpaw subcutaneous tissue after dipyrone administration was analyzed by DESI-MSI. The dipyrone administration in the subcutaneous tissue of hindpaw distributed in $[4\text{-MAA+H}]^+$ (figure 2B) and $[4\text{-MAA+Na}]^+$ (figure 2C) in a similar tissue area. No dipyrone was found distributed in hindpaw tissue.

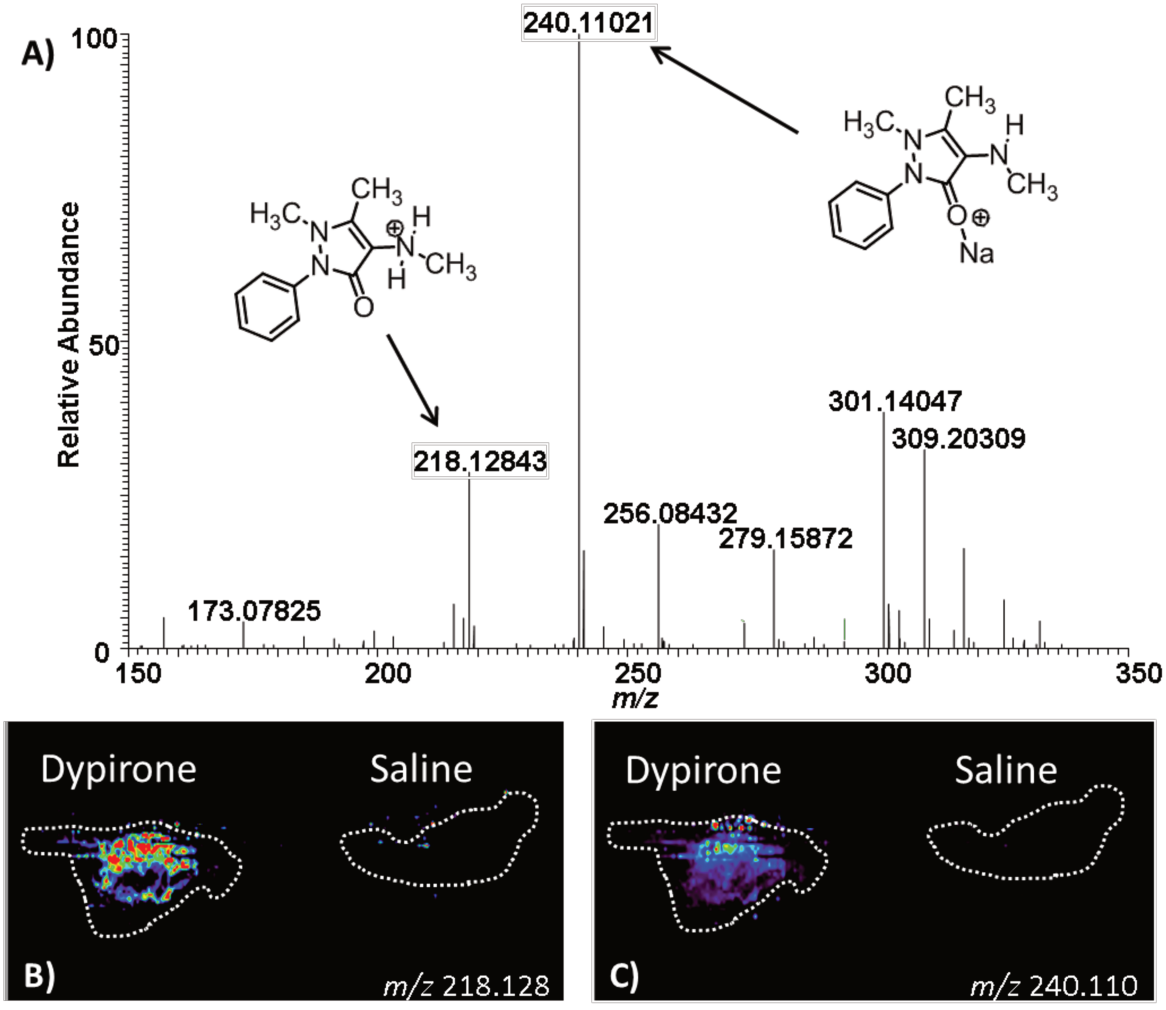

Figure 2. *Dipyrone is hydrolysate to 4-MAA in the hindpaw*.(3A) In Mass spectrum the presence of two peaks can be noticed, the first at 218 m/z (1.65 ppm) and the second 240 m/z (2.16 ppm). These peaks were assigned to the molecular peak of 4-MAA $[4\text{-MAA+H}]^+$ and the adduct of the

4-MAA with Na, $[4\text{-MAA+Na}]^+$ respectively. No signal of dipyrone (MW: 333 g/mol) was found in the mass spectrum.

3.3 The anti-hyperalgesic effect of 4-methylaminoantipyrine is mediated by CB2 and opioid receptor activation.

Once only 4-MAA was found in the peripheral tissue after local administration of dipyrone, the following experiments was performed using only 4-MAA. Local administration of carrageenan (100 µg/paw) in subcutaneous tissue of rat hindpaw induced mechanical hyperalgesia that was reduced by 4-MAA (160 µg/paw). To verify whether the analgesic effect of 4-MAA is mediated by CB2 or opioid receptors activation a selective CB2 receptor antagonist (AM630) or Naloxone an opioid non-selective antagonist were administered 30 minutes before 4-MAA. As shown in the figure 3 (panel A and B), AM630 (16.6, 50 and 150 µg/paw) and Naloxone (2, 10 and 20 µg/paw) reversed the analgesic effect of 4-MAA in a dose dependent manner (one-way ANOVA, Tukey test, $P<0.05$). The administration of vehicle of naloxone or AM630 did not change the mechanical withdraw threshold by itself (One-way ANOVA, Tukey′s test, $P>0.05$)

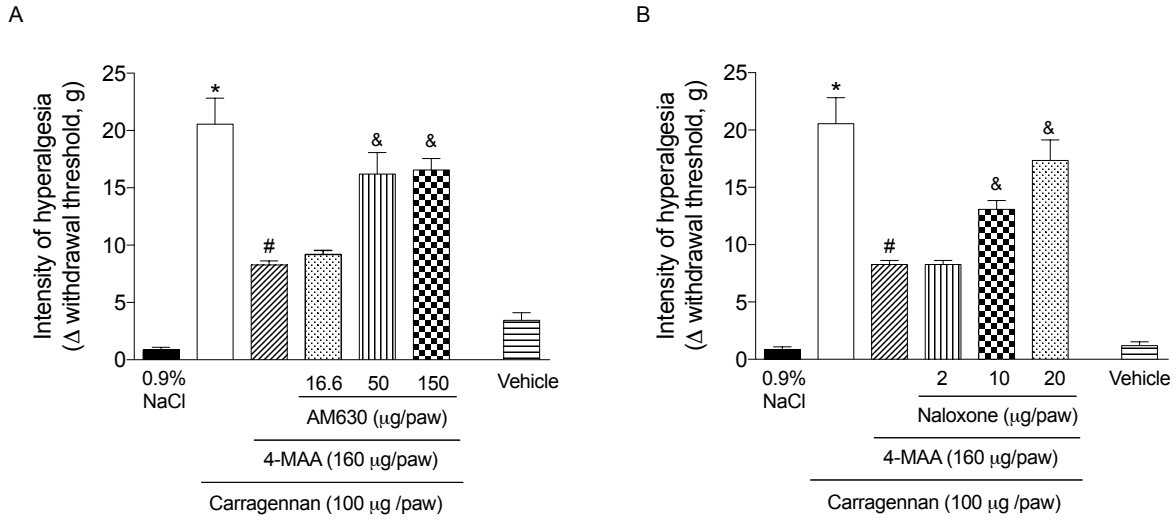

Figure 3. *Anti-hyperalgesic effect of 4-methylaminoantipyrine (4-MAA) is mediated by CB_2 and opioid receptor activation.* Local administration of carrageenan in rat′s hindpaw induced mechanical hyperalgesia 3 h after, which is decreased with 4-MAA administered 2.5 h after carrageenan. This anti-hyperalgesic effect was reversed by AM630 (**3A** 50 and 150 µg/paw) or Naloxone (3**B** 2, 10 and 20 µg/paw). The symbol "*" means different from control group (0.9% NaCl; 50 µL), "#" means different from carrageenan group and "&" means different from

carrageenan plus 4-MAA (one-way ANOVA, Tukey test, P<0.05). The administration of vehicle does not change the mechanical withdraw threshold by itself.

3.4 The anti-hyperalgesic effect of 4-methylaminoantipyrine is mediated by Kappa opioid receptor activation.

Because the analgesic effect of 4-MAA is mediated by opioid receptors activation we sought what kind of opioid receptor is involved in this effect. To analyze the involvement of kappa, mu and delta opioid receptors in the analgesic effect of 4-MAA a selective antagonist of these receptors was administered 30 minutes before 4-MAA. As show in the figure 4A the pretreatment with nor-BNI a selective kappa-opioid receptor antagonist (5, 10 or 50 µg/paw) reversed the analgesic effect of 4-MAA in a dose dependent manner (one-way ANOVA, Tukey test, P<0.05). However, CTOP, a selective mu-opioid receptor antagonist (Figure 4B; 8, 20 and 32 µg/paw) or Naltrindole, a selective delta-opioid receptor antagonist (Figure 4C; 1, 3 and 9 µg/paw) administered 30 min before 4-MAA injection did not reverse its analgesic effect (one-way ANOVA, Tukey test, P>0.05).

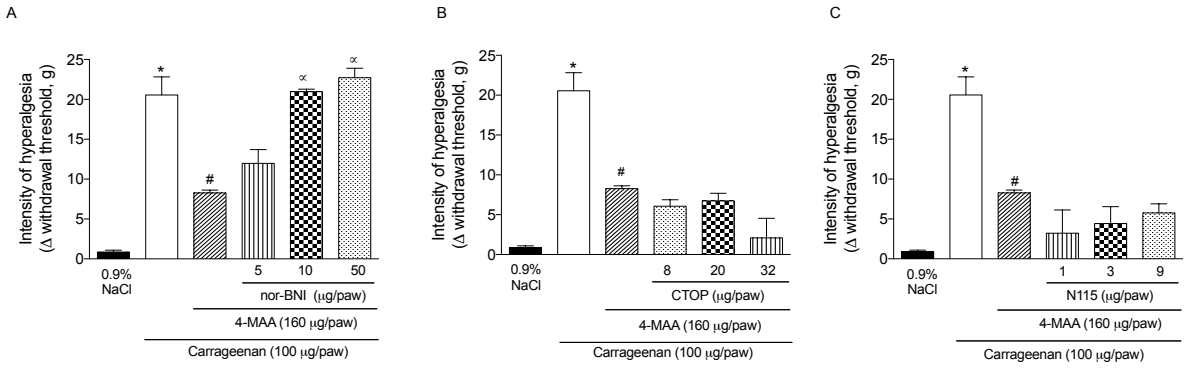

Figure 4. *Anti-hyperalgesic effect of 4-methylaminoantipyrine (4-MAA) is mediated by kappa opioid receptor activation.* Local administration of carrageenan in rat´s hindpaw induced mechanical hyperalgesia 3 h after, which is decreased with 4-MAA administered 2.5 h after carrageenan. This anti-hyperalgesic effect was reversed by nor-BNI (**4A** 5, 10 and 50 µg/paw) but not N115 (**4B** 1, 3 and 9 µg/paw) or CTOP (**4C** 8, 20 and 32 µg/paw). The symbol "*****" means different from control group (0.9% NaCl; 50 µL), "#" means different from carrageenan group (one-way ANOVA, Tukey test, P<0.05). The administration of vehicle does not change the mechanical withdraw threshold by itself.

4. Discussion

We have recently demonstrated that dipyrone and its bioactive metabolite 4-MAA has a similar analgesic effect and both induced activation of L-argenine-NO-K_{ATP} pathway (Dos Santos et al., 2014). In the current study data showed that dipyrone is quickly hydrolyzed to 4-MAA in the peripheral tissue suggesting that dipyrone's analgesic effect could be mediated by 4-MAA. Also, 4-MAA's analgesic effect is dependent of CB2 and κ-opioid receptors activation.

Our results demonstrated that dipyrone or 4-MAA locally administered in peripheral tissue inhibits the hyperalgesia induced by carrageenan, corroborating with previous studies that has been demonstrated dipyrone's analgesic effect in various pain models (Beirith et al., 1998; Dos Santos et al., 2014; Edwards et al., 2010; Rogosch et al., 2012; Vazquez et al., 2005).

Although dipyrone is majorly metabolized in two bioactive compounds, 4-MAA and 4-AA, data of this study demonstrated that both dipyrone or 4-MAA completely prevented the hyperalgesia induced by the inflammatory agent carrageenan, suggesting that the metabolite 4-MAA is enough to induce effective analgesic effect despite the 4-AA. Furthermore, dipyrone was completely hydrolyzed to 4-MAA in the peripheral tissue, confirming our hypotheses that dipyrone's analgesic effect is mediated by its hydrolysis to 4-MAA (Dos Santos et al., 2014). In fact, some studies have been shown that dipyrone's hydrolyze to 4-MAA is a non-enzymatic reaction and depends on concentration, pH and temperature (Cohen et al., 1998; Pierre et al., 2007). The findings of this study also demonstrated that 4-MAA can be detected in the peripheral tissue in two forms: protonated $[4\text{-MAA+H}]^+$ and sodiated $[4\text{-MAA+Na}]^+$. Although the ionized form seems to be more intense than sodiated form, it could be explained by a high concentration of sodium in the medium. However, independently of the form that the molecule is detected the product of hydrolysis is the same, the 4-MMA.

Our results have also shown that 4-MAA's analgesic effect is dependent on cannabinoid CB_2 and Kappa opioid receptors activation. It has been well demonstrated that CB2 receptor activation can modulate the immune response in peripheral tissue (Lunn et al., 2006). However, the data of this study demonstrated that 4-MAA completely prevented the hyperalgesia induced by carrageenan, an inflammatory agent that could be prevented by kappa opioid antagonist. This data suggested

that 4-MAA induced analgesic effect through CB2 receptor activation that not depends on the immune modulation. In fact, CB2 receptor appears to be a non-neuronal receptor (Freund et al., 2003), and also it has been demonstrated that CB2 receptors activation induces analgesia by the release of endogenous opioid that trigger a hyperpolarization of the afferent nociceptor (Negrete et al., 2011).

Because the anti-hyperalgesic effect of 4-MAA could be prevented by local administration of Kappa opioid receptor antagonist, it is plausible to hypothesize that 4-MAA does not induce anti-hyperalgesic effect directly, but instead, mediates the release of endogenous opioid by immune cells. In fact, our data demonstrated that CB2 receptor activation is involved in anti-hyperalgesic effect of 4-MAA in carrageenan-induced hyperalgesia, but not by PGE_2-induced hyperalgesia (dos Santos et al., 2014). These data suggest that the involvement of CB2 receptor activation in analgesic effect of dipyrone depends on the presence of immune cells in the peripheral tissue. This cross-talk between CB2 receptor and subsequently kappa opioid receptor activation is already shown for other drugs. The analgesic effect induced by crotalphine, a non-opioid peptide, or even CB2 agonist induces analgesic effect by releasing of dynorphin A and subsequently κ-opioid receptor activation (Machado et al., 2014; Negrete et al., 2011)

Taking together, our results provides data that suggest a new mechanism of action for dipyrone, in particular for 4-MAA that involves an interaction between peripheral CB2 and opioid systems. This interaction appears as new strategy to treat inflammatory pain without inducing central nervous system-mediated undesirable effects and pointed up to this bioactive metabolite as new strategy to treat inflammatory pain with low side-effects.

Acknowledgements

This work was funded by CAPES (Coordination of Improvement of Higher Education Personnel).

Conflict of interest

The authors declare no conflict of interest

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III – CAPÍTULO 2

O presente artigo foi submetido no periódico “Brithish Journal of Pharmacology”.

Cannabinoid type 1 receptor-dependent capsaicin receptor desensitization contributes to the analgesic effect of dipyrone in inflamed tissue

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[2]Ruihui Li - performed calcium-imaging.
[2]Istvan Nagy – designed in vitro studies, wrote the manuscript.
[1]Cláudia Herrera Tambeli[1] – designed the study and analyzed the data
[1*]Carlos Amilcar Parada – designed the study, wrote the manuscript and led the project.

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Running title: TRPV1 desensitisation by dipyrone

Acknowledgements

This work was funded by CAPES (Coordination of Improvement of Higher Education Personnel).

Conflict of interest statement

The authors declare no conflict of interest.

ABSTRACT

Background and Purpose: While Dipyrone is a widely used analgesic, its mechanism of action is not completely understood. Recently we have reported that one of Dipyrone's metabolite 4-aminoantipirine (4-AA) reduces prostaglandin E2 (PGE_2)-induced pain-related behaviour through the cannabinoid type 1 (CB1) receptor. Here, we ascertained, in naive and PGE_2-induced "inflamed" conditions both *in vivo* and *in vitro*, the effect of 4-AA on responses mediated by the transient receptor potential ion channel, subfamily V, member 1 cationic channel (TRPV1) that is indispensable for the development of inflammatory heat hyperalgesia,

Experimental Approach: *In vivo*, the effect of local administration of 4-AA (160 μg/paw) on capsaicin (0.12 μg/paw) injection-induced pain-related behaviour was assessed. *In vitro*, changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) was measured to assess 4-AA's effects on TRPV1-mediated excitation in cultured primary sensory neurons.

Key Results: 4-AA reduced capsaicin-evoked nociceptive behaviour in both conditions through the CB1 receptor. In primary sensory neurons, 4-AA (100 μM) reduced capsaicin-induced increase in $[Ca^{2+}]_i$ in a CB1 receptor-dependent manner when PGE_2 was not present. Following PGE_2 application, 4-AA (1-50μM) increased $[Ca^{2+}]_i$ through TRPV1 that lead to TRPV1 desensitisation. The CB1 receptor antagonist reduced both the excitatory and desensitising effects.

Conclusions and Implications: These findings indicate that the CB1 receptor-mediated inhibition on TRPV1 and CB1 receptor-dependent TRPV1 desensitisation contribute to the antinociceptive effect of 4-AA in naive and inflamed condition, respectively. These finding also suggest the usefulness of certain double CB1/TRPV1 agonists as putative analgesics particularly in inflammatory conditions.

Keywords: dorsal root ganglion, calcium imaging, fura-2, peripheral, sub-cutaneous.

Abbreviations list

CB1- cannabinoid type 1 (CB1) receptor

TRPV1 - transient receptor potential ion channel, subfamily V, member 1 cationic channel

4-MAA- 4-methylaminoantipyrine

4-AA- 4-aminoantipirine

PGE_2 - prostaglanding E2

CAP – Capsaicin

NaCl – Sodium chloride

DMSO - Dimethyl sulfoxide

DRGs – Dorsal root ganglia

AM251 – CB1 antagonist receptor

Ca^{2+}-influx – Calcium influx

$[Ca^{2+}]_{I-}$ intracellular calcium concentration

RTX - resiniferatoxin

3. INTRODUCTION

Dipyrone is an antipyretic and analgesic drug marketed for more than hundred years. The clinical use of Dipyrone is based on its high analgesic efficacy and low toxicity, although the mechanism of action is not fully understood (Edwards et al., 2010; Dos Santos et al., 2014). After administration, Dipyrone is quickly hydrolyzed to 4-methylaminoantipyrine (4-MAA) which is then metabolized to 4-formylaminoantipyrine (4-FAA), 4-aminoantipyrine (4-AA), and 4-acetylaminoantipyrine (4-AAA) (Pierre et al., 2007). As 4-MAA and 4-AA have the same analgesic effect as Dipyrone, they are called bioactive metabolites (Pierre et al., 2007; Rogosch et al., 2012).

4-MAA and 4-AA are found in the plasma and cerebrospinal fluid 30 minutes after Dipyrone administration (Cohen et al., 1998). In accordance, Dipyrone produces analgesic effect both through the peripheral and the central nervous system (CNS) (Lorenzetti and Ferreira, 1985; Vazquez et al., 2005). We have shown recently that while the peripherally-mediated anti-nociceptive effects of Dipyrone and 4-MAA, on prostaglanding E2 (PGE_2)-evoked pain-related behaviour, do not, those of 4-AA do involve the activation of the cannabinoid type 1 (CB1) receptor that is expressed predominantly by neurons (Di Marzo et al., 2014; Dos Santos et al., 2014).

Activity of the transient receptor potential ion channel, vanilloid sub-family, member 1 (TRPV1) that is indispensible for the development of inflammatory heat hyperalgesia contributes to the development of the PGE_2-induced pain as, through phosphorylation, PGE_2 sensitises TRPV1(Caterina et al., 1997, 2000; Davis et al., 2000; Moriyama et al., 2005; Btesh et al., 2013). Both TRPV1 and the CB1 receptor are expressed by the overwhelming majority of nociceptive primary sensory neurons and they exhibit a huge degree of co-localization (Ahluwalia et al., 2000; Veress et al., 2013; Chen et al., 2016). In addition to the co-localization, the CB1 receptor and TRPV1 exhibit a complex relationship though which the CB1 receptor may inhibit or sensitize TRPV1 (Ahluwalia et al., 2003; Hermann et al., 2003; Fioravanti et al., 2008; Chen et

al., 2016). Therefore, the effect of 4-AA on TRPV1-mediated responses, especially in inflammatory condition, is of particular interest. Accordingly, here we assessed, both *in vivo* and *in vitro*, the effects of 4-AA on capsaicin-evoked effects both with and without apriori PGE_2 application

4. MATERIALS AND METHODS

4.1 Animals

A total of 84 Male Wistar rats (8-weeks-old, 200-250g) were used in this study; 69 obtained from the Multidisciplinary Center for Biological Research (CEMIB - UNICAMP, Campinas, Brazil) and 15 Male Wistar rats from Charles River, UK. Animals (specific pathogen free - SPF) were separated randomly and housed in standard plastic cages with soft bedding (four per cage) on a 12:12 light cycle, with food (commercial chow for rodents) and filtered water available *ad libitum*, in a temperature-controlled room (± 23° C). Testing sessions took place during the light phase (09:00 AM - 5:00 PM) in a quiet room maintained at 23º C . Experimental protocols were approved by the Committee on Animal Research of the State University of Campinas (protocol number: 3372-1) and by veterinary services (Central Biological Services) at Imperial College London, UK. Experiments were conducted in accordance with the International Association for the Study of Pain (IASP) guidelines (Zimmermann, 1983), UK Animals (Scientific Procedures) Act 1986, the revised National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. Good Laboratory Practice and ARRIVE guidelines were observed. Every effort was taken to minimize the number of animals used.

2.1.2 Drugs and treatments *in vivo*

We used PGE_2 (100 ng/paw) as acute inflammatory stimuli and Capsaicin (0.12 µg/paw) as nociceptive stimuli, 4-aminoantipirine [4-AA, 160 µg/paw, (Alves and Duarte, 2002; Vivancos et al., 2004)], and AM251 as a selective cannabinoid CB_1 receptor antagonist (50 µg/paw (Dos Santos et al., 2014). PGE_2 was dissolved in alcohol 100% to a concentration of 10 mg/ml and then dissolved in 0.9% NaCl; Capsaicin was firstly dissolved in ethanol to a concentration of 0.2

mg/ml and then dissolved in 0.9% NaCl to a final concentration for behavior or in vitro test. AM251 was dissolved in propylene glycol and 10% DMSO; 4-AA was dissolved in 0.9% NaCl.

Drugs or vehicle were injected subcutaneously (50 µl) in the rat hindpaw (intraplantar) with aid of a BD *Ultra-Fine ® (30 gauge)* needle.

2.2 Behaviour measurements

Rats were placed separately in acrylic boxes for habituation period of 15-30 minutes before testing. Following capsaicin injection and the number of flinches and licking were counted during a 5 minutes period. A "flinch" was defined as a rapid jerk of the injected paw and "licking" as the time spent licking the hindpaw. To analyze PGE_2-induced TPRV1 sensitization, PGE_2 (100 ng/paw) was inject 3 hours before capsaicin. Control experiments were performed in all studies using the capsaicin vehicle (50% ethanol and 50% saline); the vehicle did not produce any significant nociceptive behaviour when injected into the hind paw at the same volume. All behaviour tests were done by a blind examiner.

2.3 Primary sensory neuron culture

Cultures were prepared as described by Chen and co-workers (Chen et al., 2016). Briefly, dorsal root ganglion DRGs (C1 to the S1 segment) were dissected and placed into Ham's nutrient F12 culture medium (Sigma, UK) supplemented with 1 mM L-glutamine (Invitrogen, UK), 5000 IU/ml penicillin (Invitrogen, UK), 5000 µg/ml streptomycin (Invitrogen, UK) and 2% Ultroser G (Biospectra, France). Ganglia were incubated in type IV collagenase (Lorne Diagnostics, UK, 300 U/ml) for 3 hours and in trypsin (0.25%) for 30 minutes at 37 °C at 5% CO_2. Following washes, ganglia were triturated, and dissociated cells plated on poly-DL-ornithine (Sigma, UK)-coated glass coverslips in the supplemented medium. Cells were grown in the supplemented medium in the presence of 50 ng/ml nerve growth factor (NGF, Promega, USA) for 12-24 hours.

2.3.1 Drugs *in vitro*

PGE_2 (1 µM, Mistry et al., 2014); Capsaicin (500 nM, Mistry et al., 2014); 4-AA (100, 50, 25, 10 and 1 µM (Nassini et al., 2015); AM251 (10 µM,) and capsazepine (10 µM). AM251, capsaicin and and capsazepine stock solutions were prepared with DMSO. PGE_2 stock solution was

prepared in ethanol. Other drugs were dissolved in 0.9% NaCl. Capsazepine and AM251 were brought from Tocris and all other drugs were obtained from Sigma-Aldrich® (MO, USA).

2.4 Ca^{2+}-imaging

For assessing changes in the $[Ca^{2+}]_i$, primary sensory neurons cultured for 1 day were loaded with Fura-2 acetoxymethyl ester (Fura-2 AM, 5 μM; Molecular Probes, Inc., Eugene, OR) in the presence of 2 mM probenecid (Molecular Probes) for 30 minutes at 37 °C in a HEPES-buffered saline (in mM): NaCl 122; KCl 3.3; $CaCl_2$ 1.3; $MgSO_4$ 0.4; $KH_2PO_4$1.2; HEPES 25; glucose 10; adjusted with NaOH to pH 7.4. Coverslips were placed in a laminar flow perfusion chamber (Warner Instrument Corp., UK) and superfused with extracellular solution (in mM: NaCl 160; KCl 2.5; $CaCl_2$ 1; $MgCl_2$ 2; HEPES 10; glucose 10; pH 7.4) continuously via a six-channel perfusion system. The following test solutions were applied to the cells: PGE_2 (1 μM, for 5 minnutes), AM251 (10 μM, for 2 minutes), Capsazepine (10 μM, for 2 minutes) 4-AA (100, 50, 25, 10, 1 μM, for 2 minutes), capsaicin (500 nM, for 30 seconds) and KCl (50 mM, for 30 seconds). The outlet of the perfusion system was positioned about 2-3 mm from the group of neurons from which the recordings were obtained. Application of drugs was controlled manually. Experiments were performed at 37 °C. Only one field of view was tested on each coverslip.

Images of Fura-2 loaded cells with the excitation wavelength alternating between 355 and 380 nM were captured with a Peltier element-cooled slow scan charge-coupled camera (optiMOS, QImaging, Canada) connected to a PC which ran the WinFluor software package (Dr Dempster, Strathclyde University, UK). Following subtraction of the background fluorescence, the ratio of fluorescence intensity at the two wavelengths as a function of time (rate 0.5 Hz) was calculated automatically ($R = F355/F380$) by the WinFlour software package. Data were further analyzed by the pClamp 10 software package (Molecular Devices, USA).

2.5 Statistical analyzes

The significance of differences between groups was established with unpaired *t*-Test, one-way ANOVA followed by Tukey's test or Fischer's exact test as appropriate. A value of $p<0.05$ was

set as the threshold of statistical significance. Statistical analyzes were performed in the GraphPad Prism v5.00 for Windows® (GraphPad Software). "n" refers to the number of animals for in vivo experiments whereas it refers to the number of neurons for in vitro measurements. In vitro, each drug application protocol was repeated in cultures from at least 3 animals.

3 RESULTS

3.1 4-AA reduces capsaicin-induced pain-related behaviour as well as capsaicin-induced Ca^{2+}-influx into cultured primary sensory neurons through CB1 receptor activation.

Subcutaneous capsaicin injection (0.12 µg/paw) into the rat hind paw induced pain-related behaviour (Figure 1 A). The capsaicin-induced pain-related behaviour was significantly reduced by the administration of 4-AA (160 µg/paw, Figure 1 A) *prior* capsaicin injection. To analyze the involvement of cannabinoid CB1 receptor in the anti-nociceptive effect of 4-AA, a cannabinoid CB1 receptor antagonist (AM251, 50 µg/paw) was administered 15 minutes before 4-AA. As shown in the Figure 1A, AM251 completely blocked the anti-nociceptive effect of 4-AA (one-way ANOVA, Tukey's test, $P<0.0001$).

In order to confirm the CB1 receptor-mediated inhibitory effects of 4-AA on TRPV1, next we measured the $[Ca^{2+}]_i$ on cultured DRG neurons. As expected, administration of capsaicin (500 nM) induced a significant increase in the $[Ca^{2+}]_I$ in a major sub-population of the cells (Figure 1B-D). Application of 4-AA alone, did not produce any measurable response, or affect the responsiveness of DRG neurons to KCl (not shown). However, pre-treatment of the cells with 4-AA (100 µM) significantly reduced capsaicin-evoked responses (Figure 1C; one-way ANOVA, Tukey's test, $P<0.0001$). Application of AM251 (10 µM) prevented the inhibitory effect of 4-AA (Figure 1D-E; one-way ANOVA, Tukey's test, $P<0.0001$). Further, 4-AA also reduced the number of capsaicin-responsive cells (Figure 1F; Fisher's exact test, $P<0.05$) and AM251 prevented this reduction without affecting the number of responsive cell to KCl (Figure 1F; Fisher's exact test, $P<0.007$).

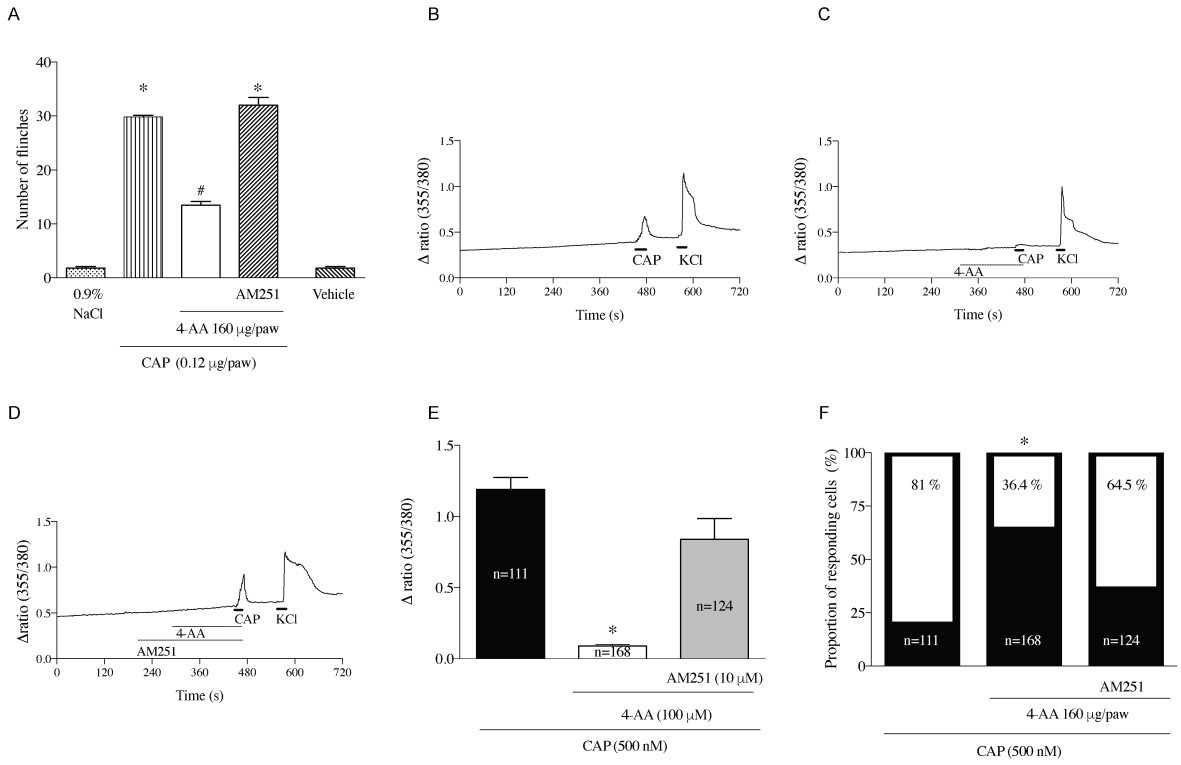

Figure 1. 4-aminoantipyrine (4-AA) reduces the excitatory effect of capsaicin through CB1 receptor activation. (A) Local administration of capsaicin (0.12 µg/paw) induces flinches. The capsaicin-induced nociceptive behaviour (0.12 µg/paw) is reduced by the administration of 4-AA (160 µg/paw). AM251(50 µg/paw) reverts the anti-nociceptive effect of 4-AA. (B and C) Capsaicin (500 nM) induces a rise in the intracellular $[Ca^{2+}]_i$ in cultured primary sensory neurons that is reversed by 4-AA (100 µM). (D-E) AM251(10 µM) reverts the inhibitory effect of 4-AA on the capsaicin-induced calcium-influx (one-way ANOVA, Tukey test, $P<0.05$). (F) 4-AA reduces the proportion of neurons that respond to capsaicin and AM251 reduces the effect of 4-AA.

In (**A)** "*" indicates difference from 0.9% NaCl, vehicle and capsaicin *plus* 4-AA. "#" indicates difference from 0.9% NaCl, vehicle, capsaicin, capsaicin *plus* 4-AA *plus* AM251. In (**E-F)** "*" indicates difference from capsaicin and capsaicin *plus* 4-AA *plus* AM251.

3.2 4-AA produces different effects on TRPV1-mediated responses following PGE2-induced sensitization in vivo and in vitro.

The inhibitory effect of 4-AA on TRPV1-mediated responses is relevant if it is maintained in inflammatory conditions. During inflammation, inflammatory mediators sensitize TRPV1. PGE_2 a major component of the inflammatory soup, has one of the most powerful sensitizing effect on TRPV1 (Moriyama et al., 2005; St-Jacques & Ma, 2013). In agreement with previous findings (Lin et al., 2006) pre-treatment of the rat paw with PGE_2 (100 ng, injected subcutaneously, 3

hours before capsaicin injection) significantly increased the capsaicin-induced pain-related behaviour (Figure 2A and 2B; one-way ANOVA, Tukey's test, $P<0.001$).

The sensitizing effect of PGE_2 was also evident *in vitro*, because cultured primary sensory neurons pre-treated with PGE_2 (1µM, 5 minutes before capsaicin application) exhibited significantly greater responses to capsaicin (500 nM, for 30 s) than without PGE_2 pre-treatment (Figure 2C, D and E; one-way ANOVA, Tukey's test, $P<0.0001$). However, the proportion of capsaicin-responding neurons was not changed by PGE_2 pre-treatment (Figure 2F; Fisher's exact test, $P>0.4$). Further, although PGE_2 *per se* produced some small change in the $[Ca^{2+}]_i$ in some cells, on average the change in the $[Ca^{2+}]_i$ did not reach the level of significance (Figure 2C and D; one-way ANOVA, Tukey's test, $P>0.05$).

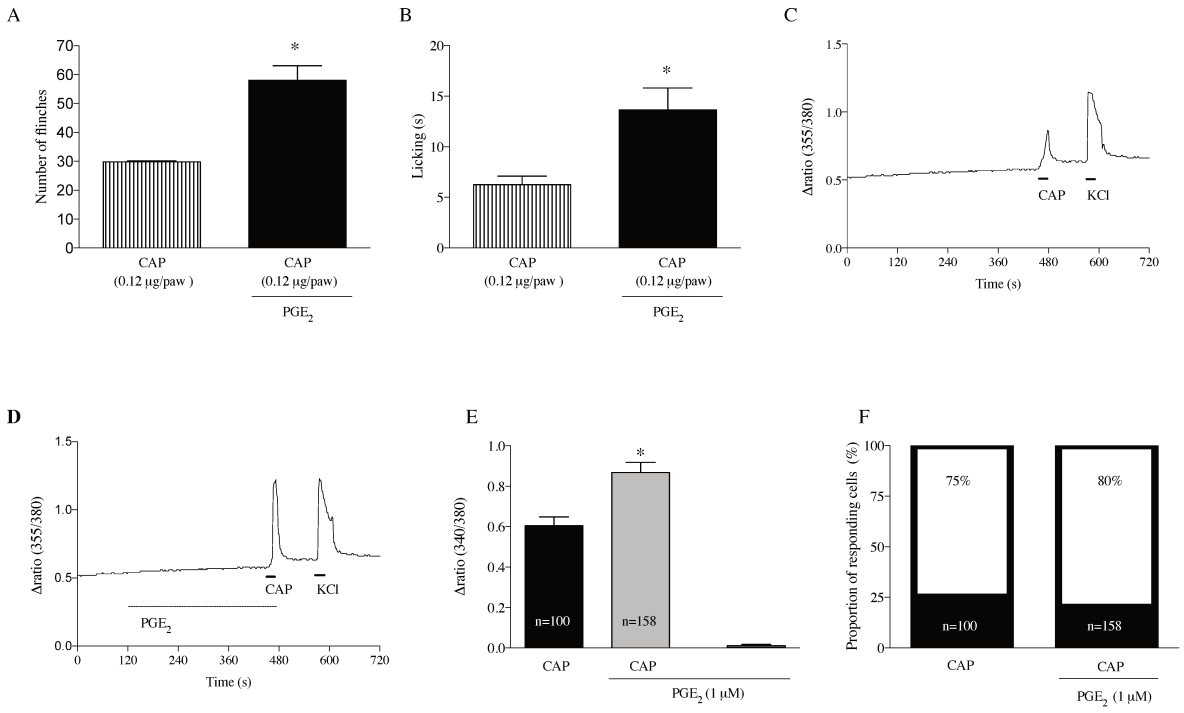

Figure 2. PGE_2 increases capsaicin-induced pain-related behaviour and capsaicin-induced Ca^{2+}-influx in primary sensory neurons. ***(A) and (B)*** Local administration of capsaicin (0.12 µg/paw) in subcutaneous tissue of hind paw induces pain-related behaviour (nociception). Pre-treatment with PGE_2 (100nM, 150 minutes) increases the capsaicin-induced nociception. (C, D and E) The pre-treatment with PGE_2 (1µM, for 5 minutes) increases capsaicin-induced calcium-influx. (F)

However, PGE_2 pre-treatment does not affect the number of capsaicin responsive neurons. (**F**). In (**A**), (**B**) and **(C),** "*" indicates difference from capsaicin alone (one-way ANOVA, Tukey test).

After confirming that PGE_2 indeed sensitizes TRPV1 both *in vivo* and *in vitro*, next we assessed the effect of 4-AA following PGE_2 administration. *In vivo*, 4-AA (160 μg) was injected 3 hours after PGE_2 injection, which was followed by capsaicin administration 30 minutes later. Assessing pain-related behaviour following 4-AA injection did not reveal any excitatory effect of 4-AA following PGE_2 injection (Figure 3A and B; one-way ANOVA). However, similarly to that observed in the naive condition, 4-AA did reduce the capsaicin-induced pain-related behaviour (Figure 3A and B; one-way ANOVA, one-way ANOVA, Tukey's test, $P<0.0001$). To find whether this inhibitory effect was mediated through the CB1 receptor, we administered AM251 (10 μM) 2 minutes before 4-AA injection. Similarly, to that found in naive condition, AM251 completely prevented the anti-nociceptive effect of 4-AA (Figure 3A and B; one-way ANOVA, Tukey's test, $P<0.0001$).

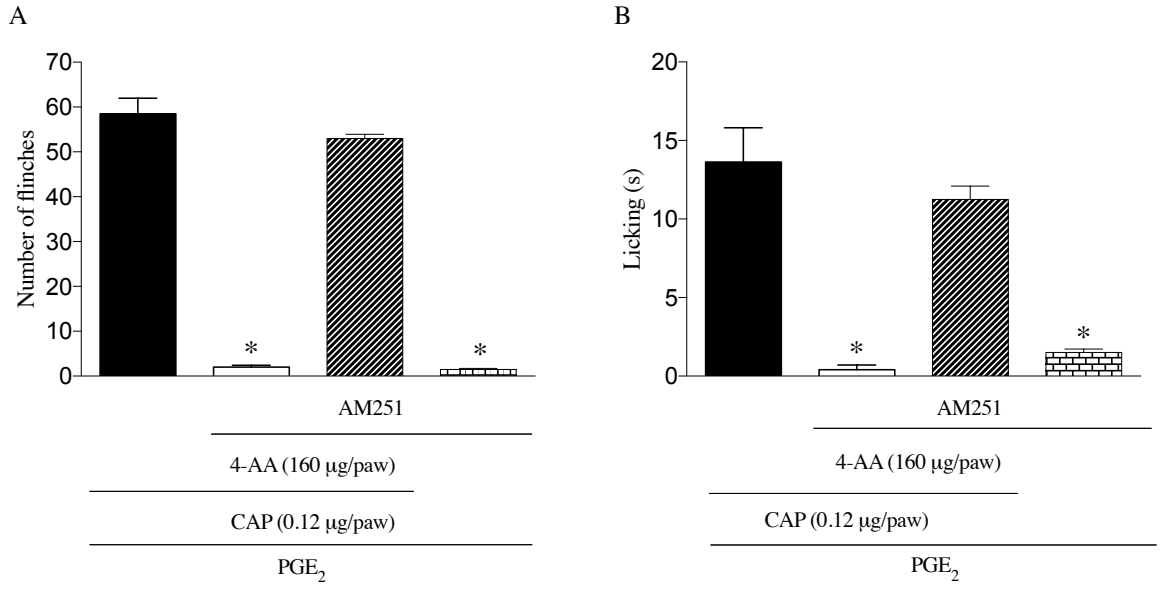

Figure 3. 4-AA reduces capsaicin-induced pain related behaviour in peripheral tissues sensitized by PGE_2. (A) and (B) 4-AA reduces capsaicin-induced pain-related behavioural responses following pre-treated with PGE_2 (Unpaired t-test; $P < 0.05$, $P<0.05$). "*" indicates difference from PGE_2 *plus* capsaicin.

To confirm the CB1 receptor-mediated inhibitory effect of 4-AA on sensitized TRPV1 *in vitro*, we pre-treated cultured primary sensory neurons with PGE_2 (1 μM for 5 minutes) and then

administered 4-AA for 2 minutes. Unexpectedly, 4-AA (50, 25, 10 and 1 μM), in a concentration-dependent manner, increased the $[Ca^{2+}]_i$ in primary sensory neurons in this condition (Figure 4A,B and C; one-way ANOVA, Tukey's test, $P<0.002$). To analyze if the 4-AA-induced increase in the $[Ca^{2+}]_i$ was mediated by TRPV1, capsazepine, a TRPV1 receptor antagonist (10 μM, 270 seconds) was applied before 4-AA (10 μM, 2 minutes). As shown in Figure 4 D and E, the 4-AA-induced increase in the $[Ca^{2+}]_i$ was completely blocked by capsazepine (one-way ANOVA, Tukey's test, $P<0.0001$).

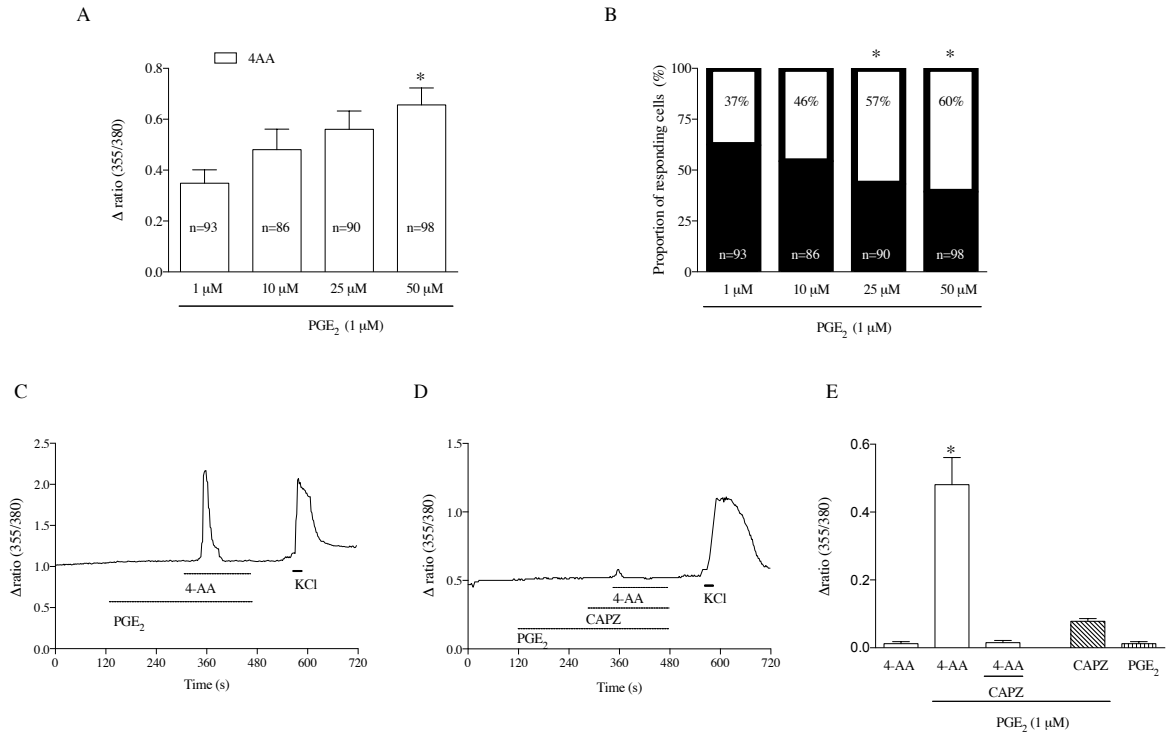

Figure 4. 4-AA increases $[Ca^{2+}]_i$ *through TRPV1 activation in neurons sensitized by* PGE_2*.* *(A and C)* 4-AA (50, 25, 10 and 1 μM) increases $[Ca^{2+}]_i$ in primary sensory neurons sensitized by PGE_2. (B) The proportion of 4-AA-responding cells is also increased following PGE_2 pre-treatment. (D) and (E) The 4-AA increases $[Ca^{2+}]_i$ was completely reversed by capsazepine (CAPZ). "*" indicates difference from the other treatments.

3.4. 4-AA desensitizes TRPV1with the contribution of CB1 receptor.

When 4-AA was applied for two minutes in cultured primary sensory neurons, the change in $[Ca^{2+}]_i$ after reaching a peak exhibited a rapid recovery indicating TRPV1 desensitisation (Figure 5B). Indeed, the increase in the $[Ca^{2+}]_i$ by 4-AA (10, 25, and 50 μM) significantly reduced

subsequent responses to capsaicin (Figure 5A; one-way ANOVA, Tukey's test, respectively $P<0.03$, $P<0.04$ and $P<0.001$).

In order to find out whether the CB1 receptor has any role in the 4-AA-increased $[Ca^{2+}]_I$ next we pretreated the cells with AM251 (10 μM for 2 minutes) before applying 4-AA. The increase in the $[Ca^{2+}]_i$ induced by 4-AA (10 μM) was significantly reduced by this pre-treatment (Figure 5C and 5D, one-way ANOVA, Tukey test, $P<0.007$). In addition, TRPV1 desensitization was also significantly reduced (Figure 5C and 5D; one-way ANOVA, Tukey test, $P<0.04$). Further, 4-AA reduced the number of capsaicin responsive neurons to 27% when compared to CAP and the pre-treatment with CB1 receptor antagonist increase the number of responsiveness neurons to capsaicin to 55% (Figure 5E; Fisher's exact test, $P<0.005$). AM251 did not change the number of responsive cells to 4-AA (Figure 5E; Fisher's exact test, $P>0.2$).

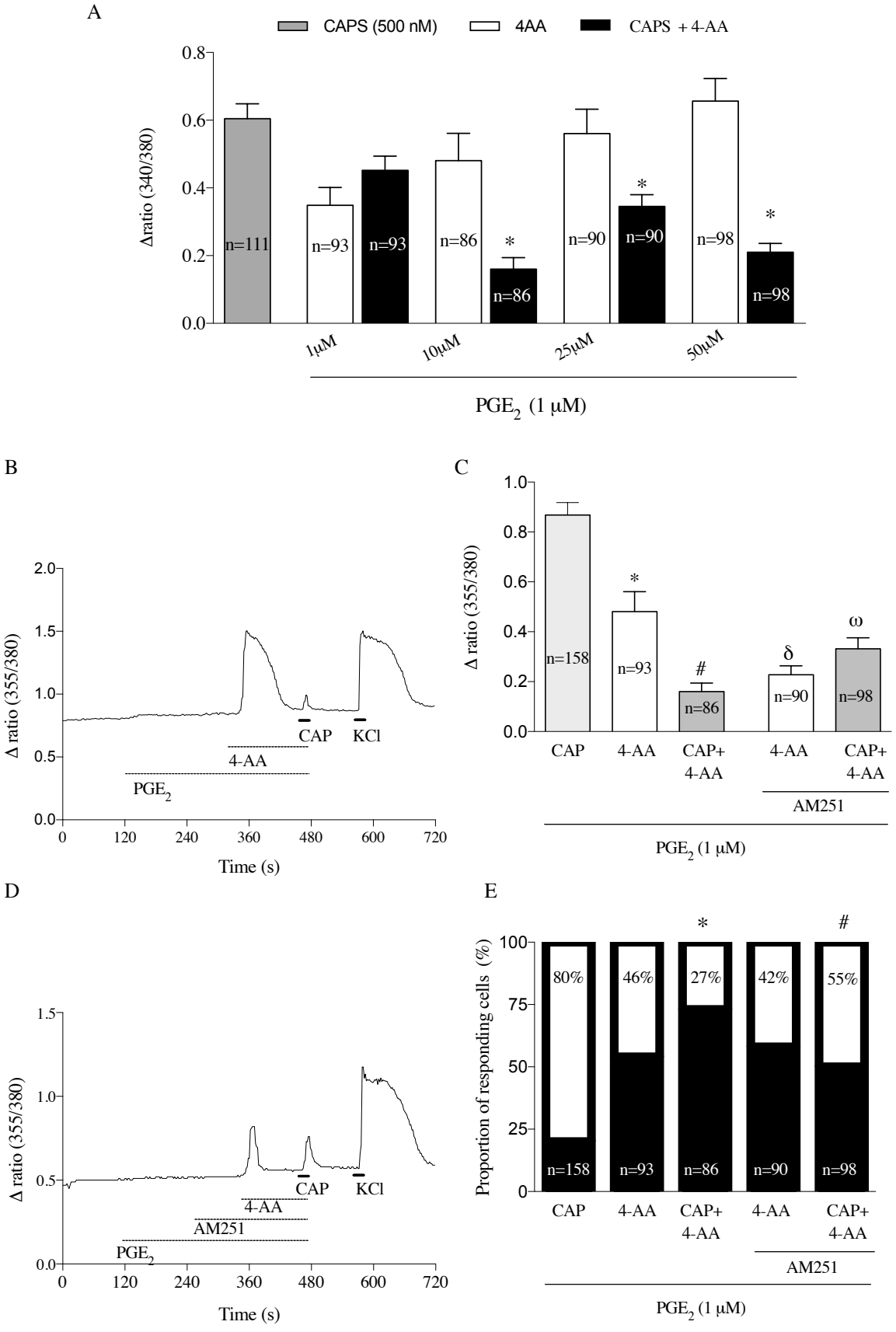
A
CAPS (500 nM)
4AA
CAPS + 4-AA
Δratio (340/380)
n=111
n=93
n=93
n=86
n=86
n=90
n=90
n=98
n=98
1μM
10μM
25μM
50μM
PGE2 (1 μM)
B
Δ ratio (355/380)
CAP
KCl
4-AA
PGE2
Time (s)
C
Δ ratio (355/380)
n=158
n=93
n=86
n=90
n=98
CAP
4-AA
CAP+ 4-AA
4-AA
CAP+ 4-AA
AM251
PGE2 (1 μM)
D
Δ ratio (355/380)
CAP
KCl
4-AA
AM251
PGE2
Time (s)
E
Proportion of responding cells (%)
80%
46%
27%
42%
55%
n=158
n=93
n=86
n=90
n=98
CAP
4-AA
CAP+ 4-AA
4-AA
CAP+ 4-AA
AM251
PGE2 (1 μM)

Figure 5. 4-AA desensitizes TRPV1 with contribution of CB1 receptor. (A) and (B) 4-AA (10, 25 and 50 μM) increase in the $[Ca^{2+}]_i$ that is followed by reduced responses to capsaicin (500 nM). (C and D) 4-AA induced increase in the $[Ca^{2+}]_i$ that is reduced by AM251. (D) AM251 (10 μM) reduces the and impairs the 4-AA-induced TRPV1 desensitization. (E) AM251(10 μM) restores the number of capsaicin responsive neurons but did not change the number of responsive cells to 4-AA. (A) "*" indicates difference from CAP; (C) "*" and "#" indicates difference from CAP; "δ" and "ϖ" indicates difference from 4-AA and CAP+4-AA respectively. (E) "*" and "#" indicates difference from CAP.

4. DISCUSSION

We recently have shown that the CB1 receptor is involved in 4-AA's analgesic effect after PGE_2 administration. (Dos Santos et al., 2014). Here, we report that 4-AA also reduces capsaicin-induced nociceptive behaviour through the CB1 receptor. Our *in vitro* findings confirm that 4-AA, through activating the CB1 receptor indeed reduces capsaicin-evoked TRPV1 activity in cultured primary sensory neurons. The CB1 receptor-mediated inhibitory effect on TRPV1-mediated responses is in agreement with a series of previous *in vivo* and *in vitro* data (Mahmud et al., 2009; Sántha et al., 2010). Although, both the CB1 receptor and TRPV1 might be expressed on cells other than peripheral terminals of a major sub-population of primary sensory neurons (Caterina et al., 1997; Ahluwalia et al., 2000; Sacerdote et al., 2000; Basu and Srivastava, 2005; Bodó et al., 2005; Ständer et al., 2005; Veress et al., 2013; Chen et al., 2016), our findings together indicate that 4-AA, following local application, acts directly on primary sensory neurons in naive condition.

Similarly to naive condition, 4-AA also reduced capsaicin-evoked responses in inflammatory condition both *in vivo* and *in vitro*. However, while in naive condition, the mechanisms of action are the same, in inflamed condition, those appear to differ, *in vitro* and *in vivo*; *In vivo*, 4-AA inhibited capsaicin-evoked nocifensive behaviour in a CB1 receptor-dependent manner, whereas *in vitro*, 4-AA induced TRPV1 desensitisation following a brief TRPV1-mediated excitation after PGE_2 application though, these effects also depended on the CB1 receptor. Considering molecular events, we propose that the mechanisms of action are the same *in vitro* and *in vivo*, in inflammatory condition.

As reported previously , those molecular events include PGE_2-induced TRPV1 sensitization, which leads increased responsiveness of the ion channel to a series of activators including noxious heat, capsaicin and the endogenous TRPV1 activator anandamide (Lopshire and Nicol, 1997, 1998; Moriyama et al., 2005; Singh Tahim et al., 2005). The finding that following PGE_2 application *in vitro*, 4-AA induces TRPV1-mediated excitatory responses indicates that 4-AA is also a TRPV1 activator, which exhibits very low efficacy and potency in naive condition. Although, we have not specifically studied, our findings do indicate that the PGE_2-evoked TRPV1 sensitization results in a significant increase both in the efficacy and potency of 4-AA on TRPV1; 100 μM had no excitatory effect in naive condition, whereas 1 μM produced a significant increase in the $[Ca^{2+}]_i$ through TRPV1 in the inflamed condition.

TRPV1-mediated Ca^{2+} influx induces TRPV1 desensitization (Koplas et al., 1997). We demonstrated *in vitro* that 4-AA also desensitises TRPV1 in inflamed condition. Hence, it is feasible to propose that similar TRPV1 desensitization by 4-AA also occurs *in vivo*. However, while TRPV1 desensitization is most often preceded by excitatory responses such as pain-related behaviour *in vivo* (LaMotte et al., 1991), we did not observe any nociceptive responses following the injection of 4-AA in the inflamed condition. The development of nociceptive responses is initiated by action potentials generation in nociceptive primary sensory neurons. Therefore, the most plausible explanation for the apparent lack of any nociceptive response following 4-AA injection is that although 4-AA activated TRPV1, that activation did not result in action potential generation in nociceptive primary sensory neurons. Such lack of action potential generation could occur if the rate of depolarization is too slow for the activation of voltage-gated sodium channels (Hille, 2001). Previously, the ultrapotent TRPV1 activator resiniferatoxin (RTX), has been shown to induce slow depolarization, which then results in the generation of very few action potentials in primary sensory neurons hence no significant pain-related behaviour at low or high RTX concentrations (Raisinghani et al., 2005). While we have not specifically studied, 4-AA, similarly to RTX, may produce slow depolarization *per se*. Alternatively, by acting also on the CB1 receptor, 4-AA may reduce the rate of depolarization for example by increasing K^+ currents (Vásquez et al., 2003; Di Marzo et al., 2014; Mackie et al., 1995).

Our findings provide evidence that, in addition to TRPV1, the CB1 receptor is also involved in 4-AA's effects in inflammatory condition, as following the administration of the CB1 receptor antagonist AM251, no inhibitory effects by 4-AA on capsaicin-induced nociceptive responses were observed. Importantly, AM251 pre-treatment also reduced the 4-AA-induced excitatory effect as well as the subsequent TRPV1 desensitization *in vitro*. As a result, the capsaicin-evoked increase in the $[Ca^{2+}]_i$ was significantly greater with than without AM251 pre-treatment. These findings are in agreement with previous findings that the CB1 receptor contributes to the sensitization of TRPV1 (Hermann et al., 2003; Fioravanti et al., 2008; Chen et al., 2016). Notably, while such CB1 receptor-mediated increase in TRPV1-mediated responsiveness is evident in naive conditions (Fioravanti et al., 2008; Chen et al., 2016), our findings suggest that it may become significantly more pronounced in inflammatory conditions.

Taken together, our *in vivo* and *in vitro* findings strongly suggest that 4-AA induces the following events both *in vitro* and *in vivo* in inflammatory condition; inflammatory mediators and the CB1 receptor activated by 4-AA sensitize TRPV1. That sensitization results in significantly increased excitatory efficacy and potency of 4-AA on TRPV1. Activation of TRPV1 by 4-AA induces Ca^{2+} influx, which in turn, desensitize the TRPV1 receptor. As a result, capsaicin-evoked responses are reduced.

In addition, to the better understanding of 4-AA's mechanism of action on nociceptive primary sensory neurons, these findings lead, at least, to three further important implications. First, our data show that there is a "switch" from an exclusively CB1 receptor-mediated, to a mixed CB1 receptor- and TRPV1-mediated, inhibitory effect of 4-AA on capsaicin-evoked responses during the transition from naive to inflammatory condition at the peripheral terminals of nociceptive primary sensory neurons. Importantly, a similar switch has been reported at the central terminals of nociceptive primary sensory neurons, as we found that endogenous anandamide-induced inhibitory effects on spontaneous excitatory postsynaptic currents in dorsal horn neurons are mediated through the CB1 receptor and the CB1 receptor together with TRPV1 in naive and inflamed conditions, respectively (Nerandzic et al., 2017). The molecular mechanism of this switch is not known at present. However, recently it was showed that the CB1 receptor and TRPV1 are engaged in a complex and currently largely unknown cross talk in a major sub-

population of primary sensory neurons (Chen et al., 2016). Our findings suggest that PGE_2 may produce a major change in that cross talk leading to pronounced CB1 receptor-mediated TRPV1 sensitization.

In the context of above mentioned cross talk, the second implication of this work is that the CB1 receptor – TRPV1 relationship appears to play a major role in regulating TRPV1 activity both in naive and inflamed conditions. However, the outcome of the cross talk differs in physiological and inflamed conditions. While in the naive condition the CB1 receptor is able to reduce TRPV1 activity (Mahmud et al., 2009; Sántha et al., 2010), in the inflamed condition, it appears to have a predominantly TRPV1 sensitizing effect.

The third implication of this work is that agents, which do not pass the blood brain barrier and able to desensitize TRPV1 without the initiation of pain could be of significant clinical importance to control TRPV1-mediated excitation hence inflammatory pain without inducing central nervous system-mediated undesirable effects. Our data suggest that CB1 receptor agonist activity of such agents would provide significant benefit as that effect could increase the TRPV1 agonist efficacy and at the same time may reduce the rate of depolarization through increasing K^+ currents in order to avoid action potential generation (Vásquez et al., 2003; Di Marzo et al., 2014; Mackie et al., 1995). Non-pungent TRPV1 agonists such as capsaicinoids do exist and some, for example olvanil and palvanil, are indeed able to reduce nocifensive/pain-related behaviour (Brand et al., 1987; De Petrocellis et al., 2011; Hoffmann et al., 2012; Alsalem et al., 2016). Some capsaicinoids such as olvanil, arvanil and palvinil, though with various potency and efficacy, activate both TRPV1 and the CB1 receptor, and TRPV1 desensitization by these agents is thought to be a major mechanism for their anti-nociceptive effects (Brand et al., 1987; Melck et al., 1999; De Petrocellis et al., 2011; Hoffmann et al., 2012; Alsalem et al., 2016). However, the majority of the already known TRPV1/CB1 receptor dual agonists, in addition to TRPV1 and the CB1 receptor, also have significant effects on other molecules of the endocannabinoid/endovanilloid system (Melck et al., 1999), and those effects may not be beneficial. At present it is not known whether 4-AA has any effect on other components of the endocannabinoid/endovanilloid system. Nevertheless, our data extend the list of TRPV1/CB1 receptor dual agonists with a compound that structurally does not relate to the already known

agents, and the widespread use of Dipyrone indicates that 4-AA may be used a starting point to develop highly potent and effective analgesics acting through desensitizing TRPV1 without inducing pain.

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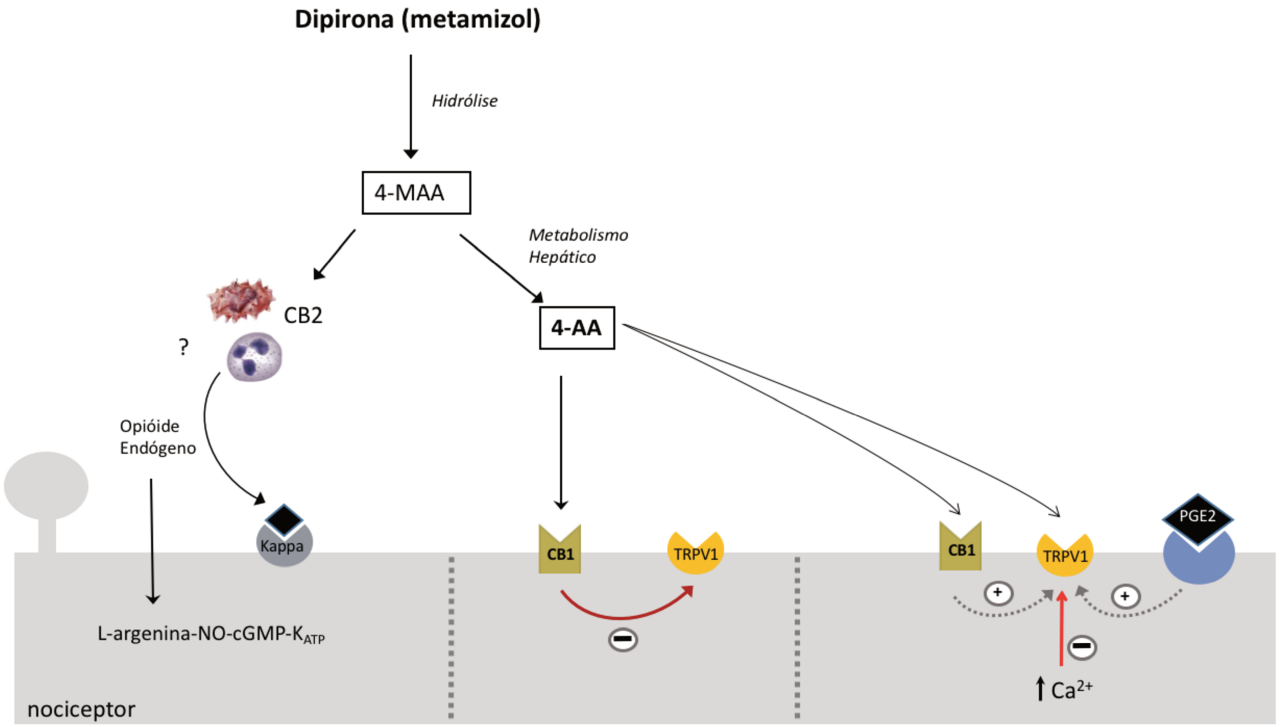
RESUMO E CONCLUSÃO

Os resultados apresentados neste estudo em ratos sugerem que:

- O efeito analgésico da dipirona é mediado por uma hidrólise local para o metabólito 4-metil-aminoantipirine (4-MAA).
- O metabólito 4-MAA reduz de maneira dose dependente a hiperalgesia mecânica induzida por carragenina.
- O efeito analgésico do metabólito 4-MAA é mediado por receptor canabinoide do tipo 2 (CB2).
- O efeito analgésico do metabólito 4-MAA é mediado por receptor kappa opióide.
- O metabólito 4-aminoantipirine (4AA) reduz a nocicepção induzida por capsaicina através da ativação de receptor canabinoide do tipo 1 (CB1).
- Prostaglandina E2 (PGE2) induz sensitização do receptor vanilóide de potêncial transiente (TRPV1) sem alterar o número de células responsivas.
- O metabólito 4-AA reverte completamente a nocicepção induzida por capsaicina quando os ratos são previamente sensibilizados com PGE2.
- O metabólito 4-AA de maneira dose dependente induz influxo de cálcio pela ativação de TRPV1 em neurônios sensibilizados por PGE2.
- O influxo de cálcio induzido por 4-AA leva desensitização de TRPV1 em neurônios sensibilizados por PGE2.
- CB1 esta envolvido no influxo de cálcio induzido por 4-AA contribuindo assim para a desensitização de TRPV1 em neurônios sensibilizados por PGE_2.

Sumarizando: Como esquematizado abaixo, os resultados do presente trabalho demonstram que a dipirona é localmente metabolizada em 4-MAA. Os dados sugerem que este metabólito promove analgesia pela interação entre o sistema canabiniode e opióide em particular pela ativação dos receptores CB2 e Kappa opioide. Por sua vez, o metabólito 4-AA reduziu a atividade das fibras TRPV1+ (fibras C) por dessensibilizarem os receptores TRPV1 via ativação dos receptores canabinoides CB1. Os dados deste trabalho também sugerem que a eficácia da dipirona em promover analgesia aumenta durante condições inflamatórias do tecido periférico.



Em conclusão, os dados deste estudo sugerem que as interações entre os sistemas canabinoide e opioide e os sistemas canabinoide e vanilóide no tecido periférico são alvos promissores no controle da dor inflamatória e a dipirona por ter como mecanismo de ação estas interações no tecido periférico comprova ser um medicamento eficaz e seguro no controle da dor inflamatória com poucos efeitos indesejáveis quando comparada a outros medicamentos atualmemte usados tais como antiinflamatórios, opioides e analgésicos de ação no sistema nervoso central.

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ANEXOS

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **Papel do monóxido do carbono sintetizado por hemo-oxigenase 1 sobre a ação anti-hiperalgésica da dipirona e seus metabolitos bioativos durante a dor inflamatória : Mecanismos implicados** (protocolo CEUA/UNICAMP nº **3772-1**), de responsabilidade do **Prof. Dr. Carlos Amilcar Parada** e **Gilson Gonlçalves Dos Santos**, teve o título alterado para **Analise da interação dos receptores canabinóides CB1 e CB2 com receptores TRPV1 e opióides no efeito analgésico da dipirona e seus metabólitos bioativos**.

Este documento é válido apenas se apresentado junto com o certificado emitido originalmente pela CEUA/UNICAMP em 08/04/2015.

Campinas, 15 de agosto de 2016.

Profa. Dra. Liana M. C. Verinaud
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CERTIFICADO

Certificamos que o projeto **"Papel do monóxido do carbono sintetizado por hemo-oxigenase 1 sobre a ação anti-hiperalgésica da dipirona e seus metabolitos bioativos durante a dor inflamatória : Mecanismos implicados"** (protocolo nº **3772-1**), sob a responsabilidade de **Prof. Dr. Carlos Amilcar Parada / Gilson Gonlçalves Dos Santos**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **08 de abril de 2015**.

Campinas, 08 de abril de 2015.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Mecanismo de ação analgésica da dipirona: Envolvimento dos receptores canabinóides CB1 e CB2 no tecido periférico**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 02 de julho de 2018.

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