UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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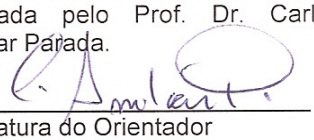
"MECANISMOS ENVOLVIDOS NA AÇÃO ANTI-HIPERALGÉSICA DO AGONISTA OPIÓIDE MU NO TECIDO PERIFÉRICO"

TESE apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Odontologia, Área de concentração em Fisiologia Oral.

Orientador: Prof. Dr. Carlos Amílcar Parada

Este exemplar corresponde à versão final da Tese defendida pela aluna Karla Elena Torres Chávez, e orientada pelo Prof. Dr. Carlos Amilcar Parada.



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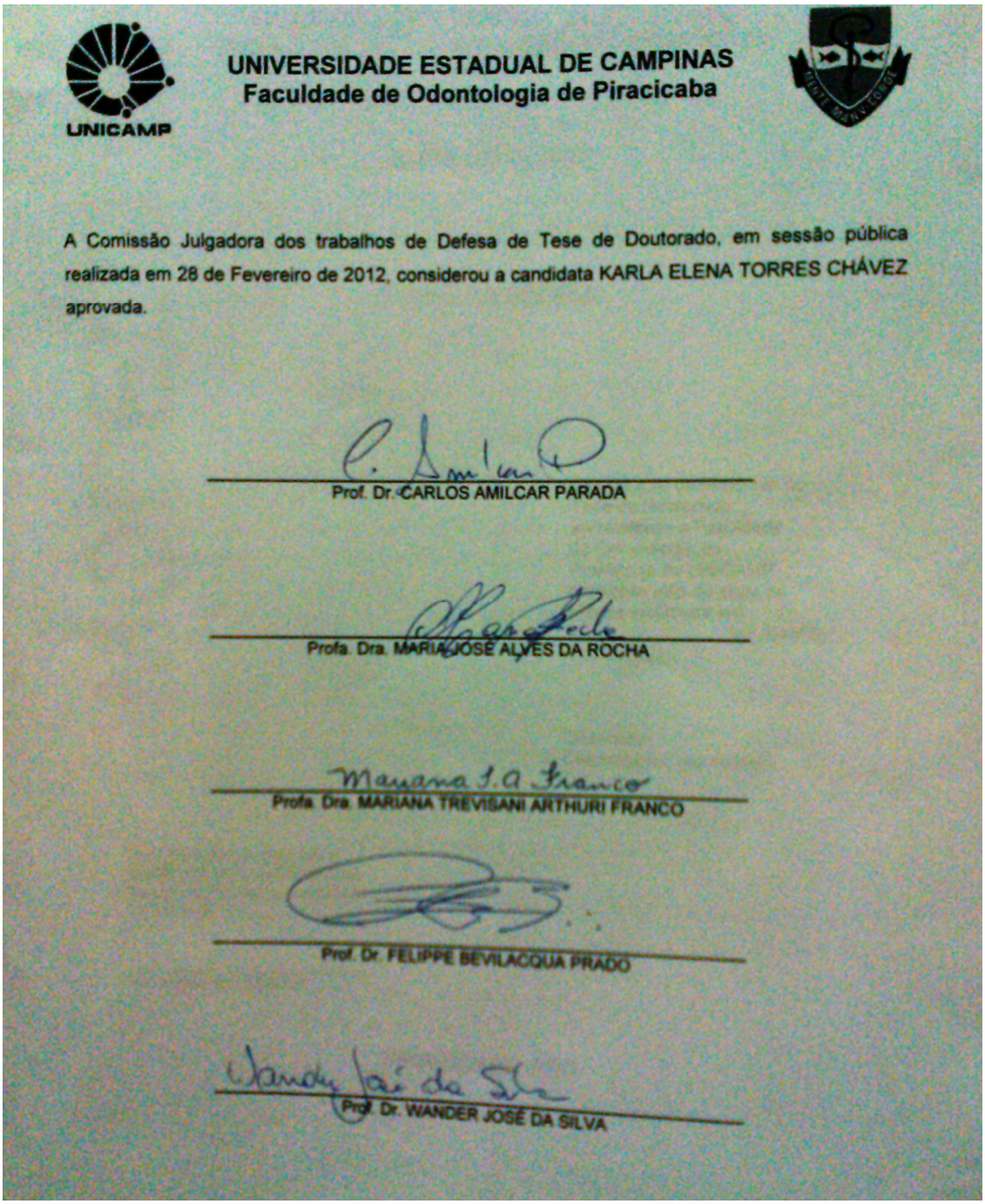

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A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 28 de Fevereiro de 2012, considerou a candidata KARLA ELENA TORRES CHÁVEZ aprovada.

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Dedico este trabalho com todo meu ser a Deus

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RESUMO

Os objetivos deste estudo foram: (1) Verificar se a administração local de prostaglandina E_2 (PGE_2) no tecido periférico aumenta o efeito anti-hiperalgésico da ativação do receptor opióide mu e se este efeito é mediado por um aumento da expressão de receptor opióide mu (2) Testar se o efeito anti-hiperalgésico da ativação do receptor opióide mu no tecido periférico está associada com a diminuição da excitabilidade das fibras-C. De acordo com o objetivo (1): A administração local de PGE_2 no tecido periférico de pata posterior de rato aumentou a expressão do receptor opióide mu em neurônios aferentes primários, bem como o efeito anti- hiperalgésico do agonista do receptor opióide um, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), administrado localmente no tecido periférico. (2): Uma pequena dose (0,5 μg) de capsaicina (agonista TRPV1) administrada na pata traseira de ratos induziu uma imediata (10 minutos) resposta nociceptiva comportamental como consequência do influxo de cátions nas fibras-C. A administração local de DAMGO reduziu o efeito anti-nociceptivo induzido pela capsaicina, que dependeu do aumento da expressão do receptor opióide mu no neurônio aferente primário. Tolbutamida, um bloqueador seletivo para a sub-unidade do receptor sulfonilurea – 1 (SUR-1) de canais de potássio adenosina trifosfato dependentes (K^+-ATP), reverteu o efeito anti-nociceptivo de DAMGO. Em conclusão, os dados deste estudo sugerem que a ativação de receptores opióides disponíveis nas terminações periféricas dos neurônios aferentes primários não são suficientes para promover analgesia, no entanto, a liberação de mediadores inflamatórios, tais como PGE_2, aumenta a expressão destes receptores, permitindo a ação analgésica dos opióides no tecido periférico. Finalmente, os dados deste estudo também sugerem que o efeito analgésico de opióides no tecido periférico está associado a uma diminuição da excitabilidade das fibras-C.

PALAVRAS – CHAVE: Hiperalgesia, Fibras C, PGE_2, DAMGO.

ABSTRACT

The aims of this study were:(1) To verify whether local administration of E_2 prostaglandin (PGE_2) in peripheral tissue increases the anti-hyperalgesic effect of mu opioid receptor activation and whether this effect is mediated by an increased expression of mu opioid receptor (2) To test if the anti-hyperalgesic effect of the activation of mu opioid receptor in peripheral tissue is associated with the decrease of C-fibers excitability. According to the objective(1): Local administration of PGE_2 in the peripheral tissue of rat hind-paw increased the mu opioid receptor expression on primary afferent neurons, as well as the anti-hyperalgesic effect of mu opioid receptor agonist, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin, (DAMGO) administrated locally in peripheral tissue. (2): A small dose (0,5 μg) of capsaicin (TRPV1 agonist) administrated in the rat hind-paw induced an immediate (10 minutes) nociceptive behavioral as a consequence of the influx of cations in C-fibers. The local administration of DAMGO reduced the anti-nociceptive effect induced by capsaicin that depends on the increase of mu opioid receptor expression in primary afferent neuron. Tolbutamide, a selective blocker of SUR-1 sub-unit of ATP-dependent potassium channel (K^+-ATP), reversed the anti-nociceptive effect of DAMGO. In conclusion, data of this study suggest that the activation of opioid receptors available on the peripheral endings of primary afferent neurons are not enough to promote analgesia, however the release of inflammatory mediators, such asPGE_2,increases the expression of these receptors, allowing the analgesic action of opioids in peripheral tissue. Finally, data of this study also suggest that the analgesic effect of mu opioid receptor activation in peripheral tissue is associated with a decrease do C-fibers excitability.

KEY WORDS: Hyperalgesia, C-fibers, PGE_2, *mu* opioid receptor

LISTA DE ABREVIATURAS E SIGLAS

PGE_2	- prostaglandina E_2
DAMGO	- [D-Ala2, N-MePhe4, Gly-ol]-enkephalin
SUR	- receptor sulfonilurea
K^+-ATP	- canais potássio adenosina trifosfato dependentes
SP	- substância P
CGRP	- peptídeo relacionado ao gene da calcitonina
TRPV	- receptores de potencial transitório vaniloide
GRD	- gânglio da raiz dorsal
SNC	- sistema nervoso central
TTXr	- de canais de sódio tetrodotoxina-resistentes
AMPc	- adenosín monofosfato cíclico
L-arginina-NO-GMPc	- via L-arginina-óxido nítrico-guanosina monofosfato cíclico
KIR	- canais de potássio retificadores de influxo
GIRK	- potássio acoplados a proteína G

SUMÁRIO

I. INTRODUÇÃO

A dor é uma experiência sensorial e emocional desagradável, associada a dano presente ou potencial, ou descrita nesses termos (IASP - International Association for the Study of Pain). Embora seja um sinal de alerta importante para preservar a integridade do organismo, quando é de grande magnitude ou se torna recorrente pode comprometer a qualidade de vida pelo desconforto, distração, ou diminuição da volição (Melzack, 1999). Para o controle da dor aguda severa, os fármacos mais utilizados são os opióides. No entanto, seu uso prolongado é dificultado pelos seus efeitos colaterais tais como a depressão do sistema respiratório, náuseas, constipação intestinal, adição e/ou tolerância (Kieffer and Gaveriaux-Ruff, 2002; Stein *et al.*, 2003). O sistema opióide promove analgesia através de diversos mecanismos, dentre eles a ativação das vias descendentes e a inibição da transmissão nociceptiva no corno dorsal da medula espinal (Fields *et al.,* 1977; Fields and Heinricher, 1989; Proudfit and Yeomans, 1995; DeLeo, 2006).

Foi demonstrado que o tráfego de receptores opióides do gânglio da raiz dorsal (GRD) para as terminações nociceptivas periféricas aumenta quando ocorre um processo inflamatório no tecido periférico(Antonijevic et al., 1995; Stein *et al*., 1995).

Os receptores opióides, são expressos tanto em neurônios do sistema nervoso central (SNC) quanto periférico, além de células do sistema endócrinológico (hipófise, glândula supra-renal), I imunológicos, e células ectodérmicas (Zollner and Stein, 2007). Existem três tipos principais de receptores opióides: mu (μ), kappa (κ) e delta (δ), os quais estão presentes também em áreas envolvidas no controle da dor, tal como a substância cinzenta periaquedutal, o corno dorsal da medula espinal e nos nervos aferentes primários (Fields *et al*., 1980; Stein *et al.,* 1995; Coggeshall *et al.*, 1997; Phillips and Currier, 2004). Alguns autores propõem que esses receptores são expressos em neurônios do GRD de pequeno, médio, e grande diâmetro (Mansour *et al*., 1994; Buzas and Cox, 1997; Coggeshall et al., 1997; Chen *et al*., 1997; Zhang *et al.*, 1998a; Zhang *et al*., 1998c; Wang and Wessendorf, 2001; Silbert *et al.*, 2003; Rau *et al.*, 2005; Gendron *et al.*, 2006), outros que são expressos em neurônios peptidérgicos de pequeno diâmetro, associados com a expressão de substância P (SP) e peptídeo relacionado ao gene da calcitonina (CGRP) (Minami *et al*., 1995; Li *et al.*, 1998; Zhang *et al.,* 1998b; Zhang *et al.,* 1998c; Stander *et al.,* 2002; Mousa

et al., 2007a; Mousa *et al.,* 2007b). Receptores opióides são transportados para os terminais do nervo periférico (Hassan *et al.*, 1993; Li *et al.*, 1996; Mousa *et al.*, 2001), onde são acoplados a proteínas Gienvolvidas com sinais efetores da inibição daadenilato ciclase e modulação de canais iônicos (Zollner and Stein, 2007). A diminuição de correntes de Ca^2parece ser um mecanismo importante para a inibição das funções dos neurônios sensoriais (Akins and McCleskey, 1993). Recentemente, canais de potássio acoplados a proteína G (GIRK) foram identificados em terminações nervosas sensoriais na epiderme(Khodorova *et al.*, 2003), mas nenhuma evidência direta de acoplamento funcional ou modulação da corrente de potássio nos reurônios do GRD foi fornecida até o momento. No entanto, receptores opióides nos neurônios do GRD suprimimem a correntes iônicas de canais de sódio tetrodotoxina-resistentes (TTXr) (Ingram and Williams, 1994), bem como de receptores de potencial transitório vaniloide 1 (TRPV1) via inibição de adenosina monofosfato cíclico (AMPc) (Endres-Becker *et al.*, 2007). Os receptores tipo *mu* são responsáveis pela maior parte da analgesia induzida por um opióide no tecido periférico, no entanto os receptores *k* e δ também podem contribuir para esta analgesia (Stein, 1993; Holden *et al.,* 2005).

A ação da morfina no SNC diminui o limiar nociceptivo mecânico (Wang *et al.*, 2006), porêm estudos experimentais demonstraram que a administração de morfina ou outros agonistas de receptor opióide no tecido periférico não inflamado, em doses que possuam apenas ação periférica, não alteram o limiar nociceptivo mecânico da pata de ratos (Ferreira and Nakamura, 1979a). Estudos também indicam que os opióides atuam diretamente nos neurônios nociceptivos periféricos (Ferreira and Nakamura, 1979a; Duarte et al., 1992) para produzir um efeito anti-hiperalgésico revertendo a sensibilização dos neurônios nociceptivos primários pela ativação da via L-arginina-óxido nítrico-guanosina monofosfato cíclico (L-arginina-NO-GMPc) (Ferreira et al., 1991; Duarte *et al.,* 1992). Assim também, a ação analgésica dos opióides no tecido periférico, estaria ligada apenas à redução da sensibilização dos neurônios aferentes primários, decorrente do processo inflamatório. No processo de sensibilização do neurônio nociceptivo periférico (Sharma *et al.*, 1975; Makman *et al*., 1988; Levine and Taiwo, 1989), a ativação dos receptores opióides inibe a adenilciclase, e conseqüente reduz a concentração intracelular de AMPc(Levine and Taiwo, 1989; Ferreira *et al.*, 1991; Duarte *et al.*, 1992).

Parte dos resultados originados deste trabalho de tese, que se encontram em fase de preparação do manuscrito, sugere que a administração de morfina no tecido periférico, não altera o limiar nociceptivo mecânico. Esse fato é evidenciado pelo limiar nociceptivo mecânico basal, não ser transmitido, pelo menos na sua maioria, por fibras nociceptivas tipo C. Embora este evento nunca tenha sido demonstrado sistematicamente por meio de experimentos, sabe-se que as fibras nociceptivas mielinizadas Aδ possuem uma velocidade de condução bem superior às fibras não mielinizadas do tipo C (Djouhri *et al.*, 1998; Basbaum and Jessell, 2000; Julius and Basbaum, 2001). Baseado nesta diferença entre as velocidades de condução dos estímulos nociceptivos nas fibras C e Aδ, é conceitualmente aceito que os estímulos nocivos que resultam em uma sensação de dor rápida, fina e bem localizada em geral, refletem a ativação de fibras Aδ e a nocicepção difusa e lenta, em queimação, é desencadeada por fibras C, as quais estão mais intimamente relacionadas com a hiperalgesia inflamatória.

É importante também salientar o fato do receptor TRPV1 ser um receptor ionotrópico, cujo canal catiônico acoplado é permeável aos íons Na^+ e Ca^{2+} e são predominantemente expressos em neurônios sensoriais (fibras tipo C) do GRD de mamíferos (Caterina *et al.*, 1997; Cortright et al., 2001). Tem sido sugerido que o receptor TRPV1 funcione como um amplificador molecular no neurônio nociceptivo durante a transmissão da dor inflamatória. Portanto, agonistas dos receptores TRPV1, tais como a capsaicina podem ser utilizados para investigar o papel das fibras tipo C na transmissão da dor. Também tem sido descrito, que o tratamento com a capsaicina pode resultar em dessensibilização ou apoptose das fibra sensorial TRPV1provávelmente (NÃO TEM ACENTO NO A) envolvendo a dessensibilização do canal (Szallasi and Blumberg, 1999) ou toxicidade celular devido ao prolongado influxo de cálcio (Tominaga, 2007).

Estudos in vivo, demonstraram que o inibidor de K^+-ATP, glibenclamida, reverte o efeito anti-hiperalgésico mediada(O) por opióides ou pela ativação da via L-arginina-NO-GMPc(Rodrigues and Duarte, 2000). A ativação dos canais K^+-ATP revertem a sensibilização induzida pela PGE_2, evidenciando uma provável importância destes canais na modulação no aumento da excitabilidade neuronal, resultado de condições inflamatórias ou injuriantes (Chi *et al.*, 2007).

Os canais K^+-ATP estão distribuídos amplamente numa variedade de tecidos e tipo de células onde eles induzem alterações metabólicas em decorrência da atividade elétrica da membrana plasmática (Ashcroft and Gribble, 1998). A abertura dos canais K^+-ATPpermite o deslocamento do potencial da membrana para um potencial de equilíbrio dos íons potássio (Aguilar-Bryan *et al.*, 1998; Rodrigues and Duarte, 2000), evidenciando que estes canais, poderiam apresentar um papel no controle da excitabilidade neuronal (Hille, 1992; Yamada and Inagaki, 2005).

Os canais K^+-ATP são formados por duas subunidades, uma relativa ao canal seletivo ao íon K^+ do tipo canais de potássio retificadores de influxo (Kir) 6.1 ou Kir 6.2 e, uma subunidade regulatória (SUR) 1 ou SUR2 (Noma, 1983; Aguilar-Bryan *et al.*, 1995; Aguilar-Bryan *et al.*, 1998; Liss and Roeper, 2001). Embora tenha sido demonstrado que o K^+-ATP está envolvido na ação anti-hiperalgésica dos opióides no tecido periférico, sugerindo que a abertura destes canais poderiam promover a hiperpolarização das fibras nociceptivas sensibilizadas Assim também, um estudo eletrofisiológico e populacional de células do GRD, demonstrou que estes canais são expressos apenas em quantidades diminutas nas membranas citoplasmáticas de alguns poucos neurônios nociceptivos primários o que aparentemente não justificaria o efeito anti-hiperalgésico dos opióides no tecido periférico (Ristoiu *et al.*, 2002). De fato, na maioria das células, os K^+-ATP são expressos nas mitocôndrias, exceção feita a algumas células, tais como as células betas pancreáticas e células do miocárdio (Ocana *et al.*, 1990; Soares *et al.*, 2000). Embora se saiba que os receptores opióides são expressos quase exclusivamente nas fibras de pequeno diâmetro~~,~~ tipo C (Przewlocki and Przewlocka, 2005) ainda não existem dados quanto à localização dos canais K^+-ATP nas fibras tipo C, nem quanto à modulação destes canais pelos mediadores inflamatórios, a exemplo do que ocorre com os próprios receptores opióides.

Entretanto, considerando que a inflamação aumenta o tráfego neuronal de receptores opióides para tecido periférico (Hassan *et al.*, 1993; Stein, 1995) e que a PGE_2 é um importante mediador inflamatório associado com o aumento da sensibilização dos neurônios nociceptivos primários (Ferreira, 1972; 1979; Ferreira and Nakamura, 1979b; Smith et al., 1982), é plausível que um aumento da densidade de receptores opióides nas fibras nociceptivas periféricas seja importante para o efeito anti-hiperalgésico ou anti-

nociceptivo dos opióides nos neurônios nociceptivos primários do tecido periférico. Portanto, os objetivos deste estudo foram: (1) Verificar se a administração da PGE_2 no tecido periférico incrementa a expressão do receptor opióide mu nos neurônios aferentes primários, permitindo um efeito anti-hiperalgesico; (2) Verificar se o efeito anti-hiperalgésico da ativação do receptor opióide μ no tecido periférico esteja associado com a diminuição da excitabilidade das fibras-C.

CAPITULO 1.

Prostaglandin E_2 increases the expression of mu opioid receptor in primary afferent neuron allowing the effect anti-nociceptive and anti-hyperalgesic of opioids.

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Abstract – The mechanism of action of morphine in the peripheral terminal of primary afferent neurons involves the opening of ATP sensitive potassium channel (KATP). The anti-hyperalgesic effect of morphine in the peripheral tissue is generally associated only with the reduction of primary afferent neurons sensitization, because the administration of morphine does not change the nociceptive threshold in non-inflamed tissue. Given that the anti-hyperalgesic effect of morphine involves mainly the activation of mu opioid receptors, the aim of this study was to test the hypothesis that the anti-hyperalgesic effect induced by the activation mu opioid receptors in inflamed peripheral tissue is mediated by an increase in the expression of mu opioid receptor in primary afferent neuron by prostaglandin E_2 allowing mu opioid receptor agonists decrease the transmission of nociceptive stimuli through C-fibers. Local administration in the rat hind-paw of 100 ng of Prostaglandin E_2 (PGE_2), but not 30 ng, increased the expression of mu opioid receptor in the saphenous nerve quantified by Western blot 3h and 24h after PGE_2 administration. DAMGO (0.001; 0.01; 0.1; 1 and 5 ng / paw), a mu opioid receptor agonist, locally administrated in hind-paw, inhibited in a dose-dependent manner the mechanical hyperalgesia induced by 100 ng, but not

by 30 ng of PGE_2. The mechanical nociceptive threshold was measured 3h after PGE_2 administration and DAMGO was administrated 30 minutes before the measurement. Capsaicin (0.5; 1 and 3 ng / paw) administrated in the normal hind-paw induced nociceptive behavior (flinches) quantified during 10 minutes following its administration. DAMGO (5 ng / paw) greatly reduced the number of flinches induced by capsaicin (0.5 μg / paw) administrated in hind-paw pre-treated with PGE_2 (100 ng / paw) 3h or 24h before, when compared with the number of flinches induced by capsaicin administrated in normal not-inflamed hind-paw. In conclusion, the data of this study strongly suggest that the anti-hyperalgesic effect mediated by morphine in inflamed peripheral tissue is associated with an increased of mu opioid receptor on primary afferent neurons induced by PGE_2 and with the decreased of excitability of C-fibers ($TRPV1^+$) mediated by mu opioid receptor activation.

Key words: Prostaglandin E_2, mu opioid receptor, anti-nociceptive, anti-hyperalgesic, opioid, C-fibers.

INTRODUCTION

Opiods are the most powerful and the most used drugs to control severe pain, however their prolonged use is difficult because of their side effects (Kieffer and Gaveriaux-Ruff, 2002). Opioids promote analgesia by acting in both Central and Peripheral Nervous System (Fields et al., 1977; DeLeo, 2006). In the Central Nervous System opioid receptors are coupled to a variety of effectors, including G protein-activated potassium channels (GIRK) (DeLeo, 2006). GIRK channels have been shown to be involved in opioid-induced analgesia by inducing membrane hyperpolarization of the neurons as a consequence of potassium ions efflux that ultimately reduces neural excitability. However, in the peripheral tissue the anti-hyperalgesic effect of opioids would be associated only to the reduction of primary afferent neurons sensitization. Indeed, the activation of opioid receptors inhibits adenylatecyclase activity and the subsequent reduction of cyclic AMP, which is involved in the sensitization of primary afferent neurons. In this vein, most studies demonstrate that local administration of morphine or other opioid receptors agonists, on normal peripheral tissue, does not alter the mechanical nociceptive threshold on rats hind paw (Gonzalez-Rodriguez *et al.*, 2010). However, it has been demonstrated that opioid

receptor activation on primary afferent neurons also reverts the hyperalgesia by activating L-argenina-NO-GMPc signaling pathway, which promotes the opening of ATP sensitive potassium channel (KATP) (Ferreira et al., 1991; Duarte et al, 1992). Once opened, KATP would facilitate efflux of potassium ions that, similarly to Central Nervous System opioid receptors, ultimately would reduce neural excitability (Ferreira et al., 1991; Duarte et al., 1992). Nevertheless, it is not clear whether this mechanism is involved in the anti-hyperalgesic effect of opioid receptors activation on the peripheral tissue.

In addition, inflammatory agents, such as CFA, increases the traffic of opioid receptors from the Dorsal Root Ganglia (DRG) to the peripheral nociceptive terminals (Stein, 1995), suggesting that inflammatory mediators increase the expression of opioid receptors on primary afferent neurons.

Therefore, the aims of this study were verify whether Prostaglandin E_2 administrated in peripheral tissue increases the expression of opioid mu receptor on primary afferent neurons enabling its effect anti-hyperalgesic, and test the hypothesis that the anti-hyperalgesic effect of opioid mu receptor activation in the peripeheral tissue is associated with the decrease of neuronal excitability of C-fibers.

EXPERIMENTAL PROCEDURES

Animals

Male albino Wistar rats weighing 180 – 280 g obtained from CEMIB/UNICAMP (Centro de Bioterismo - State University of Campinas - UNICAMP, Campinas, SP, Brazil) were used. Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of IASP on using laboratory animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas - UNICAMP. Animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 07:00 A.M.) with food and water available *ad libitum*. They were maintained on a temperature-controlled room (± 23ºC) for a 15 min habituation period prior to the test.

Drugs

Drugs used in this study were obtained from Sigma (St. Louis, MO): Prostaglandin E_2 (PGE_2; 10, 30, 100 and 300µg/paw) and capsaicin (0,5; 1 and 3µg/paw), and from Research Biochemicals Int.: ([D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin; DAMGO; 0,01; 1; 5; 10ng/paw). DAMGO was dissolved in saline (0.9% NaCl). The stock solution of PGE_2 (0.5 µg/µl) was prepared in 10% ethanol, in 10% tween 80 and the final diluted were in 0.9% NaCl (saline) immediately before use to different doses as indicated the final concentration of ethanol was ≤1%.

Subcutaneous Injections

All drugs or their vehicle were administrated locally (subcutaneous s.c.) of the rat's hind paw, between the five distal footpads, by a 30-gauge needle connected to a catheter of polyethylene (PE-50; Intramedic, Clay Adams, Becton-Dickinson, Franklin Lakes, NJ, USA) and also to a 50 µl syringe (Hamilton, Reno, NV, USA). Animals were briefly restrained and the volume of injection was 50µl.

Mechanical nociceptive test: Electronic Pressure Meter

The paw hyperalgesia was also measured with an electronic pressure-meter. The rats were placed in acrylic cages (12 × 20 × 17 cm high) with a wire grid floor 15 min before beginning the tests. During this adaptation period, the paws were poked two to three times. Before paw stimulation, the animals should be quiet, without exploratory or toilet movements, and not resting over the paws. In these experiments a pressure-meter, which consisted of a hand-held force transducer adapted with a 0.7-mm^2 polypropylene tip (electronic von Frey anesthesiometer; IITC Inc. Life Science Instruments, Woodland Hills, CA), was used. The investigator was trained to apply the polypropylene tip perpendicularly in between the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hindpaw. The stimulus was automatically discontinued and its intensity recorded when the paw was withdrawn. The maximum force applied was 60 g. The stimulus was repeated (up to six times, usually three) until the animal presented similar measurements (differences <10%). The end point was characterized by the removal of the paw in a clear flinch response after the paw

withdrawal. The animals were tested before and after treatments. The results are expressed by the Δ withdraw threshold (in g) that was calculated by subtracting the average of the last three measurements after the treatments from the average of three measurements before treatments.

Measurement of behavioral nociceptive responses

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23 °C (Rosland, 1991). Before the experiments, each animal was manipulated for 7 days to be habituated to the experimental manipulation. On the day of the experiment, each animal was individually placed in a test chamber (30x30x30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. Following the s.c. injection, the behavioral nociceptive response was quantified by counting the number of spontaneous flinches of the injected paw during 10 min. The recording time was divided into five blocks of 3 min. Rats did not have access to food or water during the test and each animal was used once.

Western blot analysis

Animals are initially anesthetized intraperitoneally with a mixture of urethane and α-chloralose (100 mg / kg and 50 mg / kg, respectively). To determine the analgesic efficacy mu opioid receptor with its concentration in primary afferent neurons (was removed a portion of the saphenous nerve 5.0 mm, 1.0 cm above the bifurcation of the saphenous nerve at the knee, immediately frozen in liquid nitrogen, and stored al -80^{o}C). For protein analysis, each sample were individually homogenized in 3 ml per gram of tissue in RIPA buffer containing TAPS (0.5 mM TAPS, 0.2 M EDTA, 20% glycerol), sucrose (0.25 M) and imidazole (3 mM) at pH 7.5 and a combination of protease inhibitors (2 µg / ml aproptinin, 2 mM phenylmethylsulfonyl fluoride, 50 µg/mL leupeptin), using a sonicator (Branson Sonifier, model 185, Danbury, CT, USA) operated at full speed for 30 seconds. The quantification of total protein was performed by the method of BCA Kit, Pierce. The samples of the protein were treated with Laemmli buffer containing 100 mM of DTT, and heated at 65^{o}C for 15 min. Then 25-50 µg of protein was applied in 8% polyacrylamide gel electrophoresis in an apparatus of the mark or Biorad Novex. Protein markers (Novex)

including myosin (205 kd), BSA (98 Kd), glutamic dehydrogenase (64 kd), carbonic anhydrase (36 kD), myoglobin (30 kD), lizosima (16 Kd) apropitina (6 kd) chain insulin (4 kd), were subjected to electrophoresis to estimate molecular weight. Protein electrophoresis gel by electroelution nitrocellulose is carried out at 30 V for 75 minutes. Nonspecific binding of antibodies in the nitrocellulose paper is reduced by pre-incubating the membranes in a blocker containing 5% milk for 30 min. The membrane is subsequently incubated at 4 ° C overnight, using the antibody specific for mu opioid receptors, (Anti-OPRM1). The next morning the "blots" were washed in washing buffer (50 mM Na2PO4, 150 mM NaCl and 0.05% Tween 20) for 60 minutes and then incubated at room temperature for 1h with anti-imunogobulina , rabbit conjugated to peroxidase (Dako, Carpinteria, CA, USA), used at a dilution of 1:2500. New washing was carried out as described above. To detect immunoreactive bands, the "blots" are exposed to chemiluminescence solution (LumiGlo chemiluninescense substrate, Kirkegard and Perry Gaithersubrg, MD. USA) for 1 minute, followed by exposure to an X-ray film-OMAT AR (Eastman Kodak Co , Rochester, NY, USA). After developing the film the "blots" are washed with India ink (1 µL/100 ml) for 2hrs to visualize the protein bands and confirm the equivalence of them. The densities of the bands on film and samples will be scanned and "saved" on computer disks for subsequent quantification of optical densitometry using the NIH image system 1.57 program. The expression of alpha-tubulin is used as a positive control the expression of the mu opioid receptor for each sample and normalized relative to ponceau. The data were analyzed by paired t-test.

Experimental design

To establish the dose of PGE_2 able to sensitize the nociceptors of rats hind paw, was initially performed a dose-response curve of PGE_2 (300, 100, 30, 10 ng/ paw) to sensitize nociceptors of the intraplantar (i.pl.) of the rat hind paw. Once established the sub-maximal doses (100 ng/paw) and intermediate dose (30 ng/paw) of PGE_2, was verified the anti-hyperalgesia effect of PGE_2 through dose-response curves of DAMGO (0.01, 0.5, 1, 5 ng/paw). The systemic effect was tested by contralateral injected of DAMGO 5 or 10 ng/paw in contralateral paw. To evaluated the expression of mu-opioid receptor in peripheral nerve was used the inmunobloting test. To test the role of mu opioid receptor on

nociception, we tested the DAMGO effect on capsaicin-induced flinches for 10 minutes after its administration. The maximum effective and local dose of DAMGO was used to verify its effect anti-hyperalgesic and anti-nociceptive. DAMGO was administrated 30 min before the measurement of PGE_2-induced hyperalgesia or capsaicin administration.

Statistical analysis

The significant difference among treatment groups was determined by One-way ANOVA following by the post-hoc Tukey test ($P < 0.05$).The effectiveness of the anti-hyperalgesic effect of DANGO was determined by comparing the slope of different inhibitory curves. The used parameters of inhibitory regression curves were standard deviation of sum of squares and R^2. Data are expressed in figures as the decrease with paw-withdrawal threshold or number of flinches, and presented as means ± S.E.M.

Results

Mechanical hyperalgesia induced by administration of PGE_2 in peripheral tissue

We tested whether the administration of PGE_2, on peripheral tissue increased the opioid mu receptor expression on rats saphenous nerve. As shown on figure 1, this increase of opioid mu receptor expression was observed.

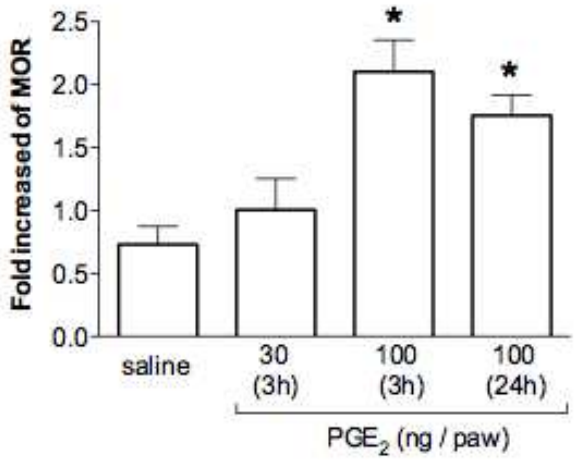


Fig. 1. Prostaglandin E_2 (PGE_2) 100 ng/ paw, but not 30 ng /paw, locally administrated in the rat hind-paw, increased the expression of mu opioid receptor (MOR) on the saphenous nerve 3h and 24 h after its administration.The symbol "*" means

significantly different from saline control group ($p < 0.05$; ANOVA and post-hoc Tukey test; n = 4 samples per group).

To establish the dose of PGE_2 able to sensitize the nociceptors of rats hind paw, it was made a dose-response curve of PGE_2. The dose of 100 ng was considered sub-maximal and the dose of 30 ng was considered intermediate (figure 2, panel A). Once stablished the sub-maximal and the intermediate doses of PGE_2, the next step was to verify, through dose-response curves, the effect of different DAMGO doses on the hiperalgesic effect of PGE_2 on the doses of 30 ng and 100 ng. Administration of DAMGO (0,01; 0,5; 1 ou 5ng) did not alter the 30 ng PGE_2-induced hiperalgesia (figure 2, panel B). However, same doses of DAMGO, administrated on the same conditions, reduced on a dose-dependent manner the 100 ng PGE_2-induced hiperalgesia (figure 2, panel B).

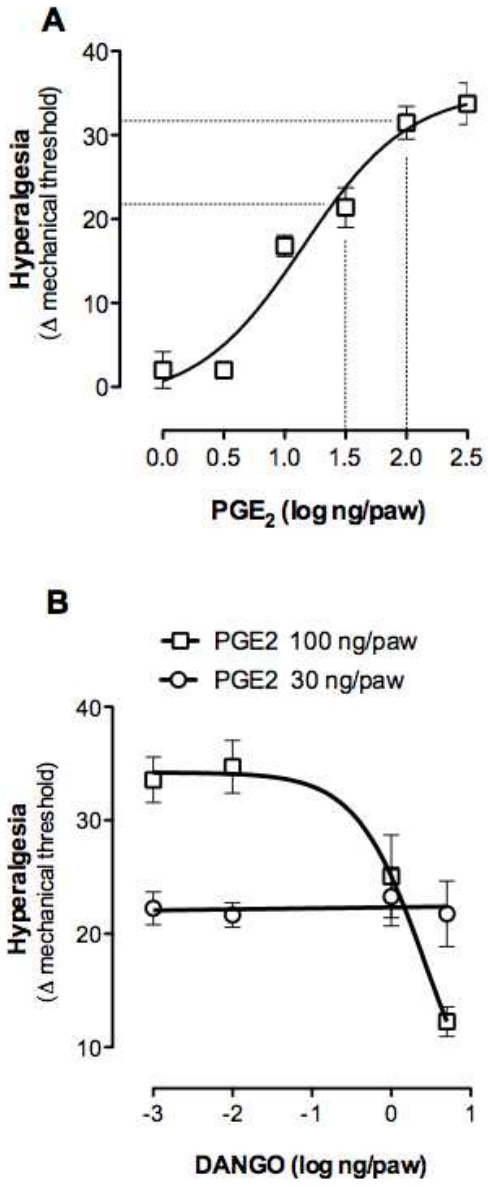
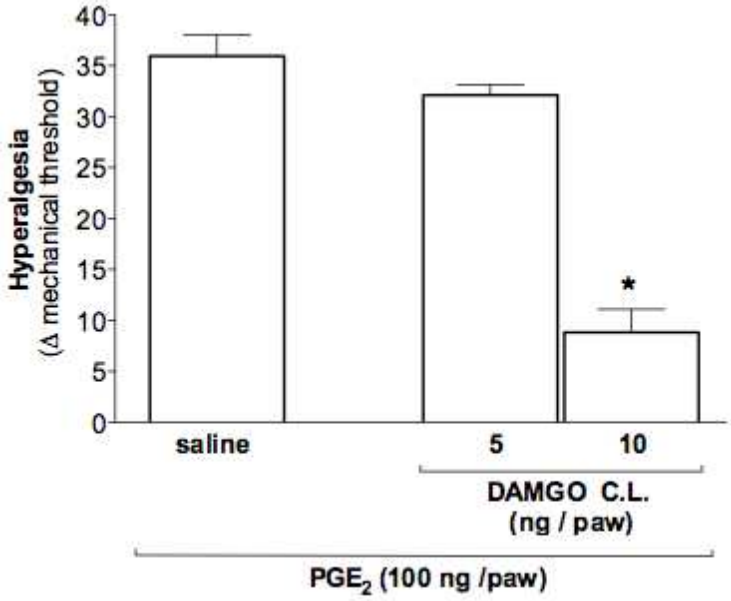


Fig. 2. PGE_2-inducedhyperalgesia and its inhibition by DANGO **(A)** The administration of prostaglandin E_2 (PGE_2; 1, 3, 10, 30, 100 and 300 ng /paw) induced mechanical hyperalgesia in a dose-dependent manner expressed as a non-linear regression curve (R^2 = 0.887; Standard deviation of sum of squares, Sy.x = 4.68). (**B)** The local administration of DAMGO (0.001, 0.01, 1 and 5 ng / paw) decreases in a dose-dependent manner the hyperalgesia induced by 100 ng but not 30 ng of PGE_2. Data are expressed as an inhibitory regression curve to 100 ng of PGE_2 (R^2 = 0.997; Sy.x = 0.897) or linear regression to 30 ng of PGE_2 (R^2 = 0.04; Sy.x = 0.886. Slope does not differ from zero, *P* = 0.793). The hyperalgesia was measured 3h after PGE_2 administrations (n = 5 - 9 per group).

To rule out a systemic effect of DAMGO, the doses of 5 ng and 10 ng were administrated on the contra-lateral paw. Only the dose of 10 ng had an anti-hiperalgesic effect, showing that this dose is capable of reducing hiperalgesia by activating opioid receptors of the Central Nervous System (figure 3A). As shown on figure 3B, the doses of 5 ng of DAMGO or morphine reduced the PGE2-induced hyperalgesiawith a similar magnitude ($P > 0.05$; ANOVA, Tukey test).

A



B

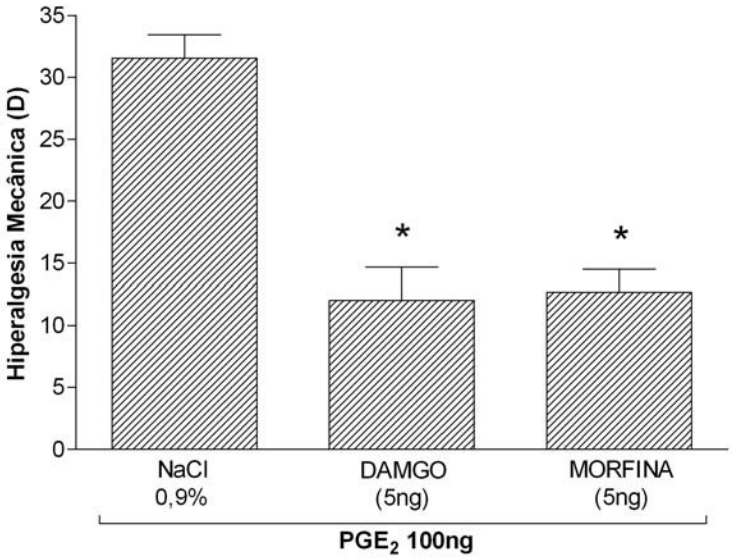


Fig. 3. (A) Local administration of 10 ng / paw, but not 5 ng / paw of DAMGO in the contra-lateral paw (C.L.) blocked the hyperalgesia induced by PGE_2 (100 ng /paw). The symbol "*" means significantly different from saline control group ($p < 0.05$; ANOVA and post-hoc Tukey test; n = 5 - 6 per group). **(B)** DAMGO (5ng) or morphine (5ng) reduced the PGE_2-induced hyperalgesiawith a similar magnitude. The symbol "*" means significantly different from saline control group ($p < 0.05$; ANOVA and post-hoc Tukey test; n = 6 samples per group).

Then, to test the role of mu opioid receptor on nociception mediated by C-fibers, we tested the DAMGO effect on capsaicin. Seeing that capsaicin induces nociception mediated exclusively by C fiber (TRPV1), we evaluated the capsaicin dose according to the nociceptive response (flinches) immediately after its administration, ruling out nociceptive responses from sensitized neurons. To avoid apoptosis, it was used the minimal effective capsaicin dose (0,5 µg/pata) that was able to induce immediate nociceptive response (figue 4, panel A). Once stabilished the capsaicin dose used on the study, we tested the anti-nociceptive effect of DAMGO (figure 4, panel B).

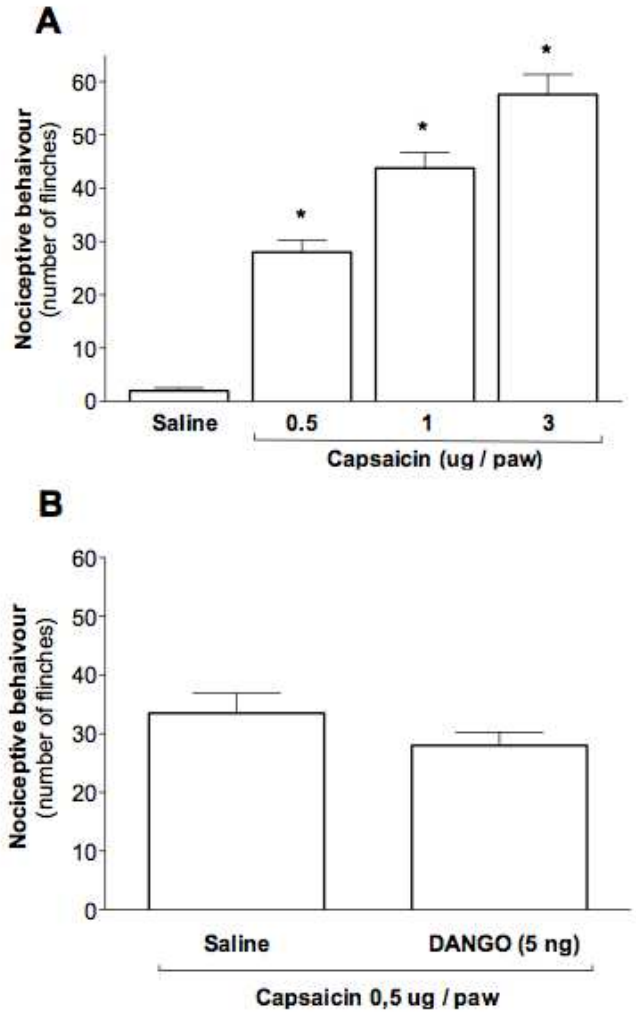


Fig. 4. Localadministration of capsaicin (0.5, 1 or 3 µg / paw) in the hind-paw induces flinches quantified for 10 minutes following its administration (**A**) Local administration of DAMGO (5 ng / paw) did not change the number of flinches induced by capsaicin (0.5 µg / paw) administrated 30 minutes before. **(B)** Anti-nociceptive effect of DAMGO (5 ng / paw). The symbol "*" means significantly different from saline control group (ANOVA and post-hoc Tukey test; n = 6 per group).

As shown on figure 4, the administration of DAMGO (5 ng) 30 minutes before capsaicin administration did not alter nociceptive response induced by capsaicin (0,5 µg/paw). Since we had seen that PGE_2 increases opioid receptors traffic, we tested whether

PGE_2 alters the antinociceptiveeffect of opioid mu agonist. As shown on figure 5, PGE_2 increased the opiod mu agonist effectiveness, reducing the nociception to a lower level than the basal response. These data show that the antinociceptive effect of opioid agonists depends on the increase of receptors expression on the primary afferent neuron.

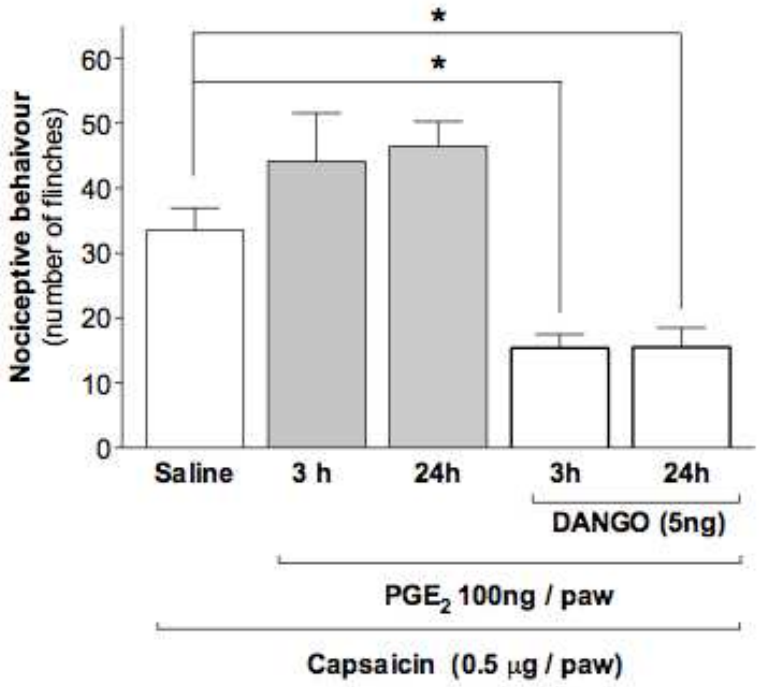


Fig. 5. Local administration of DAMGO (5 ng / paw) decreased the number of flinches induced by capsaicin in hind-paws pre-treated with Prostaglandin E_2 (PGE_2; 100 ng /paw) 3h or 24h before, when compared with capsaicin administrated in normal hind-paws. DAMGO was administrated 30 minutes before capsaicin. The symbol "*" means significantly different from saline control group ($p < 0.05$; ANOVA and post-hoc Tukey test; n = 6 - 8 per group).

To test whetherthe anti-nociceptive effect of DAMGO was mediated by K^+-ATP, tobultamide, a selective antagonista of the SUR-1 sub-unit of K^+-ATP was administrated 5 minutes before DAMGO administration. As showed in the figure 6, tobultamide (80, 160 or 320 μg) reverted in a dose-depend manner the effect anti-nociceptive of DAMGO (5 ng/paw).

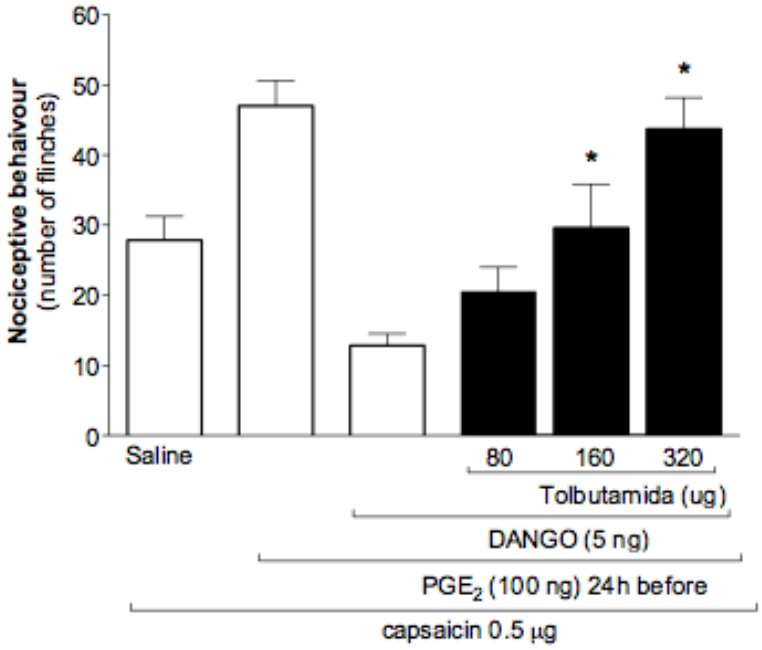


Fig. 6. Local administration of DAMGO (5 ng / paw) decreased the number of flinches induced by capsaicin in hind-paws pre-treated with Prostaglandin E_2 (PGE_2; 100 ng /paw) 24h before, when compared with capsaicin administrated in normal hind-paws. DAMGO was administrated 30 minutes before capsaicin. Tolbutamide (80, 160 or 320 µg /paw) administrated 5 minutes before DAMGO reverted its effect. The symbol "*" means significantly different from DAMGO group ($p < 0.05$; ANOVA and post-hoc Tukey test; n = 5 - 8 per group).

DISCUSSION

This study suggests that the anti-hyperalgesic effect mediated by morphine in inflamed tissue depends on the increase of mu opioid receptor on primary afferent neurons induced by inflammatory mediators, such as PGE_2 and the anti-hyperalgesic mechanism of action of morphine in peripheral tissue is associated with decreasing of excitability of C-fibers.

Although there were no data demonstrating that PGE_2 is able to increase mu opioid receptor expression in primary afferent neurons, it has been demonstrated that inflammatory agents, such as CFA, administrated in the peripheral tissue, increases the

synthesis and /or the axonal transport of opioid receptors in primary afferent neuron, resulting in their up-regulation (Hassan et al., 1993; Antonijevic et al., 1995; Jeanjean et al., 1995; Ji et al., 1995; Stein, 1995). Indeed, many studies demonstrated that local administration of opioids decrease inflammatory hyperalgesia indifferent models of inflammation, such as subcutaneous (Binder et al., 2001; Furst et al., 2005; Pacheco et al., 2005) , articular (Bakke et al., 1998; Clemente et al., 2004) and muscular (Nunez et al., 2007). However, the data of this study suggest that the activation of mu opioid receptor expressed in normal non-sensitized nociceptor is not enough to induce analgesia, because the local administration of DAMGO did not change the mechanical hyperalgesia induced by 30 ng of PGE_2, but greatly decreased the hyperalgesia induced by 100 ng of PGE_2. At the same time, 100 ng, but not 30 ng of PGE_2, greatly increased the expression of mu opioid receptor in the saphenous nerve. It is important to point out that, the activation of either mu, kappa or delta opioid receptor on peripheral tissue reduces inflammatory hyperalgesia(Stein, 1993; Holden et al., 2005), however, the activation of mu opioid receptor is the main responsible for the analgesia induced by morphine on peripheral tissue (Fang et al., 1986). Demonstrating previous results (Fang et al., 1986), the anti-hyperalgesic effect induced by local administration of 5 ng of DAMGO did not differ from that one induced by 5 ng of morphine (data not shown).Therefore, the findings of this study strongly suggest that the inflammatory process is essential to the opioid-induced analgesia in peripheral tissue.

In another set of experiments, the data of this study demonstrated that local administration of DAMGO, at the same dose that decreased PGE_2-induced hyperalgesia, also decreased the nociceptive behavior (flinches) induced by capsaicin, when the peripheral tissue was challenged with PGE_2 (100 ng), either 3 or 24h before. We also demonstrated that Tolbutamide(Henquin et al., 2011), a blocker of potassium channel ATP-sensitive (KATP) reverted the anti-nociceptive effect of DAMGO. Local administration of capsaicin acts on TRPV-1 receptor of C-fibers inducing an immediate influx of Ca^{2+} and Na^{+}(Szallasi et al., 2007). We assumed that during the time analyzed in this study (10 minutes), capsaicin almost exclusively induced depolarization of C-fiber and flinches behaviors of rat hind-paw (nociception), without sensitization. Previous experiments from our laboratory demonstrated that neither PKA inhibitor (AKAPI, 1μg / paw), nor PKC

inhibitor (PKCepsilon, 1µg / paw) (Sachs et al., 2009) reduces the capsaicin-induced flinches, which was blocked only by local administration of lidocaine 2% (data not shown). Take together these data suggest that mu opioid receptor activation on primary afferent neuron decreases the transmission of nociceptive stimuli through C-fiber by opening K^+-ATP.

It has been demonstrated that the anti-hyperalgesic mechanism of action of opioids administrated on peripheral tissue involves, at least the inhibition of cAMP (Levine and Taiwo, 1989) and L-argenine/NO/cGMP pathway activation (Ferreira and Nakamura, 1979a). Subsequent studies demonstrated that the activation of L-argenine/NO/cGMP pathway ultimately induces the opening of K^+-ATP that is involved in the anti-hyperalgesic effect of opioids administrated in the peripheral tissue (Duarte et al., 1992).

More recently the participation of K^+-ATP on anti-hyperalgesic effect of opioids has been questioned because studies have demonstrated that the expression o this potassium channel in the membrane of DRG cells is minimal (Ristoiu et al., 2002). However, parallel with the increase of mu-opioid receptor expression induced by PGE_2 and demonstrated in this study, recent data from our laboratory also demonstrated that PGE_2 can differently modulate the expression of K^+-ATP on the membrane of DRG cells (manuscript in preparation).

Agreeing with our findings, the lower expression of mu opioid receptor on primary afferent neurons in normal non-inflamed tissue could explain, at least in part, the reason by which administration of opioids in peripheral tissue usually does not change the mechanical nociceptive threshold (Malo et al., 1977; Bullingham et al., 1984; Schulte-Steinberg et al., 1995; Atanassoff et al., 1997; Yarussi et al., 1999; Worrich et al., 2007). It is also important to comment that another recent findings from our laboratory suggest that the mechanical nociceptive threshold in normal conditions does not depend on C fibers which ones are associated with inflammatory sensitization mediated by PGE_2 (manuscript in preparation).

In conclusion, the findings of this study support the hypothesis that the anti-hyperalgesic effect of opioids in the peripheral tissue during inflammation is associated with increased of mu opioid receptor on primary afferent neurons induced by PGE_2. Also,

the anti-hyperalgesic effect of opioids in the peripheral tissue depends on, at least in part, of the decreased of C-fibers ($TRPV1^+$) excitability.

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CONCLUSÃO

A ativação de receptores opióides disponíveis nas terminações periféricas dos neurônios aferentes primários não é suficiente para promover analgesia. No entanto, a liberação de mediadores inflamatórios, tais como PGE_2, aumenta a expressão destes receptores, permitindo a ação analgésica dos opióides no tecido periférico.

O efeito analgésico de opióides no tecido periférico está associado a uma diminuição da excitabilidade das fibras C.

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ANEXOS 1

APROVAÇÃO DO COMITE DE ÉTICA

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **1530-1**, sobre **"Mecanismos envolvidos na ação anti-hiperalgésica do agonista opióide *mu* no tecido periférico"**, sob a responsabilidade de **Prof. Dr. Carlos Amilcar Parada / Profa. Dra. Claudia Herrera Tambeli / Karla Elena Torres Chávez**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **19 de maio de 2008**.

C E R T I F I C A T E

We certify that the protocol nº **1530-1**, entitled **"Mechanisms underlying the anti-hyperalgesic effect of *mu* opioid agonists in the peripheral tissue"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **May 19, 2008**.

Campinas, 19 de maio de 2008.

Profa. Dra. Ana Aparecida Guaraldo
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