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Pharmacological evidence for the activation of Ca^{2+}-activated K^+ channels by meloxicam in the formalin test

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Abstract

The possible participation of K^+ channels in the antinociceptive action of meloxicam was assessed in the 1% formalin test. Local peripheral administration of meloxicam produced a dose-dependent antinociception only during the second phase of the formalin test. K^+ channel blockers alone did not modify formalin-induced nociceptive behavior. However, local peripheral pretreatment of the paw with charybdotoxin and apamin (large- and small-conductance Ca^{2+}-activated K^+ channel inhibitors, respectively), 4-aminopyridine and tetraethylammonium (non-selective voltage-dependent K^+ channel inhibitors), but not glibenclamide or tolbutamide (ATP-sensitive K^+ channel inhibitors), dose-dependently prevented meloxicam-induced antinociception. It is concluded that meloxicam could open large- and small-conductance Ca^{2+}-activated K^+ channels, but not ATP-sensitive K^+ channels, in order to produce its peripheral antinociceptive effect in the formalin test. The participation of voltage-dependent K^+ channels was also suggested, but since non-selective inhibitors were used the data await further confirmation.

Keywords: Meloxicam; Antinociception; K^+ channels; Apamin; Charybdotoxin; 4-aminopyridine; Tetraethylammonium; Glibenclamide; Tolbutamide; Formalin test

1. Introduction

Meloxicam, a non-steroidal anti-inflammatory drug (NSAID) derived from enolic acid, is a preferential inhibitor of cyclooxygenase-2 (COX-2) (Pairet et al., 1998) that possesses potent anti-inflammatory and antinociceptive activity together with minor gastrointestinal and renal damage in preclinical (Ogino et al., 1997; Pairet et al., 1998) and clinical (Noble and Balfour, 1996; Ogino et al., 2002; Fleischmann et al., 2002) studies.

Previous evidence has shown that the antinociceptive activity of meloxicam observed after systemic administration is mainly due to a peripheral action at or near the nociceptor endings (Laird et al., 1997; Santos et al., 1998; Aguirre-Bañuelos and Granados-Soto, 2000), although a spinal action has also been suggested (López-García and Laird, 1998). It is known that meloxicam, as other NSAIDs, inhibits the prostaglandin synthesis in the peripheral tissue and central nervous system (López-García and Laird, 1998; Gupta et al., 2002) in order to produce its antinociceptive effect. However, this inhibition is not enough to completely account for the efficacy of this agent in several models of pain in animals. In this sense, it has been shown that meloxicam is able to reduce the migration of leukocytes under shear stress and the leukocyte adhesion in the diabetic retina (Hofbauer et al., 1999; Joussen et al., 2002). Moreover, meloxicam is able to interfere with the activation state of αIIbβ3 integrin and to inhibit the platelet primary aggregation (Domínguez-Jiménez et al., 1999). Furthermore, some reports suggest that release of acetylcholine (Ach) in the spinal cord (Pinardi et al., 2003) could be

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involved in the mechanism of action of meloxicam. Meloxicam also reverses thymulin-induced c-*fos* in the spinal cord of rats (Saade et al., 1999) and it reduces retinal tumor necrosis factor (TNF-α) and endothelial nitric oxide synthase (eNOS) levels (Joussen et al., 2002). We have previously demonstrated the involvement of the nitric oxide (NO)-cyclic GMP pathway in the local peripheral antinociceptive effect produced by meloxicam in the rat formalin test (Aguirre-Bañuelos and Granados-Soto, 2000). More recently, we have found that antinociception induced by NSAIDs, which activates the NO-cyclic GMP pathway, can be diminished by K^+ channel inhibitors (Lázaro-Ibáñez et al., 2001; Ortiz et al., 2002, 2003a), suggesting that these drugs could modulate K^+ channels through the activation of the NO-cyclic GMP pathway in order to produce their peripheral antinociceptive effect. Therefore, this work was undertaken to determine the possible participation of K^+ channels on the peripheral antinociception induced by meloxicam. For this purpose, we tested the actions of charybdotoxin (an inhibitor of large-conductance Ca^{2+}-activated K^+ channels; Stretton et al., 1992), apamin (an inhibitor of small-conductance Ca^{2+}-activated K^+ channels; Romey et al., 1984), 4-aminopyridine and tetraethylammonium (non-selective voltage-dependent K^+ channel inhibitors; Cook and Quast, 1990), glibenclamide and tolbutamide (ATP-sensitive K^+ channel blockers; Edwards and Weston, 1993) on meloxicam-induced antinociception in the 1% formalin test.

2. Materials and methods

2.1. Animals

Female Wistar rats aged 8–10 weeks (weight range, 180–200 g) from our own breeding facilities were used in this study. Animals had free access to food and drinking water before experiments. Efforts were made to minimize animal suffering and to reduce the number of animals used. Rats were used once only. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983). Additionally, the study was approved by the Institutional Animal Care and Use Committee (Departamento de Farmacobiología, México, D.F., Mexico).

2.2. Measurement of antinociceptive activity

Rats were placed in open Plexiglas observation chambers for 30 min to allow them to adjust to their surroundings; then they were removed for formalin administration. Fifty microliters of diluted formalin (1%) were injected subcutaneously (s.c.) into the dorsal surface of the right hind paw with a 30-gauge needle. Animals were then returned to the chambers and nocifensive behavior was observed immediately after formalin injection. Mirrors were placed to enable unhindered observation. Nocifensive behavior was quantified as the number of flinches of the injected paw during 1 min-period every 5 min up to 60 min after injection (Wheeler-Aceto and Cowan, 1991; Malmberg and Yaksh, 1992). Flinching was readily discriminated and was characterized as rapid and brief withdrawal or flexing of the injected paw. Formalin-induced flinching behavior is biphasic. The initial acute phase (0–10 min) is followed by a relatively short quiescent period, which is then followed by a prolonged tonic response (15–60 min). At the end of the experiment the rats were sacrificed in a CO_2 chamber.

2.3. Drugs

Meloxicam was a gift of Laboratorios Promeco S.A. (Mexico City). Glibenclamide (glyburide), tolbutamide, charybdotoxin, apamin, 4-aminopyridine and tetraethylammonium were purchased from Sigma (St. Louis, MO, USA). Charybdotoxin, apamin, 4-aminopyridine and tetraethylammonium were dissolved in saline. Glibenclamide and tolbutamide were dissolved in dimethylsulfoxide (DMSO) 20%.

2.4. Study design

Rats received appropriate vehicle (50 μl) or increasing doses of meloxicam (50–200 μg/paw in 50 μl) in their right paw 20 min before formalin injection into the same paw. To determine whether meloxicam acted locally, meloxicam was administered to the left (contralateral; 200 μg in 50 μl) paw 20 min before formalin was injected into the right paw, and the effect assessed. In order to determine whether meloxicam-induced antinociception was mediated by the K^+ channel activation, effect of pre-treatment (10 min before) with the appropriate vehicle (DMSO 20% for glibenclamide and tolbutamide or saline for charybdotoxin, apamin, tetraethylammonium and 4-aminopyridine) or apamin (0.1–2 μg), charybdotoxin (0.1–1 μg), 4-aminopyridine (10–50 μg), tetraethylammonium (25–100 μg), glibenclamide (25–100 μg) or tolbutamide (25–100 μg) on the antinociceptive effect induced by meloxicam (200 μg) was assessed. Drugs were injected in a volume of 50 μl. Doses and drug administration schedule of K^+ channel inhibitors and meloxicam for peripheral administration were selected based on previous reports (Lázaro-Ibáñez et al., 2001; Ortiz et al., 2002, 2003a) and on pilot experiments in our laboratory. Rats in all groups were tested for possible side effects observed as a reduction of righting, stepping, corneal and pinna reflexes as previously described (Malmberg and Yaksh, 1992).

2.5. Data analysis and statistics

All experimental results are given as the mean ± SEM for 6 animals per group. Curves were constructed plotting the number of flinches as a function of time. The area under the number of flinches against time curves (AUC), an expres-

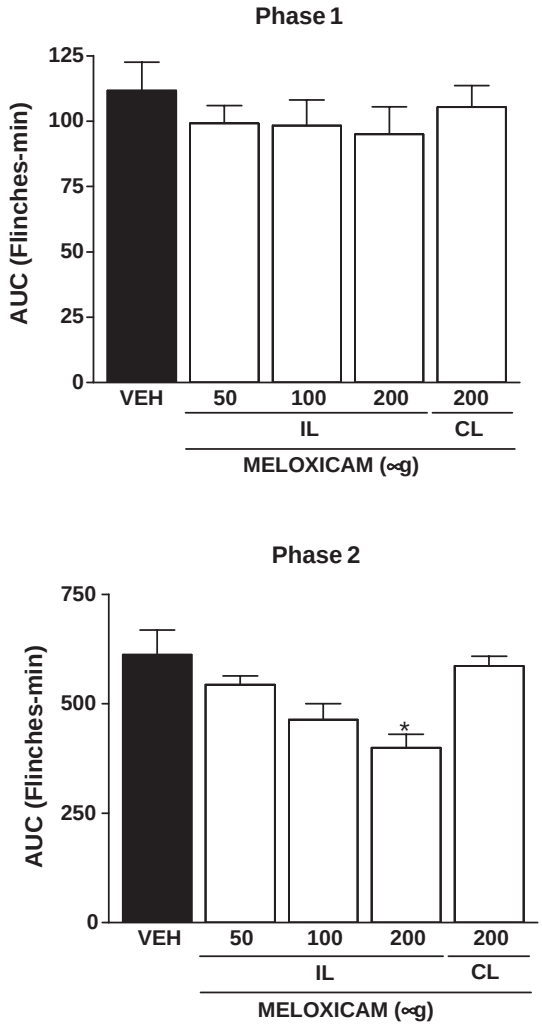

Fig. 1. Local antinociceptive effect of meloxicam in the formalin test. Rats were pretreated with a s.c. injection of vehicle or meloxicam into either the right (ipsilateral, IL) or left (contralateral, CL) paw before formalin injection. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ± S.E.M. for 6 animals. * Significantly different from vehicle group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

sion of the duration and intensity of the effect, was calculated by the trapezoidal rule (Tallarida and Murray, 1981). Reduction of the number of flinches or AUC of the second phase is reported only, since we were not able to observe effect on phase 1. Analysis of variance (ANOVA), followed by Tukey's test was used to compare differences between treatments. Differences were considered to reach statistical significance when $P<0.05$.

3. Results

3.1. Peripheral antinociceptive effect of meloxicam

Formalin administration produced a typical pattern of flinching behavior. The first phase started immediately after administration of formalin and then diminished gradually in about 10 min. The second phase started at about 15 min and lasted until 1 h. Ipsilateral, but not contralateral, local peripheral administration of meloxicam produced a dose-dependent reduction in the flinching behavior otherwise observed after formalin injection (Fig. 1). Meloxicam significantly reduced the number of flinches during phase two ($P<0.05$), but not during phase one. No side effects were observed in either group, control or treated.

3.2. Effect of apamin, charybdotoxin, 4-aminopyridine and tetraethylammonium on the peripheral antinociceptive effect of meloxicam

Local peripheral pretreatment with the small- and large-conductance Ca^{2+}-activated K^+ channel inhibitors apamin and charybdotoxin was able to reverse ($P<0.05$) meloxicam-

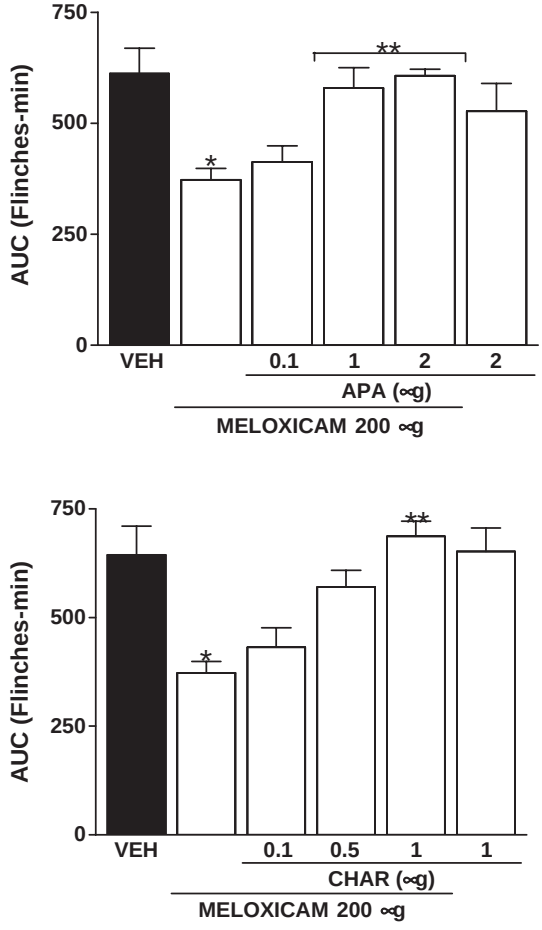

Fig. 2. Effect of small- and large-conductance Ca^{2+}-activated K^+ channel blockers apamin (APA, top panel) and charybdotoxin (CHAR, bottom panel), respectively, on the peripheral antinociception produced by meloxicam 200 μg during the second phase of the formalin test. Rats were pretreated with a s.c. injection of apamin or charybdotoxin plus meloxicam into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ± S.E.M. for at least 6 animals. * Significantly different from the vehicle group ($P<0.05$) and ** significantly different from the meloxicam group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

induced antinociception (200 μg/paw) (Fig. 2). Likewise, 4-aminopyridine and tetraethylammonium (non-selective voltage-dependent K^+ channel inhibitors) dose-dependently prevented ($P<0.05$) the antinociception produced by meloxicam (Fig. 3). K^+ channel blockers, by themselves, were not able to modify formalin-induced nociceptive behavior.

3.3. Effect of glibenclamide and tolbutamide on the peripheral antinociception induced by meloxicam

Local peripheral pretreatment with the ATP-sensitive K^+ channel inhibitors glibenclamide and tolbutamide (25–100 μg/paw) was not able to reverse ($P>0.05$) the meloxicam-induced antinociception (200 μg/paw) (Fig. 4).

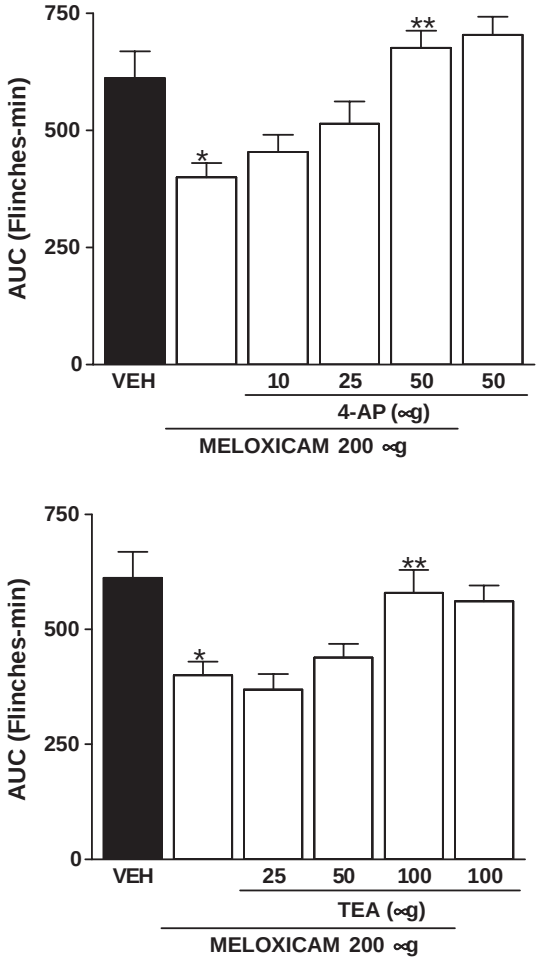

Fig. 3. Effect of voltage-dependent K^+ channel blockers 4-aminopyridine (4-AP, top panel) and tetraethylammonium (TEA, bottom panel) on the peripheral antinociception produced by meloxicam 200 μg during the second phase of the formalin test. Rats were pretreated with a s.c. injection of 4-AP or TEA plus meloxicam into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean±S.E.M. for 6 animals. * Significantly different from the vehicle group ($P<0.05$) and ** significantly different from the meloxicam group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

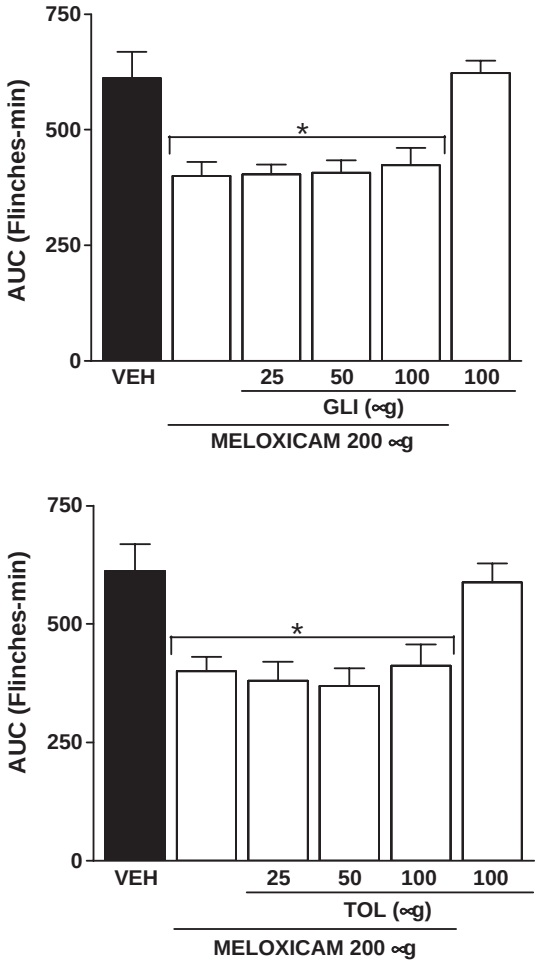

Fig. 4. Effect of ATP-sensitive K^+ channel inhibitors glibenclamide (GLI, top panel) and tolbutamide (TOL, bottom panel) on the peripheral antinociception produced by meloxicam 200 μg during the second phase of the formalin test. Rats were pretreated with a s.c. injection of glibenclamide or tolbutamide plus meloxicam into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean±S.E.M. for 6 animals. * Significantly different from the vehicle group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

At the tested doses, glibenclamide and tolbutamide did not modify the nociceptive behavior induced by formalin.

4. Discussion

In the present study, peripheral administration of meloxicam produced a dose-dependent antinociceptive effect suggesting a significant participation of a peripheral component in the action of this drug. Since meloxicam is a preferential inhibitor of the inducible isoform of cyclo-oxygenase enzyme (COX-2) (Ogino et al., 1997; Pairet et al., 1998), our results suggest a possible participation of a peripheral cyclooxygenase-2 in the nociceptive and inflammatory process in the formalin test. However, local peripheral administration of celecoxib, another selective

COX-2 inhibitor, was not able to reduce flinching behavior in the same model. Therefore, it is likely that inhibition of COX-1, by meloxicam can explain its antinociceptive effect in this model (Engelhardt et al., 1996), as it is the case for diclofenac, a non-selective cyclooxygenase inhibitor. Moreover, other mechanisms such as peripheral activation of the nitric oxide-cGMP pathway could also contribute to their actions (Aguirre-Bañuelos and Granados-Soto, 2000). Central actions of meloxicam can be discarded, as drug administration into the contralateral paw did not produce any effect, suggesting that the observed effect was restricted to the local level.

The results reported here suggest that modulation of some K^+ channels at the peripheral level represents an important step in the peripheral mechanism of antinociception induced by meloxicam. Our data demonstrate that the local administration of Ca^{2+}-activated and non-selective voltage-dependent K^+ channel inhibitors prevented the meloxicam-induced peripheral antinociception. However, glibenclamide and tolbutamide, ATP-sensitive K^+ channel inhibitors (Amoroso et al., 1990; Davies et al., 1991; Edwards and Weston, 1993), were not able to block the antinociceptive action of meloxicam, suggesting that meloxicam does not activate these channels in order to produce its antinociceptive effect at peripheral level. It is interesting to note that, at the same or lower doses, the ATP-sensitive K^+ channel blockers glibenclamide or tulbutamide were able to reverse the peripheral antinociception induced by ketorolac (Lázaro-Ibáñez et al., 2001), diclofenac (Ortiz et al., 2002) and morphine (Granados-Soto et al., 2002) in the same model. Likewise, at the doses tested in this study, tolbutamide and glibenclamide blocked morphine-, dibutyryl-cyclic GMP- or diclofenac-induced peripheral antinociception (Rodrigues and Duarte, 2000; Soares and Duarte, 2001; Alves et al., 2004) in other inflammatory pain models. Taken together, the data presented do not support the possible participation of the ATP-sensitive K^+ channels in meloxicam-induced peripheral antinociception. According to these results, it has been demonstrated by our group that the local peripheral antinociceptive effects produced by metamizol and resveratrol, which have the ability to inhibit prostaglandin synthesis, were blocked by the administration of Ca^{2+}-activated and non-selective voltage-dependent K^+ channels inhibitors, but not by the ATP-sensitive K^+ channel blockers glibenclamide, tolbutamide and glipizide (Granados-Soto et al., 2002; Ortiz et al., 2003b).

At the concentrations used in this work, the K^+ channel blockers (glibenclamide, tolbutamide, charybdotoxin, apamin, 4-aminopyridine and tetraethylammonium) used did not modify the flinching behavior of rats in comparison with that of control rats. The lack of effect of the K^+ channel blockers is consistent with the results of studies in which these compounds did not modify the nociceptive activity of thermal noxious stimuli and mechanical hyperalgesia (Welch and Dunlow, 1993; Rodrigues and Duarte, 2000), thus excluding the possibility that the prevention of meloxicam antinociception could be due to a hyperalgesic or nociceptive effect of the K^+ channel blockers used. The lack of modification of the flinching behavior by the K^+ channel modulators at concentrations able to prevent meloxicam antinociception might also indicate that the K^+ channels of primary afferent neurons involved in the modulation of pain are not tonically activated.

Our results also provide pharmacological evidence of the involvement of voltage-dependent K^+ channels in the peripheral mechanism of the action of meloxicam. The local administration of tetraethylammonium and 4-aminopyridine (inhibitors non-selective of voltage-dependent K^+ channels; Cook and Quast, 1990; Mathie et al., 1998) prevented the antinociception induced by meloxicam. Since 4-aminopyridine is also a delayed rectifier K^+ channel inhibitor (Rosati et al., 1998), the blockade of meloxicam-induced antinociception by 4-aminopyridine suggests the participation of that channel. However, as these drugs are non-specific compounds, the possibility that the observed effect could be due to the actions on other K^+ channels cannot be discharged with the present experiments. The fact that tetraethylammonium is also able to block Ca^{2+}-activated K^+ channels (Cook and Quast, 1990) further suggest the participation of these channels in the peripheral mechanism of action of meloxicam.

The analgesic action of NSAIDs can, in part, be explained by the inhibition of cyclooxygenase. This enzyme controls the synthesis of prostaglandins from arachidonic acid, all of which might be involved in nociceptor excitation and sensitization, and hence, in pain and hyperalgesia (Zimmermann, 1984). However, actions on the metabolism of arachidonic acid cannot fully explain the antinociceptive effects of these peripherally acting drugs. There is evidence that meloxicam produces antinociception through the preferential inhibition of cyclooxygenase-2, but also of cyclooxygenase-1 (Ogino et al., 1997; Pairet et al., 1998). The reduction of prostaglandins at the primary afferent neurons induced by meloxicam would diminish the depolarization of nerve terminals otherwise observed during the nociceptive process. In addition to the cyclooxygenase related action, the opening of K^+ channels (Ca^{2+}-activated and voltage-dependent) by meloxicam would further reduce the depolarization leading to antinociception. The exact mechanism of meloxicam to modulate K^+ channels in sensory neurons is at present unknown. Previously, we have reported that meloxican is able to activate the NO-cyclic GMP pathway (Aguirre-Bañuelos and Granados-Soto, 2000) in order to produce peripheral antinociception in the 1% formalin test model. More recently, Duarte and coworkers have suggested the possible participation of some types of K^+ channels on NO- and cyclic GMP-induced peripheral antinociception (Soares et al., 2000; Soares and Duarte, 2001). Therefore, meloxicam could activate the NO-cyclic GMP pathway which in turn would activate K^+ channels, as it is the case for ketorolac and diclofenac

(Lázaro-Ibáñez et al., 2001; Ortiz et al., 2003a). The fact that drugs which increase the intracellular concentration of cyclic GMP, as sildenafil, produce charybdotoxin or apamin-sensitive peripheral antinociception is in line with our suggestion (Ambriz-Tututi et al., 2005).

In conclusion, meloxicam produced peripheral antinociception in the 1% formalin test. The antinociceptive effect of meloxicam was antagonized by charybdotoxin, apamin, 4-aminopyridine and tetraethylammonium, but not by glibenclamide or tolbutamide. These results strongly suggest that modulation of several K^+ channels at the primary afferent neuron plays an important role in the peripheral antinociception of meloxicam in the formalin test.

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