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Atropine reverses the antinociception of nonsteroidal anti-inflammatory drugs in the tail-flick test of mice

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Abstract

The nonsteroidal anti-inflammatory drugs (NSAIDs) clonixin, diclofenac, piroxicam, ketoprofen, meloxicam, and paracetamol induced antinociception after intraperitoneal or intrathecal administration in mice submitted to an acute thermal algesiometric test without inflammation (tail-flick). Antinociception was evaluated by the increase in reaction time difference (Δ latency), between readings obtained before and after the administration of drugs. The antinociception induced by doses of NSAIDs producing between 20% and 30% of the maximum possible effect (MPE) 30 min after intraperitoneal and 15 min after intrathecal injections was compared with the antinociception obtained after pretreatment with 1 mg/kg atropine ip, 30 min before. Systemic atropine (1 mg/kg) significantly antagonized NSAID-induced antinociception in all cases, both after intraperitoneal and intrathecal administration. Cholinergic depletion by intracerebroventricular hemicholinium-3 (HC-3, 5 μg) 5 h before prevented the antinociceptive effect of all NSAIDs. These observations suggest that intrinsic muscarinic cholinergic facilitatory pathways represent an important modulating system in pain perception in this animal model of acute thermal pain. The results of the present work support the increasingly accepted notion that NSAIDs are effective analgesics even when inflammation is not present, acting by mechanisms that involve actions on spinal and supraspinal nociceptive transmission. It is suggested that, similar to morphine and clonidine, the active mechanism of NSAIDs may involve the release of acetylcholine (ACh) in the spinal cord.

Keywords: NSAID; Antinociception; Atropine; Tail-flick test

1. Introduction

Several drugs induce analgesia or antinociception by interfering with the neuronal pathways involved in the receipt and transmission of nociceptive information from the periphery to higher centers in the central nervous system. Several receptors, including α-adrenoceptors, 5-HT_1, 5-HT_2, and 5-HT_3 subtypes of serotonin receptors, nicotinic and muscarinic cholinoceptors, are expressed pre- and postsynaptically in neurons at spinal and supraspinal levels, and can modulate nociceptive information (Fürst, 1999). Cholinergic drugs, such as nicotinic and muscarinic agonists, induce antinociception after systemic or intrathecal administration in several algesiometric assays (Abram and O'Connor, 1995; Damaj et al., 1998; Eisenach, 1999; Guimaraes et al., 2000; Rueter et al., 2000; Damaj, 2000). In addition, controversial reports indicate that atropine, a paradigmatic cholinergic muscarinic antagonist, can produce analgesia with no effect or hyperalgesia in several different algesiometric tests (Ghelardini et al., 1990; Zarrindast et al., 1997; Coimbra et al., 2001).

On the other hand, it is well known from experimental and clinical studies that cyclooxygenases (COX-1 and COX-2) are the major targets of nonsteroidal anti-inflammatory drugs (NSAIDs), a heterogeneous group of agents with similar mechanisms of action and therapeutic effects, widely used for the treatment of fever, inflammation, and pain (Smith et al., 2000; Tulunay, 2000). However, the assumption that all NSAIDs relieve pain through an inhibition of COX and prostaglandin biosynthesis is not a complete, and satisfactory explanation of the antinociception observed in several models of acute pain paradigms with no inflammation, such as the tail-flick and hot-plate tests (McCor-

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mack and Brune, 1991; Björkman, 1995; Miranda et al., 2001b).

Considering that both NSAIDs and cholinergic agents can induce antinociception, the available knowledge on the possible interactions between the antinociceptive activity exerted by the cholinergic system and NSAIDs is scarce. The present study was undertaken to evaluate the role of the cholinergic system in the antinociceptive effect of NSAIDs using the tail-flick test in mice.

2. Materials and methods

2.1. Animals

CF-1 mice of either sex, weighing 28 ± 2 g, were used throughout the experimental work. The animals were acclimatized to the laboratory environment for at least 2 h before being used, and ethical standard guidelines were followed as previously described (Miranda et al., 1993) and were approved by the local ethical commission of the Faculty of Medicine. In particular, the duration of the experiments was as short as possible. The number of animals involved was kept to a minimum and the animals were killed by cervical dislocation immediately after the recording period. Each animal was used only once and received only one dose of the drugs tested. All observations during the assay were performed by the authors in a randomized and blinded manner and a minimum of eight animals were used for each treatment. For intraperitoneal administration, drugs were dissolved in saline and injected in a volume of 10 ml/kg. Intrathecal administration was performed according to the technique described by Hylden and Wilcox (1980) and the total dose was injected in a constant volume of 5 μl dissolved in a slightly hypertonic solution of glucose (6%) to limit diffusion. Intracerebroventricular administration was performed under slight ether anesthesia using isotonic saline as a solvent, according to a modification of the method described by Haley and McCormick (1957); a small incision was made on the skull to expose bregma, and the intracerebroventricular injection was made in a volume of 5 μl through a puncture point 2 mm caudal and 2 mm lateral to bregma, using a 10-μl Hamilton syringe with a 27-gauge needle, modified so as to penetrate the brain 2 mm from the top of the skull.

2.2. Nociceptive assay

A radiant heat, automatic tail-flick algesiometer (U. Basile, Comerio, Italy) was used to measure response latencies, according to the method originally described by D'Amour and Smith (1941). The light beam was focused on the animal's tail about 4 cm from the tip and the intensity was adjusted so that baseline readings were between 2 and 3 s. An 8-s cut-off was imposed to avoid tail damage. Control reaction time was recorded twice, with an interval of 20 min between readings, the second reading being similar to the first. Only animals with baseline reaction times between 2 and 3 s were used in the experiments. Atropine (1 mg/kg) was always injected intraperitoneally 30 min before the administration of NSAIDs, which were administered intraperitoneally or intrathecally (Miranda et al., 1993). Hemicholinium-3 (HC-3, 5 μg in 5 μl saline) was administered intracerebroventricularly 5 h before NSAIDs. The reaction time was again tested 30 min after the intraperitoneal administration and 15 min after the intrathecal administration of a dose of NSAID, which produced approximately between 15% and 30% of the maximum possible effect (MPE) and the difference in reaction time (Δ latency) was recorded. These doses were selected because they gave a consistent analgesia in the tail-flick test in mice and because higher doses tested showed a great variation in MPE, possibly due to induced behavioral and motor dysfunction in the animals. The experimental value was derived from the mean of three consecutive readings in which the light was focused on three adjacent points of the tail. Tail-flick latencies were converted to % MPE as follows: % MPE = 100 × (postdrug latency − predrug latency)/(cut-off time − predrug latency). Each animal was used as its own control.

2.3. Drugs

The following NSAIDs were used: ketoprofen, diclofenac, clonixin, piroxicam, meloxicam, and paracetamol, kindly provided by local laboratories. Atropine sulfate and hemicholinium-3 hydrobromide were purchased from RBI/Sigma (Natick, MA). All drugs were freshly dissolved in saline for intraperitoneal administration. For intrathecal administration, drugs were dissolved in a slightly hypertonic glucose solution (6%) to limit diffusion. Doses were expressed based on salts.

2.4. Statistical analysis

Results are expressed as the difference in latency time (mean ± S.E.M.) between the mean of two separate control

Table 1
% MPE in the tail-flick of mice induced by intraperitoneal and intrathecal NSAIDs administration

NSAID	Dose[a] (mg/kg ip)	% MPE	Dose[b] (mg/kg it)	% MPE
Clonixin	50	18.8	1.5	16.5
Piroxicam	50	19.4	1.5	16.4
Diclofenac	30	21.4	0.9	22.1
Ketoprofen	50	23.4	2.0	22.3
Meloxicam	13	30.5	0.4	38.0
Paracetamol	125	28.1	3.75	29.5

[a] The intraperitoneal dose was injected in a volume of 10 ml/kg to at least 10 mice per group.
[b] The intrathecal dose was injected in a fixed volume of 5 μl to at least eight mice per group.

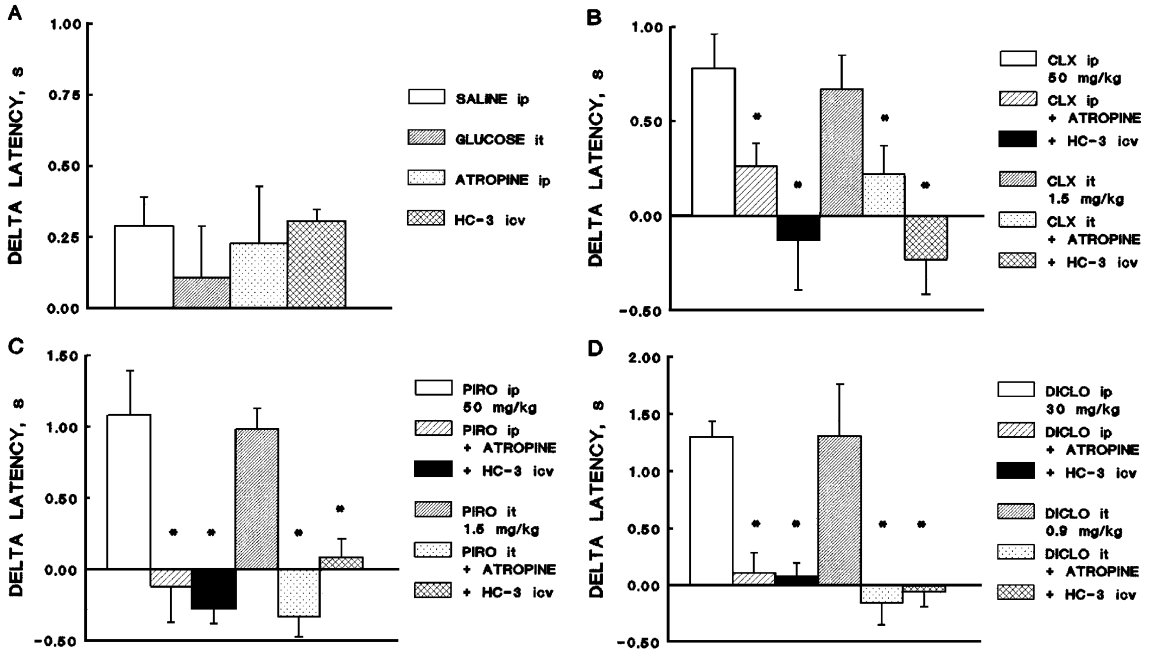

Fig. 1. (A) Control effects of intraperitoneal saline (10 ml/kg, $n=34$), intrathecal 6% glucose (5 μl, $n=23$), intraperitoneal atropine (1 mg/kg, $n=18$), and intracerebroventricular hemicholinium-3 (HC-3, 5 μg, $n=12$) on the differences in reaction time (Δ latency) in the tail-flick test of mice. (B)–(D) Effect of the intraperitoneal and intrathecal administration of clonixin (CLX), piroxicam (PIRO), and diclofenac (DICLO) alone and after atropine (1 mg/kg ip) and hemicholinium-3 (HC-3, 5 μg icv).

readings and the mean of three consecutive experimental readings of each group (Δ latency). The statistical significance between groups was assessed by ANOVA followed by Student–Newman–Keuls test to compare differences in latencies. Probability values less than .05 ($P<.05$) were considered as statistically significant.

3. Results

3.1. Antinociceptive effects

The control administration of either 0.9% saline (10 ml/kg ip) or 6% glucose solution (5 μl it) did not induce

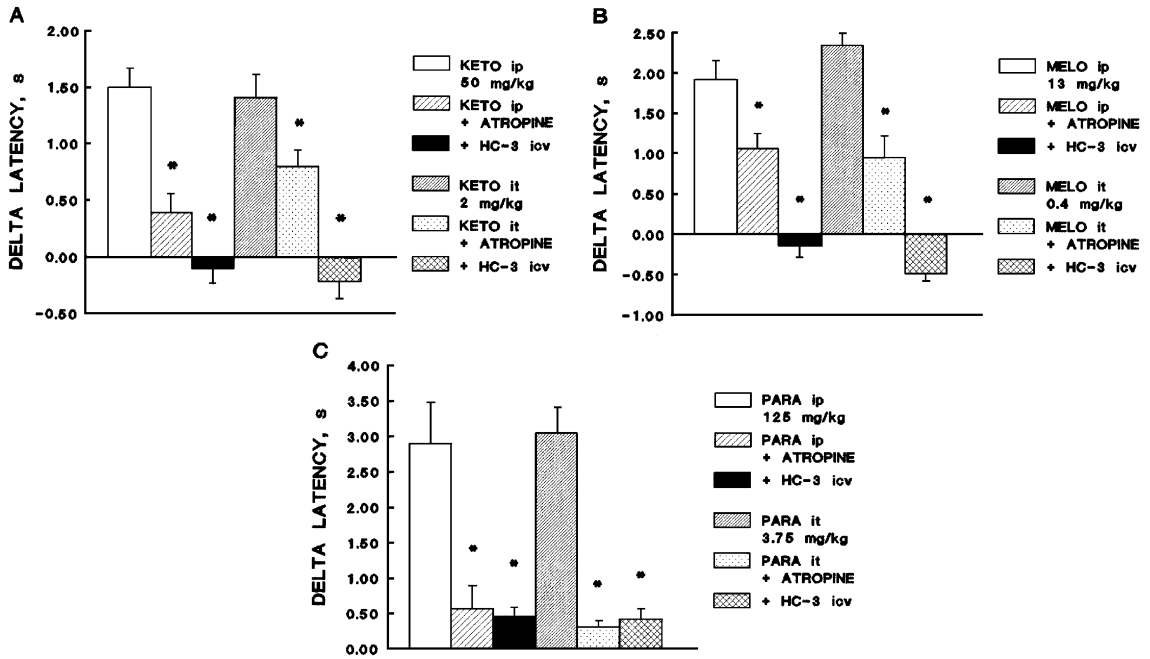

Fig. 2. Differences in reaction time (Δ latency) in the tail-flick test of mice after the intraperitoneal and intrathecal administration of ketoprofen (KETO, A), meloxicam (MELO, B), and paracetamol (PARA, C) alone and after atropine (1 mg/kg ip) and hemicholinium-3 (HC-3, 5 μg icv).

antinociception activity in the tail-flick of the mice. Atropine administered intraperitoneally (1 mg/kg) and HC-3 administered intracerebroventricularly (5 μg) did not produce a significant increase in tail-flick response latency as compared to saline or glucose control animals (Fig. 1A). The administration of atropine or HC-3 did not evoke abnormal behavior or visual motor changes in the animals.

The NSAIDs doses tested evidenced a consistent antinociceptive activity in the tail-flick acute thermal assay, as shown by a significant increase in reaction time as compared to the controls injected with the corresponding vehicle. Table 1 shows the percentage of the MPE (% MPE) for each NSAID dose used in intraperitoneal and intrathecal administrations.

3.2. Interactions between atropine and HC-3 with NSAIDs

The pretreatment of the animals with 1 mg/kg of atropine intraperitoneally significantly antagonized the antinociceptive effects of clonixin, piroxicam, diclofenac (Fig. 1B–D), ketoprofen, meloxicam, and paracetamol (Fig. 2A–C) administered either systemically or intrathecally, producing a significant decrease in the tail-flick response latency. The observed antagonism was not the same for the different drugs tested, being almost complete in the case of diclofenac, piroxicam, and paracetamol and less evident but significant in the case of ketoprofen, clonixin, and meloxicam. The pretreatment of the animals with hemicholinium (5 μg icv) 5 h before the test also resulted in a complete antagonism of the antinociceptive effect of intraperitoneal or intrathecal NSAIDs (Figs. 1 and 2).

4. Discussion

The results of the present study demonstrated that some NSAIDs, administered either systemically or intrathecally, were effective to induce antinociception in a model of acute pain, as evidenced by the significant increase in latency time in the tail-flick thermal assay. This result is important, since it has been previously assumed that NSAIDs were not effective in models of acute pain, because these drugs interact with mechanisms that develop during pathological conditions (Walker et al., 1999). The results of the present work support the increasingly accepted notion that NSAIDs are effective analgesics even when inflammation is not present, acting by additional mechanisms that involve actions on spinal and supraspinal nociceptive transmission (Cashman, 1995; McCormack and Brune, 1991; Miranda et al., 2001a; Pinardi et al., 2001).

The antinociceptive effects elicited by the systemic or intrathecal administration of NSAIDs were significantly antagonized by atropine. This antagonism indicates a muscarinic receptor-mediated interaction in the antinociceptive activity of NSAIDs and suggests a very important involvement of cholinergic mechanisms in the expression of the antinociceptive effect. The fact that the pretreatment with atropine reversed the antinociceptive effect of all the NSAIDs administered either intraperitoneally or intrathecally suggests, at least, the existence of an interaction between the analgesic activity of these drugs and a cholinergic muscarinic mechanism at spinal and/or supraspinal level. The most likely site of interaction is at spinal level, since, as shown in Table 1, approximately equieffective NSAIDs doses were much lower for intrathecal than for systemic administration. The existence of muscarinic receptor subtypes in the dorsal horn of the spinal cord has been demonstrated by autoradiographic, binding, and pharmacological studies (Gillberg and Askmark, 1991; Iwamoto and Marion, 1993; Eisenach, 1999). However, it appears that the antinociceptive action of NSAIDs in the mouse tail-flick test also involves central muscarinic cholinergic neurons, since depletion of acetylcholine (ACh) and inhibition of ACh synthesis with intracerebroventricular HC-3 blocks the antinociception at a time when ACh content is maximally depleted (Chen and Robinson, 1990). Since atropine is a nonselective cholinergic muscarinic receptor antagonist that crosses the brain–blood barrier, it can be postulated that NSAIDs in this somatic acute pain model exert an antinociceptive activity mediated by spinal and supraspinal muscarinic cholinergic receptors. These findings are concordant with previous results that indicate that activation of spinal ACh-M_1 and/or ACh-M_3 mediate antinociceptive effects (Naguib and Yaksh, 1997). The antinociception induced by NSAIDs was similar even if the inhibitory effects of atropine were slightly different for the drugs tested, suggesting that the cholinergic modulation of antinociception is probably not related to the selectivity of the NSAID for inhibition of CNS constitutive COX-1 or COX-2. The antinociceptive effect is similar in spite of different relative potencies of NSAIDs for COX isoforms' selectivity; moreover, paracetamol has little inhibitory activity on both isoforms (Vane, 2000). The cholinergic modulation of NSAIDs analgesic action seems to be independent of the inhibitory effect of these drugs on COX isoforms, as has been shown also for chemopreventive and antitumorigenic properties of NSAIDs (Baek et al., 2002).

It has been reported that ACh-M_1 receptor subtype is fundamental to induce central cholinergic analgesia (Ghelardini et al., 2000). However, there are discrepancies in relation to the relative role played by each subtype of muscarinic receptor in antinociception. By means of in vitro receptor autoradiography, saturation binding, and competition binding assays, it has been demonstrated that ACh-M_2 and ACh-M_3, but not ACh-M_1 receptor subtypes, modulate spinal antinociception (Hoglund and Baghdoyan, 1997). In addition, according to previous studies, both postsynaptic muscarinic M_1 and presynaptic M_2 receptors

are involved in supraspinal antinociception (Bartolini et al., 1992), but spinal cholinergic antinociception is mediated predominantly via the ACh-M_1 receptor (Naguib and Yaksh, 1997).

Several reports support a role for ACh in the inhibition and modulation of the transmission of nociceptive information (Eisenach, 1999; Haberberger et al., 2001). In addition, it has been demonstrated that antinociceptive agents, such as morphine or clonidine, are able to produce an increase in the spinal release of ACh. It has also been demonstrated that this endogenous ACh plays an important role in mediating the analgesic effect of morphine and clonidine (De Kock et al., 1997; Chen and Pan, 2001). On the basis of the effect of depletion of ACh by the pretreatment with HC-3 and muscarinic receptor blocking with atropine, it could be hypothesized that similar to morphine and clonidine, the active mechanism of NSAIDs may involve the release of endogenous ACh, in addition to COX and prostaglandin biosynthesis inhibition. These results suggest that the antinociception elicited by intrathecal or intraperitoneal administration of NSAIDs may depend on the existence of muscarinic cholinergic sites that modulate the transmission of afferent nociceptive information (Iwamoto and Marion, 1993). In addition, the fact that pretreatment with atropine or HC-3 antagonized the antinociception developed by NSAIDs suggest that pre- and postsynaptic mechanisms facilitating cholinergic transmission are involved in the antinociception of NSAIDs and intrinsic cholinergic facilitatory pathways represent an important modulating system in pain perception in this animal model of acute thermal pain.

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References

Abram SE, O'Connor TC. Characteristics of the analgesic effects and drug interactions of intrathecal carbachol in rats. Anesthesiology 1995;83:844–9.

Baek SJ, Wilson LC, Lee CH, Eling TE. Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): inhibition of cyclooxygenase and induction of NSAID-activated gene. J Pharmacol Exp Ther 2002;301:1126–31.

Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A. Role of muscarinic receptor subtypes in central antinociception. Br J Pharmacol 1992;105:77–82.

Björkman R. Central antinociceptive effects of non-steroidal anti-inflammatory drugs and paracetamol. Experimental studies in the rat. Acta Anesthesiol Scand 1995;(Suppl 103):1–44.

Cashman JN. The mechanisms of action of NSAIDs in analgesia. Drugs 1995;52(Suppl 5):13–23.

Chen SR, Pan HL. Spinal endogenous acetylcholine contributes to the analgesic effect of systemic morphine in rats. Anesthesiology 2001;95:525–30.

Chen R, Robinson SE. The effect of cholinergic manipulations on the analgesic response to cobrotoxin in mice. Life Sci 1990;47:1949–54.

Coimbra NC, Castro-Souza C, Segato EN, Nora JE, Herrero CF, Tedeschi-Filho W, et al. Post-ictal analgesia: involvement of opioid, serotoninergic and cholinergic mechanisms. Brain Res 2001;888:314–20.

Damaj MI. The involvement of spinal Ca (2^+)/calmodulin–protein kinase II in nicotine-induced antinociception in mice. Eur J Pharmacol 2000;404:103–10.

Damaj MI, Fei-Yin M, Dukat M, Glassco W, Glennon RA, Martin BR. Antinociceptive responses to nicotinic acetylcholine receptor ligands after systemic and intrathecal administration in mice. J Pharmacol Exp Ther 1998;284:1058–65.

D'Amour FF, Smith GL. A method for determining loss of pain sensation. J Pharmacol Exp Ther 1941;72:74–9.

De Kock M, Eisenach J, Tong C, Schmitz AL, Scholtes JL. Analgesic doses of intrathecal but not intravenous clonidine increase acetylcholine in cerebrospinal fluid in humans. Anesth Analg 1997;84:800–3.

Eisenach JC. Muscarinic-mediated analgesia. Life Sci 1999;64:549–54.

Fürst S. Transmitters involved in antinociception in the spinal cord. Brain Res Bull 1999;48:129–41.

Ghelardini C, Malmberg-Aiello P, Giotti A, Malcangio M, Bartolini A. Investigation into atropine-induced antinociception. Br J Pharmacol 1990;101:49–54.

Ghelardini C, Galeotti N, Bartolini A. Loss of muscarinic antinociception by antisense inhibition of M_1 receptors. Br J Pharmacol 2000;129:1633–40.

Gillberg PG, Askmark H. Changes in cholinergic and opioid receptors in the rat spinal cord, dorsal root and sciatic nerve after ventral and dorsal root lesion. J Neural Transm 1991;85:31–9.

Guimaraes AP, Guimaraes FS, Prado WA. Modulation of carbachol-induced antinociception from the rat periaqueductal gray. Brain Res Bull 2000;51:471–8.

Haberberger R, Scholz R, Kummer W, Kress M. M2-receptor subtypes does not mediate muscarine-induced increases in $[Ca^{2+}]$i in nociceptive neurons of rat dorsal root ganglia. J Neurophysiol 2001;84:1934–41.

Haley TJ, McCormick WG. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. Br J Pharmacol Chemother 1957;12:12–5.

Hoglund AU, Baghdoyan HA. M2, M3 and M4, but not M1 muscarinic receptor subtypes are present in rat spinal cord. J Pharmacol Exp Ther 1997;281:470–7.

Hylden JLK, Wilcox GL. Intrathecal morphine in mice: a new technique. Eur J Pharmacol 1980;67:313–6.

Iwamoto ET, Marion L. Characterization of the antinociception produced by intrathecally administered muscarinic agonists in rats. J Pharmacol Exp Ther 1993;266:329–38.

McCormack K, Brune K. Dissociation between the antinociceptive and anti-inflammatory effects of the nonsteroidal anti-inflammatory drugs. Drugs 1991;41:533–47.

Miranda HF, Sierralta F, Pinardi G. Previous administration of indomethacin or naloxone did not influence ketorolac antinociception in mice. Anesth Analg 1993;77:750–3.

Miranda HF, Sierralta F, Pinardi G. An isobolographic analysis of the adrenergic modulation of diclofenac antinociception. Anesth Analg 2001a;93:430–5.

Miranda HF, López J, Sierralta F, Correa A, Pinardi G. NSAID antinociception measured in a chemical and a thermal assay in mice. Pain Res Manag 2001b;6:190–6.

Naguib M, Yaksh TL. Characterization of muscarinic receptor subtypes that mediate antinociception in the rat spinal cord. Anesth Analg 1997;85:847–53.

Pinardi G, Sierralta F, Miranda HF. Interaction between the antinociceptive effect of ketoprofen and adrenergic modulatory systems. Inflammation 2001;25:233–9.

Rueter LE, Meyer MD, Decker MW. Spinal mechanisms underlying A-85380-induced effects on acute thermal pain. Brain Res 2000;872:93–101.

Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural cellular and molecular biology. Annu Rev Biochem 2000;69:145–82.

Tulunay FC. NSAIDs: behind the mechanisms of action. Funct Neurol 2000;15:202–7.

Vane J. Aspirin and other anti-inflammatory drugs. Thorax 2000;55(Suppl 2):S3–9.

Walker K, Fox AJ, Urban LA. Animal models for pain research. Mol Med Today 1999;5:319–21.

Zarrindast MR, Pazouki M, Nassiri-Rad S. Involvement of cholinergic and opioid receptor mechanisms in nicotine induced antinociception. Pharmacol Toxicol 1997;81:209–13.