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Probable activation of the opioid receptor-nitric oxide-cyclic GMP-K^+ channels pathway by codeine

Mario I. Ortiz [a,*], Jhanet Castro-Olguín [b], Nayeli Peña-Samaniego [c], Gilberto Castañeda-Hernández [d]

[a] *Laboratorio de Farmacología Área Académica de Medicina del Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo ExHacienda la Concepción Carr, Pachuca-Actopan s/n Tilcuautla, Hidalgo 42160, Mexico*
[b] *Facultad de Medicina, Región Orizaba-Córdoba, Universidad Veracruzana, Orizaba, Veracruz, Mexico*
[c] *Facultad de Medicina Humana, Universidad Autónoma de Nayarit, Tepic, Nayarit, Mexico*
[d] *Sección Externa de Farmacología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México, D.F. Mexico*

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Abstract

There is evidence that local peripheral administration of morphine produces antinociception through the activation of the nitric oxide (NO)-cyclic GMP-K^+ channels pathway. Therefore we evaluated the possible participation of this pathway in the antinociceptive action produced by codeine in the rat 5% formalin test. Local peripheral injection of codeine produced a dose-dependent antinociception during the first and second phases of the test. Local pretreatment of the paws with the NO synthase inhibitor N^G-L-nitro-arginine methyl ester (L-NAME), the soluble guanylyl cyclase inhibitor methylene blue, the ATP-sensitive K^+ channel inhibitors glibenclamide and tolbutamide, the non-selective voltage-gated K^+ channel inhibitors 4-aminopyridine (4-AP) and tetraethylammonium (TEA) and the opioid receptor blocker naloxone prevented codeine-induced antinociception in both phases of the test. L-NAME, methylene blue, K^+ channel blockers and naloxone by themselves did not modify formalin-induced nociceptive behavior. Our data suggest that codeine could activate the opioid receptor-NO-cyclic GMP-K^+ channels pathway in order to produce its peripheral antinociceptive effect in the formalin test.

Keywords: Codeine; Nitric oxide; Cyclic GMP; K^+ channels; Opioid receptors; Naloxone; Glibenclamide; Tolbutamide; Formalin test

1. Introduction

Opioids are some of the most efficacious analgesics used in humans (Cherny, 1996). Opioids produce analgesia by binding to specific receptors both within and outside the central nervous system (Simon, 1986; Dionne et al., 2001). Opioid analgesics are classified as full agonists, partial agonists or mixed agonist–antagonists, depending on the specific receptors to which they bind and their intrinsic activity at that receptor (Picker and Dykstra, 1989; Cherny, 1996). Pharmacologically, the opioid receptor has been classified into at least three mayor subtypes (μ, δ, and κ) on the basis of their difference in apparent affinity for ligands (Simon, 1986; Mignat et al., 1995).

Codeine is a naturally occurring opium alkaloid. Like morphine it is a constituent of the opium poppy, *Papaver somniferum*. Codeine constitutes about 0.5% of opium, which continues to be a useful source of its production, although the bulk of codeine used therapeutically is prepared by the methylation of morphine (Williams et al., 2001). Codeine possesses mild sedative, antitussive, antidiarrhoeal and analgesic effects (Cherny, 1996; Williams et al., 2001). Codeine has been considered to be effective against mild to moderate pain, but scientific studies have shown that codeine in combination with other drugs (paracetamol, diphenhydramine and other non-steroidal anti-inflammatory drugs) in optimal doses is more effective (De Craen et al., 1996; Jiménez-Andrade et al., 2003). The properties of codeine have been attributed to an action on the central and peripheral nervous systems (Cherny, 1996). Our group recently has tested that the local peripheral (intraplantar) and spinal (epidural) administrations of codeine are able to reduce the nociceptive behaviour induced by 5% formalin in the rat (Jiménez-

* Corresponding author. Tel.: +52 77 1717 2000x5105; fax: +52 77 1717 2000x5111.
E-mail address: mario_i_ortiz@hotmail.com (M.I. Ortiz).

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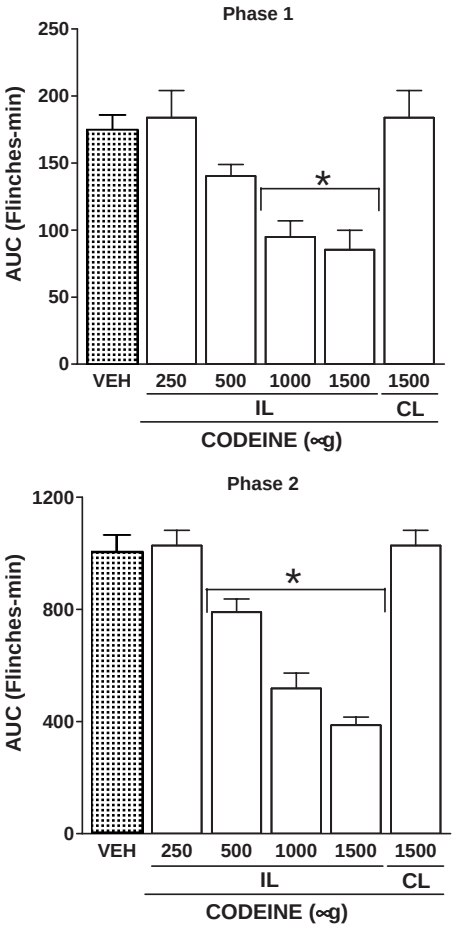

Fig. 1. Local peripheral antinociceptive effect of codeine on the 5% formalin test. Rats were pretreated with a local injection of vehicle (VEH) or codeine into the right (ipsilateral, IL) or left paw (contralateral, CL), before formalin injection. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± SEM of the data obtained in 6 animals. *Significantly different from the vehicle (VEH) group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

Andrade et al., 2003). This last result suggests that codeine is able to produce an antinociceptive effect when this one is applied of local way.

Codeine is an opiate receptor agonist and it is able to bind to the μ receptor like morphine but with a much lower affinity (Chen et al., 1991; Mignat et al., 1995). It also binds to the κ and δ receptors but again has a much lower affinity than morphine, though the difference is less marked (Chen et al., 1991; Mignat et al., 1995). Likewise, electrophysiologic and receptor binding studies showed that codeine and other opioids have noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist properties (Yamakura et al., 1999).

Although the mechanism by which codeine produces an analgesic action is by the activation of opioid receptors, there have been many reports suggesting that the systemic analgesic effect of codeine is either wholly or mostly dependent on its metabolism to morphine, codeine-6-glucuronide and norcodeine (Chen et al., 1991; Yue et al., 1991; Cleary et al., 1994).

There is experimental evidence to suggest that morphine produces peripheral antinociception through the activation of the nitric oxide (NO)-cyclic GMP pathway (Ferreira et al., 1991; Mixcoatl-Zecuatl et al., 2000). Likewise, we have demonstrated that the peripheral antinociceptive effect of morphine on the formalin test was reverted by the ATP sensitive-K^+ channels inhibitor glibenclamide and the non-selective voltage-gated K^+ channel inhibitor 4-aminopyridine (Ortiz et al., 2002). Therefore, this study was commenced to determine the possible participation of the NO-cyclic GMP-K^+ channels pathway on the peripheral antinociception induced by codeine. For this purpose, we tested the actions of N^G-L-nitro-arginine methyl ester (L-NAME, a NO synthesis inhibitor) (Rees et al., 1990), methylene blue (a soluble guanylyl cyclase inhibitor) (Moncada et al., 1991), glibenclamide and tolbutamide (ATP-sensitive K^+ channel blockers) (Edwards and Weston, 1993), 4-aminopyridine (4-AP) and tetraethylammonium (TEA) (non-selective voltage-gated K^+ channel inhibi-

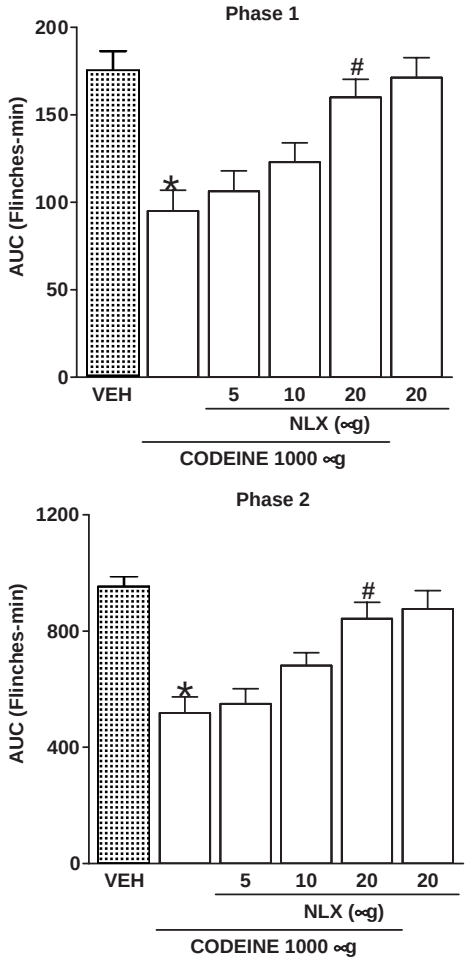

Fig. 2. Effect of local peripheral administration of naloxone (NLX) on the local peripheral antinociception produced by codeine during the first and second phases of the formalin test. Rats were pretreated with a local injection of codeine and then NLX into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± SEM of the data obtained in 6 animals. *Significantly different from the vehicle (VEH) group ($P<0.05$) and #significantly different from the codeine group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

tors) (Cook and Quast, 1990) and naloxone (an opioid receptor antagonist) (Feinberg et al., 1976) on codeine-induced antinociception in the 5% formalin test.

2. Material 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. At the end of the experiment the rats were sacrificed in a CO_2 chamber. Female rats were used because they are less aggressive than adult male rats and, thus, easier to use for behavior pain testing. Besides, we have previously made experiments with female and male rats, and we did not find significant difference between sex in the nociceptive behavior to the formalin administration. 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 (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México, D.F. Mexico).

2.2. Measurement of nociceptive activity

Rats were placed in open Plexiglas observation chambers for 30 min to allow them to accommodate to their surroundings; then they were removed for formalin administration. Fifty microliters of diluted formalin (5%) was 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. The formalin was made of commercially available 37% formaldehyde solution further diluted in isotonic saline. Nocifensive behavior was quantified as the numbers of flinches of the injected paw during 1-min periods every 5 min up to 60 min after injection (Malmberg and Yaksh, 1992). Flinching was readily discriminated and was characterized as rapid and brief with-

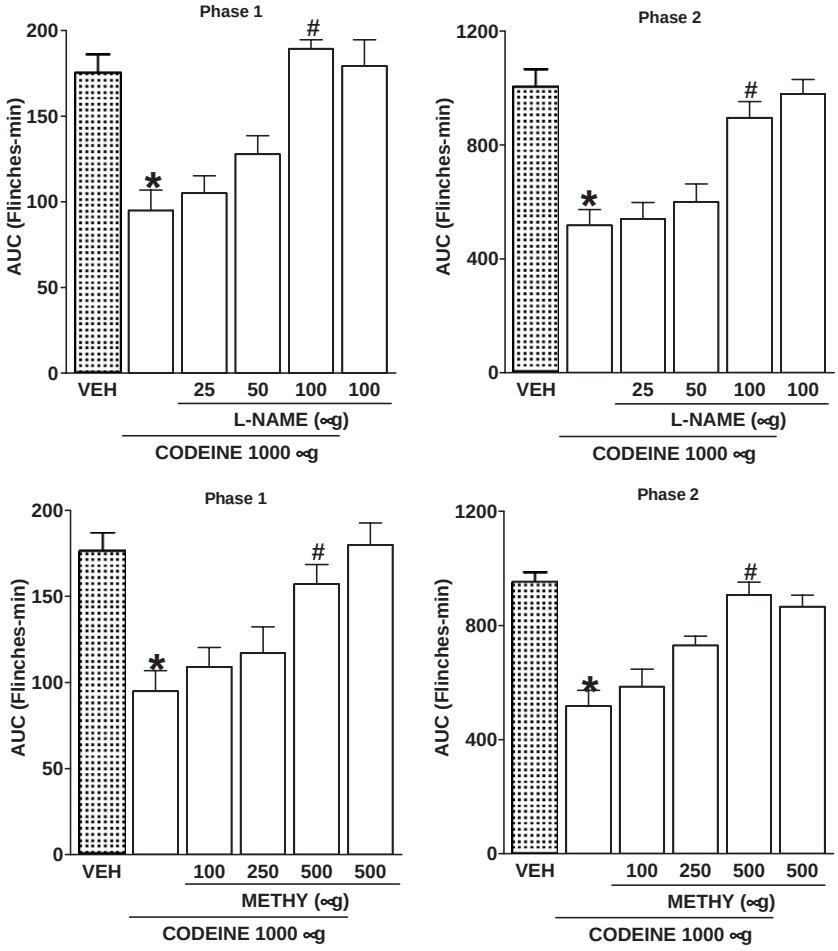

Fig. 3. Effect of L-NAME (top panels) or methylene blue (METHY; bottom panels) on the local peripheral antinociception produced by codeine during the first and second phases of the formalin test. Rats were pretreated with a local injection of codeine and then L-NAME or METHY into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± SEM of the data obtained in 6 animals. *Significantly different from the vehicle (VEH) group ($P<0.05$) and #significantly different from the codeine group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

drawal 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).

2.3. Drugs

Codeine phosphate was kindly supplied by Novartis Farmacéutica (Mexico). Glibenclamide (glyburide), tolbutamide, 4-aminopyridine (4-AP), N^G-L-nitro-arginine methyl ester (L-NAME), methylene blue, naloxone and tetraethylammonium (TEA) were purchased from Sigma (USA). Codeine phosphate, 4-AP, L-NAME, naloxone, methylene blue and TEA were dissolved in saline. Glibenclamide and tolbutamide were dissolved in dimethylsulfoxide (DMSO) 20%.

2.4. Study design

Rats received a s.c. injection (50 μl) into the dorsal surface of the right hind paw of vehicle (saline) or increasing doses of codeine (250–1500 μg/paw) 20 min before formalin injection into the ipsilateral paw. To determine whether codeine acted locally, it was administered to the left (contralaterally; 1500 μg/paw) paw 20 min before formalin was injected into the right paw, and the corresponding response on nociceptive behaviour was assessed. To determine whether codeine-induced peripheral antinociception was mediated by either the opioidergic system, NO-cyclic GMP pathway or K^+ channels, effect of pretreatment (10 min before formalin injection) with the appropriate vehicle or naloxone (5–20 μg/paw), L-NAME (25–100 μg/paw), methylene blue (100–500 μg/paw), glibenclamide (25–100 μg/paw), tolbutamide (25–100 μg/paw), 4-AP (25–100 μg/paw) and TEA (50–200 μg/paw) on the antinociceptive effect induced by local peripheral codeine (1000 μg/paw) was assessed. Drugs were injected in a volume of 50 μl. Each rat received 3 injections and appropriate controls for multiple injections and vehicles were performed before starting the formal study. Doses and drug administration schedules of inhibitors and codeine for peripheral administrations were selected based on previous reports (Rodrigues and Duarte, 2000; Ortiz et al., 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.

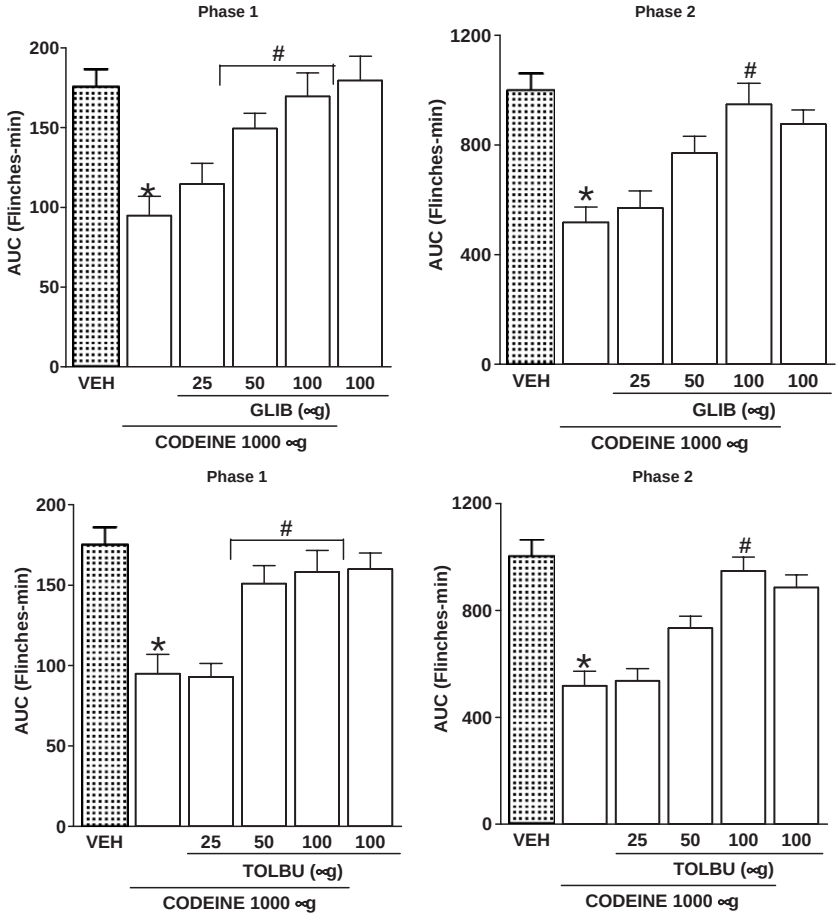

Fig. 4. Effect of glibenclamide (GLIB, top panels) or tolbutamide (TOLBU, bottom panels) on the local peripheral antinociception produced by codeine during the first and second phases of the formalin test. Rats were pretreated with a local injection of codeine and then glibenclamide or tolbutamide into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± SEM of the data obtained in 6 animals. *Significantly different from the vehicle (VEH) group ($P<0.05$) and # significantly different from the codeine group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

2.5. Data analysis and statistics

All experimental results are given as the means ± 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 expression of the duration and intensity of the effect, was calculated by the trapezoidal rule. Reduction of number of flinches or AUC of both phases is reported, since we were able to observe effect on phases 1 and 2. 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 codeine

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 administration of codeine produced a dose-dependent reduction in the flinching behavior otherwise observed after formalin injection (Fig. 1). Codeine significantly reduced the number of flinches during both phases ($P<0.05$). No reduction in the assessed reflexes were observed in either group, control or treated.

3.2. Effect of naloxone on the local peripheral antinociceptive activity of codeine

Local peripheral administration of the opioid receptor antagonist naloxone did not produce any significant effect on formalin-induced flinching behavior as compared to vehicle ($P>0.05$). Moreover, local peripheral application of naloxone was able to reduce the local peripheral antinociception induced by codeine during both phases ($P<0.05$; Fig. 2).

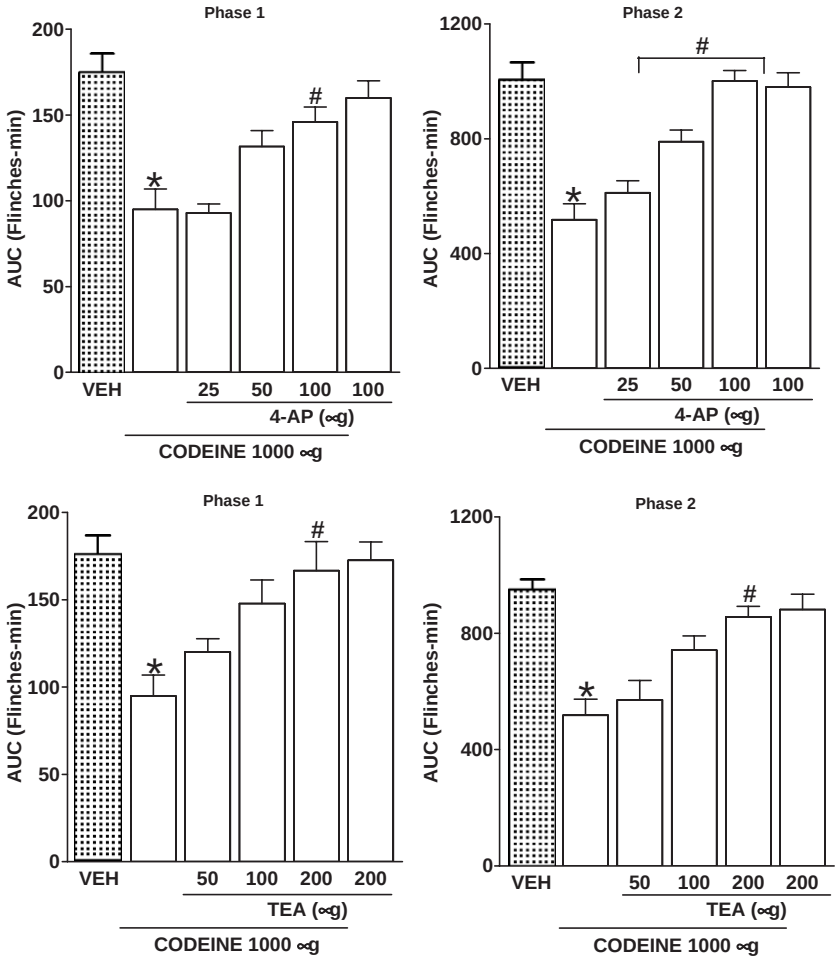

Fig. 5. Effect of 4-AP (top panels) or TEA (bottom panels) on the peripheral antinociception produced by codeine during the first and second phases of the formalin test. Rats were pretreated with a local injection of codeine and then 4-AP or TEA into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± SEM of the data obtained in 6 animals. *Significantly different from the vehicle (VEH) group ($P<0.05$) and # significantly different from the codeine group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

3.3. Effect of L-NAME and methylene blue on the peripheral effect of codeine

Local pretreatment with the NO synthesis inhibitor L-NAME or the guanylyl cyclase inhibitor methylene blue, was able to prevent codeine-induced antinociception during the first and second phases ($P<0.05$) (Fig. 3). Given alone, the inhibitors did not modify formalin-induced nociceptive behavior ($P>0.05$) (Fig. 3).

3.4. Effect of glibenclamide and tolbutamide on the peripheral effect of codeine

Local pretreatment with the ATP-sensitive K^+ channel inhibitors glibenclamide or tolbutamide was able to prevent codeine-induced antinociception during both phases ($P<0.05$) (Fig. 4). Given alone, peripheral ATP-sensitive K^+ channel inhibitors did not modify formalin-induced nociceptive behavior ($P>0.05$) (Fig. 4).

3.5. Effect of 4-AP and TEA on the peripheral effect of codeine

Local pretreatment with the non-selective voltage-gated K^+ channel inhibitors 4-AP and TEA was able to prevent codeine-induced antinociception during the phases 1 and 2 of the formalin test ($P<0.05$) (Fig. 5). Administrated alone, 4-AP and TEA did not modify formalin-induced nociceptive behavior ($P>0.05$) (Fig. 5). No reduction in the assessed reflexes was observed in either group, control or treated.

4. Discussion

In the fomalin test diluted formalin is injected subcutaneously into a hind paw, and nociceptive behavior is scored. Two phases of the response are observed: an early phase starting immediately after injection and lasting for 5–10 min and a late phase 15–60 min after injection (Dubuisson and Dennis, 1977). It is now known that the first phase is due to a direct effect of formalin on nociceptors, whereas the second phase is mediated by a combination of peripheral input and spinal cord sensitization (Tjølsen et al., 1992; Dallel et al., 1995). Opioid analgesics such as morphine and codeine seem to be antinociceptive for both phases, although the second is more sensitive to these drugs (Karim et al., 1993). In contrast, non-steroidal anti-inflammatory drugs such as indomethacin and diclofenac seem to suppress only the second phase (Malmberg and Yaksh, 1992). Codeine binds with stereospecific receptors at many sites within the central nervous system to alter processes affecting both the perception of pain and the emotional response to pain (Cherny, 1996). Precise sites and mechanism of action of codeine have not been fully determined. In the present study local peripheral administration of codeine (250–1500 μg/paw) in a formalin-injured paw produced an antinociceptive effect during both phases. It is possible that 1500 μg/paw (7.5 mg/kg for a 200 g rat) of codeine seem very high in the present study. However, the antinociceptive effect was not due to a systemic or a central action since the administration of codeine (1500 μg/paw) in the contralateral paw was inactive. Likewise, there are reports that have demonstrated doses $\geq$10 mg/kg of codeine in order to obtain analgesic efficacy (Jiménez-Andrade et al., 2003; Erichsen et al., 2005). Besides, we used the dose of 1000 μg/paw (it equals 5.0 mg/kg for a 200 g rat) in order to demonstrate the probable participation of the opioid receptor-NO-cyclic GMP-K^+ channels pathway.

There is evidence that biotransformation is required for codeine to exert analgesic properties. It has been established that codeine must be *O*-demethylated to morphine to produce analgesia in both humans and rats (Chen et al., 1991; Cleary et al., 1994). This reaction is mediated through the activity of the enzyme CYP2D6 (Vree and Verwey-van Wissen, 1992). However, *O*-demethylation of codeine to produce morphine is a minor pathway accounting only for about 5% of the codeine biotransformation, whereas its glucuronidation by UDP-glucuronosyltransferase to codeine-6-glucuronide and its *N*-demethylation to norcodeine account for approximately 80–85% and 10%, respectively (Yue et al., 1991). In our study, interestingly codeine was able to produce a local peripheral antinociceptive effect. A probable hypothesis may be that sufficient quantities of metabolites are formed within the subcutaneous tissue. It is supported by the fact that CYP2D6 and UDP-glucuronosyltransferase are expressed primarily in the liver but also in skin and in other extrahepatic tissues (Burchell and Coughtrie, 1989; Du et al., 2004). In light of these findings, it is hypothesized that the local peripheral antinociception induced by codeine is due to codeine itself and its metabolites (Chen et al., 1991; Cleary et al., 1994; Srinivasan et al., 1997; Vree et al., 2000).

In order to verify the contribution of opioid receptors, we found that the local peripheral administration of the opioid receptor antagonist naloxone was able to revert the antinociceptive action induced by codeine. These results obtained in the animal experiments agree with the published ones by Molina et al. (1983), which demonstrated that codeine has a peripheral site of action in the prostaglandin hyperalgesia test and besides this effect was blocked by the local peripheral administration of naloxone. Taken together then, these findings suggest that codeine is able to activate opioid receptors at the periphery in order to produce their antinociceptive effects on the rat formalin test.

Opioid receptors are linked to trimeric G proteins that, in turn, may modulate Ca^{2+} and K^+ channels, adenyl cyclase, and other signal transduction systems (Cadet, 2004). In the current work, the NO synthesis inhibitor L-NAME (Rees et al., 1990) and the guanylyl cyclase inhibitor methylene blue (Moncada et al., 1991) were able to reduce the antinociceptive effect produced by codeine. The participation of the L-arginine-NO-cyclic GMP pathway in the peripheral antinociception induced by opioids is supported by several observations. First of all, it has been widely demonstrated that the local peripheral administration of opioids is able to produce an antinociceptive effect on several pain tests (Ferreira et al., 1982, 1991; Molina et al., 1983; Hassan et al., 1989; Stein et

al., 1991; Rodrigues and Duarte, 2000; Ortiz et al., 2002; Rodrigues et al., 2005). Second, the local administration of NO synthesis inhibitors (L-NAME and L-NMMA) or guanylyl cyclase inhibitors (methylene blue and ODQ) reverted the peripheral antinociceptive effects produced by morphine (Ferreira et al., 1991; Mixcoatl-Zecuatl et al., 2000) and besides it has been clearly established that NO is a downstream signaling molecule released in response to morphine (Welters et al., 2000; Cadet, 2004). In the case of codeine, Ko et al. (2000) in the experimental allergic conjunctivitis, demonstrated that the instillation of codeine on the conjunctiva was able to increase the NO levels within 1.5 h. Finally, the local peripheral administration of cyclic GMP phosphodiesterase inhibitors (MY5445 and sildenafil) was able to potentiate the local peripheral response produced by morphine (Ferreira et al., 1991; Mixcoatl-Zecuatl et al., 2000). However, studies in the literature indicate that the NO-cyclic GMP pathway can have pronociceptive rather than antinociceptive effects (Aley et al., 1998). It discrepancy may be due to the different experimental pain model used, diverse tissue level and the variant NO and cyclic GMP intracelular content (Kawabata et al., 1994; Pehl and Schmid, 1997; Tegeder et al., 2002). Nevertheless, it is important to point out that in the rat formalin and the rat paw pressure models, the production of NO and cyclic GMP in subcutaneous tissue is involved in antinociceptive states (Duarte et al., 1992; Soares et al., 2000; Soares and Duarte, 2001; Lázaro-Ibáñez et al., 2001; Ortiz et al., 2003a; Alves et al., 2004; Sachs et al., 2004).

The standardised nomenclature for potassium channels recognizes four different types of K^+ channels known as voltage-gated (K_v), calcium-activated (K_{Ca}), inward rectifier (K_{ir}) and finally two-pore (K_{2P}) K^+ channels (Gutman et al., 2003). Besides Ca^{2+}, ATP and voltage, K^+ channels are also modulated by other messengers and many drugs that are able to inhibit or activate their opening (Ewald et al., 1985; Cook and Quast, 1990; Edwards and Weston, 1993; Gutman et al., 2003; Ocaña et al., 2004). In the present study, local peripheral administration of the ATP-sensitive K^+ channel inhibitors glibenclamide and tolbutamide (Edwards and Weston, 1993) was able to block the effect of codeine, suggesting that this opioid could activate these channels in order to produce its antinociceptive action in the periphery. Many reports have shown that the administration of ATP-sensitive K^+ channels blockers reduced the antinociceptive and antihyperalgesic effects induced by morphine and other opioids at supraspinal, spinal and peripheral levels (Ortiz et al., 2002; Ocaña et al., 2004). Likewise, it has been demonstrated that the supraspinal and spinal administrations of the ATP-sensitive K^+ channels activators pinacidil and cromakalim besides to produce an antinociceptive effect they were able to potentiate the response of morphine (Vergoni et al., 1992; Ocaña et al., 1996). Overall our results are supported with reports previous where the local peripheral administration of glibenclamide and tolbutamide were able to revert the antinociceptive and antihyperalgesic effects produced by morphine (Rodrigues and Duarte, 2000; Ortiz et al., 2002).

In this study we were able to find that the local peripheral administration of 4-AP and TEA reverted the antinociceptive effect produced by codeine. These findings also suggest the participation of voltage-gated K^+ channels in the action of codeine; however, since that 4-AP and TEA are non-selective voltage-gated blockers (Cook and Quast, 1990), this result awaits further investigation. Using the paw pressure test several authors have proved that the local peripheral administration of 4-AP and TEA was not able to block the antinociception produced by morphine, fentanyl and dibutyryl-cyclic GMP (Rodrigues and Duarte, 2000; Soares and Duarte, 2001; Rodrigues et al., 2005). Likewise, the i.c.v. administration of 4-AP and TEA did not modify the antinociception induced by μ-opioid receptor agonist in the hot plate and tail flick models in mice (Ocaña et al., 1990, 1995). This discrepancy may be due to the different experimental pain models used, diverse species, different tissues and the type and intensity of the injurious stimuli. However, it is important to indicate that in the rat formalin test 4-AP and TEA were able to revert the antinociceptive effect induced by resveratrol (Granados-Soto et al., 2002), morphine (Ortiz et al., 2002) and other analgesic drugs (Ortiz et al., 2003a,b, 2005).

It is substantial to indicate that the activation of the NO-cyclic GMP-ATP-sensitive K^+ channels pathway does not always participate in the antinociceptive actions induced by analgesic drugs on the formalin test. We have previously demonstrated that the antinociceptive effects produced by metamizol (Ortiz et al., 2003b), resveratrol (Granados-Soto et al., 2002) and meloxicam (Ortiz et al., 2005) in the formalin test were not reverted by the local peripheral administration of glibenclamide and tolbutamide. Similarly, indomethacin-induced antinociception was not blocked by the local peripheral administration of L-NAME, glibenclamice and tolbutamide in the same model (Ortiz et al., 2003a).

When applied alone, neither the opioid receptor-NO-cyclic GMP pathway inhibitors nor the K^+ channels blockers affected formalin behaviour. The lack of effect of these compounds is consistent with results of studies in which these drugs were not able to modify the formalin-induced nociception and mechanical hyperalgesia (Rodrigues and Duarte, 2000; Ortiz et al., 2003a; Sachs et al., 2004), thus excluding the possibility that the inhibition of codeine antinociception could be due to a hyperalgesic or nociceptive effect of the blockers used. The lack of modification of the flinching behavior by the different modulators at concentrations able to prevent codeine antinociception might also indicate that the opioid receptor-NO-cyclic GMP-K^+ channels pathway in subcutaneous tissue involved in the modulation of pain are not tonically activated.

In summary, the results showed here displayed that codeine was able to produce peripheral antinociception on the 5% formalin test during both phases. Potassium channel opening appears to be the consequence of the activation of opioid receptors and to an increase in the intracellular content of NO and cyclic GMP at the nociceptor. Taken together then, data from the present study show that the opioid receptor-NO-cyclic GMP-K^+ channel pathway is involved in the peripheral antinociceptive action induced by codeine.

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