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Role of the NO-cGMP pathway in the systemic antinociceptive effect of clonidine in rats and mice

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Abstract

The mechanism underlying the analgesic effect of clonidine, an α_2-adrenoceptor agonist, remains uncertain. Activation of α_2-adrenoceptor induces the release of nitric oxide (NO) from endothelial cells, which has led us to test the hypothesis that the observed antinociceptive effect induced by the systemic administration of clonidine depends on the NO-cGMP pathway. The possible involvement of an opioid link in the antinociceptive effect of clonidine was also evaluated. The antinociceptive effect induced by systemic administration (intravenous or intraperitoneal) of clonidine was evaluated using the rat paw formalin, mice tail-flick and writhing tests. Clonidine (3–120 μg/kg) induces a dose-dependent antinociceptive effect in the formalin, tail-flick and writhing tests. The antinociceptive effect of clonidine in a dose that had no sedative effect assessed by rota rod test, was significantly reduced by NO-synthase and guanylyl cyclase inhibition. The antinociceptive effect of morphine, but not clonidine, was inhibited by naloxone. Our current results suggest that the antinociceptive effect of systemic clonidine does not involve the opioid receptor and is modulated by the NO-cGMP pathway.
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Keywords: Clonidine; Antinociception; NO; 7-NI; ODQ; Morphine; Naloxone

1. Introduction

Clonidine is an α_2-adrenoceptor agonist, used mainly in the treatment of arterial hypertension (Mrockzek et al., 1972), also with antinociceptive (Sierralta et al., 1996) and sedative effects (Drew et al., 1979). Although the sedative effect of clonidine is not yet a routine therapeutic indication, the clinical use of clonidine in the control of pain is becoming important (Aveline et al., 2002). The antihypertensive, sedative and antinociceptive effects of clonidine probably have a common mechanism, because all these effects are inhibited by α_2-adrenoceptor antagonists (Timmermans et al., 1981; Howe et al., 1983). The analgesic effect of clonidine is observed through different methods of administration, such as intrathecal, epidural, intravenous, transdermic and oral. The mechanisms responsible for the antinociceptive effect of clonidine are not yet completely understood, and may involve central (Ossipov et al., 1985), spinal (Yaksh and Reddy, 1981) and peripheral pathways (Nakamura and Ferreira, 1988). The putative involvement of nitric oxide (NO) in the analgesic effect of intrathecal clonidine remains controversial, because the reduction of the antinociceptive effect of clonidine by NO-synthase inhibition demonstrated by Lothe et al. (1994) and Pan et al. (1998), was not confirmed by Xu et al. (1996). There are no reports of involvement of the NO-cGMP pathway in the systemic antinociceptive effect of clonidine. We have recently established that the antihypertensive (Soares de Moura et al., 2000) and sedative (Soares de Moura et al., 2001) effects of clonidine are significantly reduced by inhibiting NO-synthase and guanylyl cyclase. Our previous findings led us to the present study in which we hypothesised that a NO-cGMP pathway may also modulate

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the systemic antinociceptive effect of clonidine. In the current investigation, we assessed, in rats and mice, the role of NO-cGMP pathway and opioid mechanisms in the antinociceptive effect of clonidine.

2. Materials and methods

2.1. Animals

Male Swiss mice (20–25 g) and Wistar rats (250–350 g) were housed in the Animal Care Facility at 26 ± 1 °C and were maintained on a 12-h light/dark cycle. The animals were allowed free access to food and water until 1 h before the experiments. Animal handling and experimental procedures were approved by the Ethical Committee for Use of Experimental Animals of the State University of Rio de Janeiro. Each animal was used only once and received one dose of the drug tested. Control animals (taking vehicle) were run interspersed concurrently with drug-treated animals, which prevented all the controls from being run on a single group of animals at one time during the course of the investigation.

2.2. Measurement of antinociceptive activity

Antinociception was assessed by three different tests described below.

2.2.1. Formalin-induced hindpaw-licking method in rats

The formalin test used to study the antinociceptive effect of clonidine was adapted from Abbott et al. (1995). The animals were injected subcutaneously with formalin (50 μl; 5% v/v) into the dorsal surface of the right hindpaw. Observation started immediately after formalin injection and the duration of paw licking and biting was monitored in the periods 0–5 min (early phase) and 20–30 min (late phase). The antinociceptive effect of clonidine (10–60 μg/kg ip), injected 30 min before formalin, was assessed in rats pretreated with saline (60 min before formalin). The effect of clonidine (30 μg/kg ip) injected 15 min before formalin was also assessed in rats pretreated with 7-nitroindazole (7-NI; 30 and 60 mg/kg ip) or 1H-[1,2,4]oxadiazole [4,3-a] quinoxalin-1-one (ODQ; 2.5 mg/kg ip) and vehicle (DMSO 7.5% in saline ip), injected 30 min before formalin. The antinociceptive effects of clonidine (30 μg/kg ip) and morphine (1 mg/kg ip), injected 30 min before formalin, were assessed in rats pretreated with saline or naloxone (0.3 mg/kg ip) 60 min before formalin.

2.2.2. Acetic acid-induced writhing in mice

The mice were injected intraperitoneally with acetic acid (10 ml/kg, 0.6% v/v), as previously reported by Koster et al. (1959), and placed in a 40 × 30 × 25 cm box maintained in a quiet and illuminated room. The resulting abdominal constrictions (writhes) were counted for 10 min thereafter, starting 5 min after the administration of the acetic acid solution. A writhe was defined as a contraction of the abdominal muscles followed by an extension of the forelimbs and elongation of the animal body. Saline or clonidine (3, 10 or 20 μg/kg) was injected intravenously in the dorsal vein of the tail, 5 min before the intraperitoneal administration of the acetic acid solution. The antinociceptive effect of clonidine was also assessed in mice pretreated with 7-NI (15 and 30 mg/kg im) or ODQ (2.5 mg/kg im) and vehicle (DMSO 7.5% in saline im) injected 25 min before clonidine. The antinociceptive effect of clonidine (20 μg/kg iv) or morphine (1 mg/kg iv) was assessed in mice pretreated with naloxone (0.2 mg/kg im) or vehicle (saline im) injected 25 min before clonidine or morphine.

2.2.3. Warm-water tail-flick method in mice

The test consisted of immersing the end of the mice tails in warm water (50 ± 1 °C) and measuring the time elapsed until the tails flicked (tail-flick latency) to a maximum of 10 s cutoff time to prevent tissue damage (Sewell and Spencer, 1976). The mice were restrained individually in a plastic tube with two holes, one for ventilation and the other to permit tail exposition to the warm water. Baseline records of tail-flick latencies correspond to a mean of three measurements obtained with intervals of 10 min before administration of clonidine (10–120 μg/kg ip), morphine or saline (time zero). The animals were resubmitted to the test at 5, 15, 30, 45 and 60 min after time zero. The antinociceptive effect of clonidine (30 μg/kg ip) was also studied in mice pretreated with 7-NI (15 and 30 mg/kg ip), ODQ (10 mg/kg ip) or vehicle (DMSO 7.5% in saline) 25 min before clonidine. The antinociceptive effect of clonidine (30 μg/kg ip) and morphine (1 mg/kg ip) was assessed in mice pretreated with saline or naloxone (0.1 mg/kg ip) injected 25 min before clonidine or morphine.

2.3. Rota rod performance test

To evaluate the locomotor activity after clonidine administration, the rats (250–280 g) were tested on the rota rod apparatus. The rats were first conditioned on the rota rod (Letica Scientific Instruments) at a constant speed of 18 rpm. After 7 days of training sections, those rats that remained on the rotating spindle for two consecutive periods of 60 s or more (maximum of 180 s) were selected in the pretest. Thereafter, each rat was tested again on the accelerating rota rod in which the speed of the spindle was increased from 4 to 40 rpm over a period of 30 s and the time spent on the accelerating rota rod was determined. Under these conditions, two testing sections with 5 min of interval were performed, before and 30 min after injection of clonidine (30 μg/kg ip). Results are expressed as the time spent on the accelerating rota rod obtained from the two consecutive trials before and after clonidine injection. All tests started at approximately the same time (10 a.m.).

2.4. Statistical analysis

The results are reported as the mean ± S.E.M. A one-way analysis of variance (ANOVA) was used to compare the changes between different doses or treatments. When a significant effect was indicated, comparisons between individual means were subsequently tested using the Bonferroni procedure (Graph Pad in Stat, version 3.01). Significant differences between two groups were also determined by the Student's *t* test (two-tail *P* values) for unpaired samples. Statistical differences were considered to be significant at $P < .05$. The experimental ED_{50} values (effective dose producing a 50% reduction of nociceptive behavior response) was determined from the dose–response curves of clonidine using GraphPad InStat program.

2.5. Drugs

All drugs, except 7-NI and ODQ, were diluted in saline. 7-NI and ODQ were diluted in DMSO (7.5% in saline). Clonidine hydrochloride was a gift from Boehringer de Angeli Quimica e Farmacêutica, São Paulo, Brasil; 7-NI was purchased from Sigma, St. Louis, MO, USA. ODQ was purchased from Calbiochem-Novabiochem, Canada. Morphine was purchased from Laboratório Cristalia, São Paulo, Brasil. Naloxone was purchased from Rhodia Farmacêutica, São Paulo, Brasil.

3. Results

3.1. Antinociceptive effect of clonidine

Clonidine administered either intravenously or intraperitoneally induced a dose-dependent antinociceptive effect in both phases of the rat formalin test ($n = 6$), in the mouse tail-flick test ($n = 6$) and in the writhing test ($n = 6$; Fig. 1). The experimental effective dose resulting in a 50% reduction of control response (ED_{50}) of clonidine was 23.04 μg/kg in Phase 1 and 16.44 μg/kg in Phase 2 of formalin test. In the writhing test, the ED_{50} of clonidine was 11.75 μg/kg. It should be noted that clonidine (30 μg/kg ip) elicited its antinociceptive effect without changing motor coordination as revealed by the rota rod test. Rota rod performance in the clonidine-treated animals (20.7 ± 1 s; $n = 9$) was not significantly different from the control group (21.3 ± 1 s; $n = 9$).

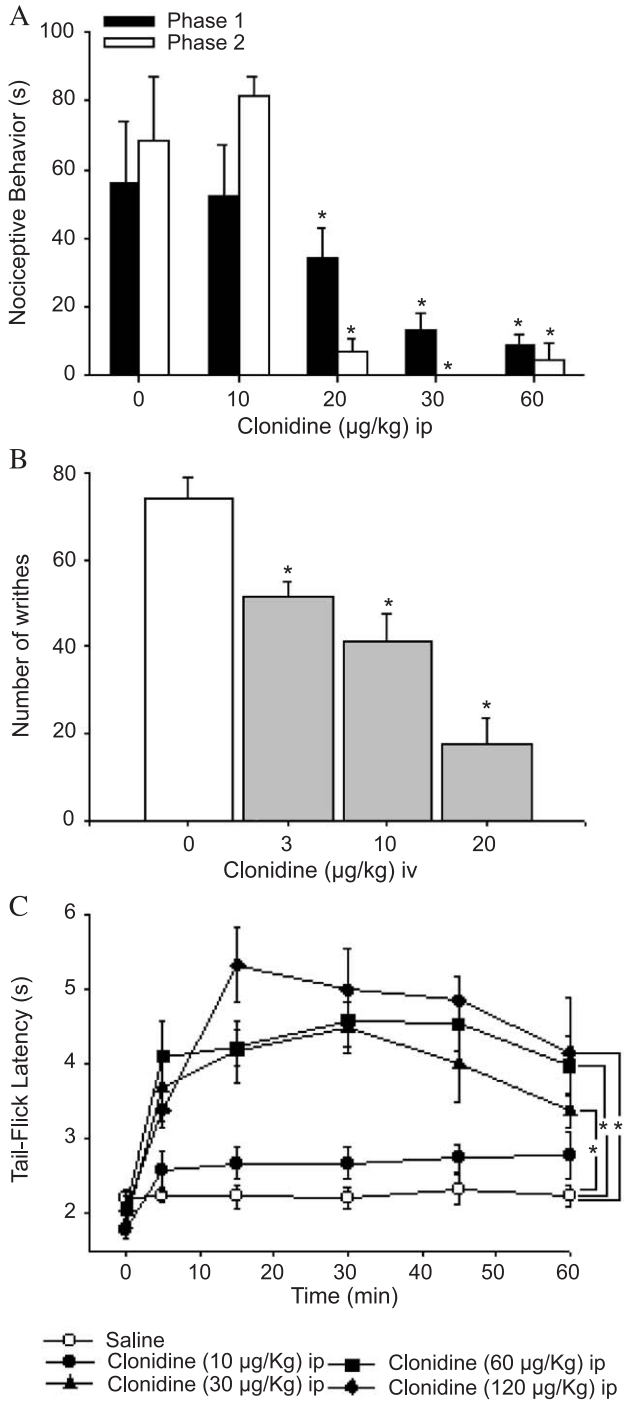

Fig. 1. Antinociceptive effect of clonidine assessed by formalin test in rat (A) or writhing (B) and tail-flick (C) tests in mice. Results are expressed as the mean ± S.E.M. of six animals for each group. * $P < .05$ as compared to saline (0, on formalin test and writhing tests; –O– on tail-flick test; ANOVA and Bonferroni's Multiple Comparison Test).

3.2. Effect of inhibition of NO-synthase and guanylyl cyclase on the antinociceptive effect of clonidine

Inhibition of NO-synthase by 7-NI did not change either the tail-flick ($n = 8$) and writhing tests ($n = 8$) or the early phase of the formalin ($n = 6$) test when compared to vehicle (DMSO in saline). As previously described (Moore et al., 1993), 7-NI significantly reduced the late phase ($n = 6$) of formalin test. Pretreatment with 7-NI significantly reduced the antinociceptive effect of clonidine (30 μg/kg ip) in both phases of the formalin test and in the writhing and tail-flick tests when compared to 7-NI alone (Fig. 2).

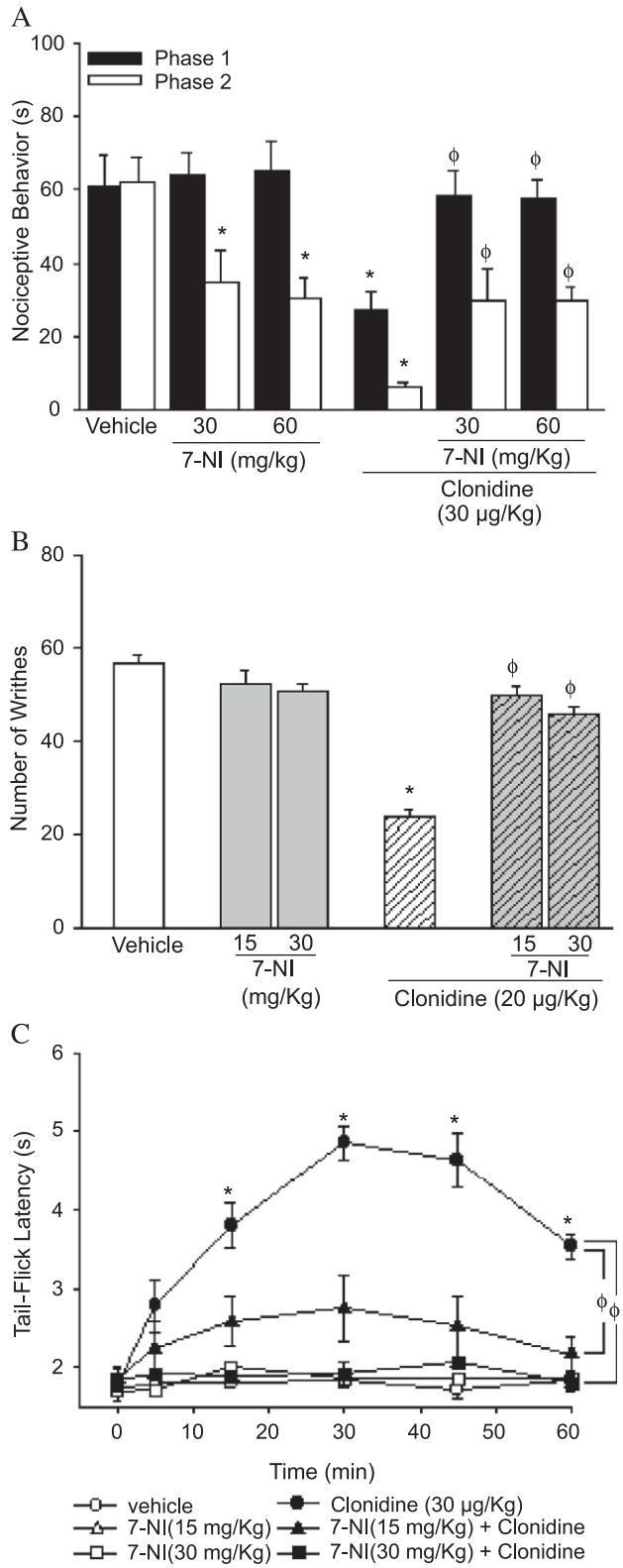

Fig. 2. Effect of 7-NI on the antinociception induced by clonidine in formalin test in rat (A) or writhing (B) and tail-flick (C) tests in mice. Results are expressed as the mean ± S.E.M. of six to eight animals for each group. * $P < .05$ compared to 7-NI vehicle (DMSO 7.5% in saline). $^{\phi}P < .05$ compared to clonidine responses without 7-NI (Student's t test).

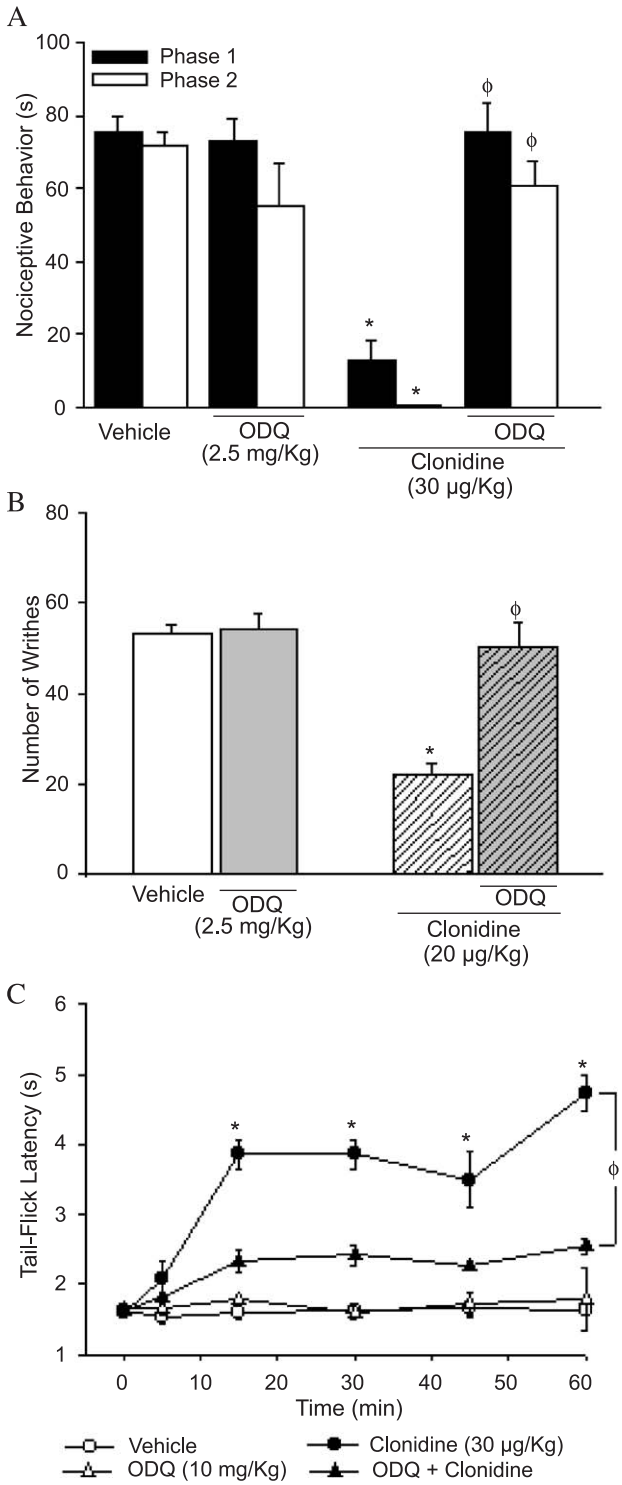

Fig. 3. Effect of ODQ on the antinociception induced by clonidine in the formalin test in rat (A) or writhing (B) and tail-flick (C) tests in mice. Results are expressed as the mean ± S.E.M. of six to eight animals for each group. * $P < .05$ compared to ODQ vehicle (DMSO 7.5% in saline). $^{\phi}P < .05$ compared to clonidine responses without ODQ (Student's t test).

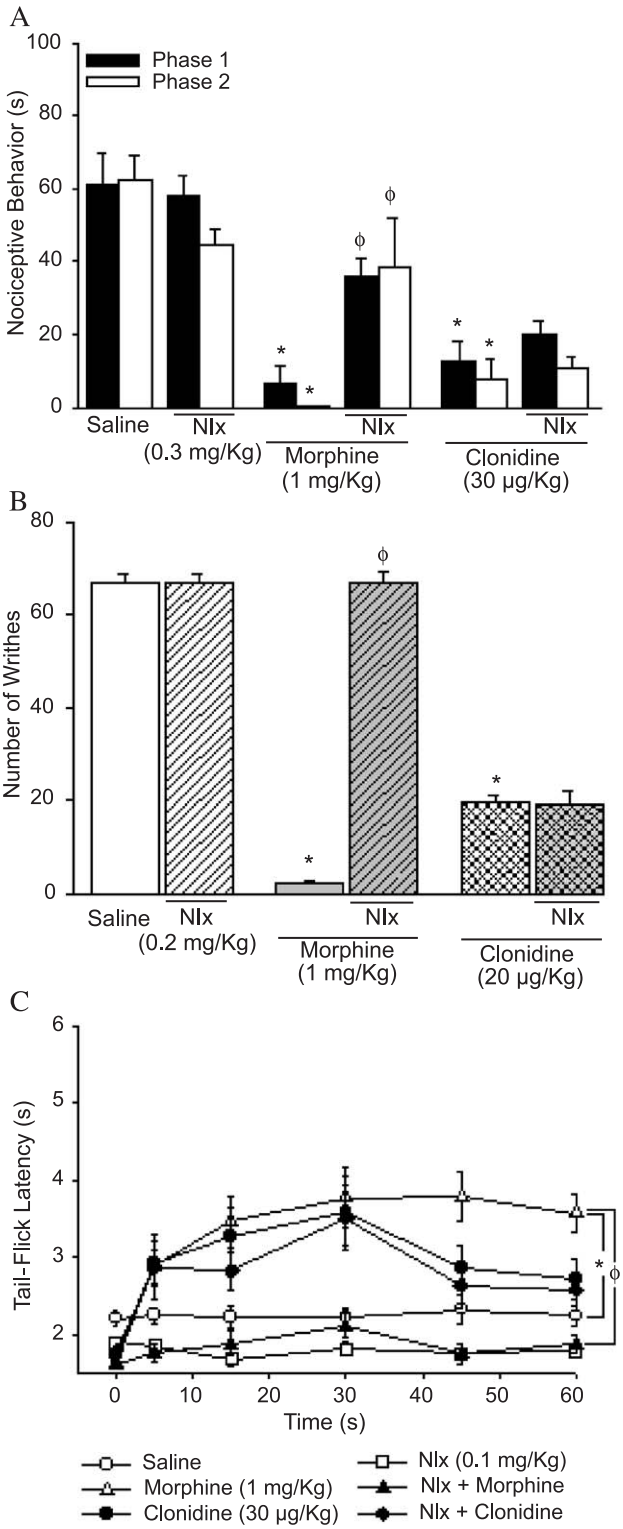

Fig. 4. Effect of naloxone (Nlx) on the antinociception induced by clonidine or morphine in formalin test in rat (A), writhing (B) and tail-flick (C) tests in mice. Results are expressed as the mean ± S.E.M. of 6–11 animals for each group. * $P<.05$ compared to saline (vehicle). $^{\Phi}P<.05$ compared to morphine responses without Nlx (Student's t test).

Inhibition of guanylyl cyclase by ODQ did not change either the tail-flick ($n=8$) and writhing tests ($n=6$) or both phases of the formalin test ($n=6$) when compared to vehicle (DMSO in saline). Pretreatment with ODQ significantly reduced the antinociceptive effect of clonidine observed in both phases of formalin test ($n=6$), writhing ($n=6$) and tail-flick tests ($n=8$; Fig. 3).

3.3. Effect of naloxone on the antinociceptive effect of clonidine

Inhibition of opioid receptors by naloxone (0.1–0.3 mg/kg ip) did not change either the formalin ($n=6$), tail-flick ($n=6$) or writhing tests ($n=6$–11) when compared to saline. The antinociceptive effect of morphine (1 mg/kg ip), used as a positive control, was significantly reduced by naloxone. On the other hand, naloxone did not change the antinociceptive effect of clonidine in all tests (Fig. 4).

4. Discussion

The present study demonstrates that the systemic administration of clonidine reduces, in a dose-dependent manner, pain-induced behaviours assessed by the subplantar injection of formalin in rats and intraperitoneal injection of acetic acid solution or thermal tail stimulation in mice. Our observations confirm previous results, that systemic administration of clonidine induces an antinociceptive effect in experimental animals (Sierralta et al., 1996). The mechanism of the antinociceptive effect of clonidine is complex and involves central (Ossipov et al., 1985), spinal (Yaksh and Reddy, 1981) and peripheral (Nakamura and Ferreira, 1988) levels. The antinociceptive effect of clonidine is probably due to the activation of α_2-adrenoceptors in the central nervous system because it is inhibited by yohimbine (Howe et al., 1983). However, the exact mechanisms linking α_2-adrenoceptors activation and the antinociceptive effects of clonidine are unknown.

The antinociceptive effect of clonidine demonstrated in the present study is not due to a sedative effect because 30 µg/kg of clonidine that induced a significant antinociceptive effect did not induce any change in the motor coordination as evaluated by the rota rod test.

Clonidine induces release of NO (Vanhoutte, 2001), a very important modulator of neuronal activity that plays a significant role in the peripheral (Duarte et al., 1992) and central mechanisms of nociception (Tseng et al., 1992), through mechanisms not yet clarified. Depending on the experimental conditions, NO increases or decreases pain (Meller and Gebhart, 1993). In addition, NO release is increased (Xu et al., 1997, 2000) or decreased (Lin et al., 2002) by intrathecal injection of clonidine. Our present results show, in agreement with a previous report (Moore et al., 1993), that the inhibition of NO-synthase by 7-NI induces a significant antinociceptive effect on the late, but not the early phase of the formalin-

induced nociceptive response, an effect that is probably due to involvement of NO in the phenomenon of "wind-up" in dorsal horn neurones following activation of sensory C fibres (Moore et al., 1993).

This study is the first to show a modulation of the systemic antinociceptive effect of clonidine by the NO-cGMP pathway in mice and rats. Our results demonstrate that the antinociceptive effect of clonidine assessed by formalin test and tail-flick or writhing tests is significantly reduced by 7-NI in a dose that inhibits NO-synthase (Moore et al., 1993). Our data is not in accordance with previous results showing that NO-synthase inhibition did not reduce the antinociception from intrathecal clonidine (Xu et al., 1996; Przesmycki et al., 1999; Lin et al., 2002). The difference in our results could be explained if the effect of clonidine injected directly into the spinal cord had different antinociceptive mechanisms from the systemic application. The NO-cGMP pathway depends on the synthesis and release of NO triggered by activation of NO-synthase, that activating guanylyl synthase induces the formation of cGMP, the most important messenger of the system. The involvement of NO-cGMP pathway in the antinociception induced by the systemic administration of clonidine is demonstrated by the inhibitory effect of 7-NI, an inhibitor of the NO-synthase and further strengthened by the observation that ODQ, in a dose that inhibits guanylyl cyclase (Da Silva-Santos et al., 2002), significantly reduced the antinociceptive effect of clonidine in the formalin, tail-flick and writhing tests.

Probably, the hypotensive effect of clonidine does not play a role in the antinociceptive effect of clonidine because we found that the hypotensive effect of clonidine in anesthetized rats (as described by Soares de Moura et al., 2000) is not inhibited by 7-NI (Soares de Moura, personal observation). The antinociceptive effect of clonidine demonstrated in the present study is not due to a sedative effect because 30 μg/kg of clonidine that induced a significant antinociceptive effect did not induce any change in the motor coordination as evaluated by the rota rod test.

Considering the present results and our previous findings demonstrating that the antihypertensive (Soares de Moura et al., 2000) and the sedative (Soares de Moura et al., 2001) effects of clonidine are antagonised by NO-synthase and guanylyl cyclase inhibition, the antihypertensive, sedative and analgesic effects of clonidine probably have a common mechanism modulated by a NO-cGMP pathway.

The mechanism of the analgesia induced by opioids involves modulation of the adrenergic system, because systemic or intracerebral administration of opiates increases norepinephrine concentration in the lumbar cerebrospinal fluid (Tyce and Yaksh, 1981). Therefore, an interaction between opioid receptors and the activation of α_2-adrenoceptors could be important in the mechanism of the antinociceptive effect of clonidine. Sierralta et al. (1996), have shown that a low dose of naloxone (1 μg/kg) inhibits the antinociceptive effect of the intracerebroventricular administration of clonidine, but at higher doses (10 mg/kg), naloxone increases the antinociceptive effect of clonidine. However, the present study showed that inhibition of opioid receptors with naloxone in a dose that significantly reduced the antinociceptive effect of morphine (1 mg/kg) did not decrease the systemic antinociceptive effect of clonidine in the formalin test and in the tail-flick or writhing test, therefore suggesting that the systemic analgesic effect of clonidine is independent on opioid receptors.

In summary, the data obtained in the present work suggest that NO-cGMP pathway plays a very important role in the systemic antinociceptive effect of clonidine in rodents. Furthermore, the systemic antinociceptive effect of clonidine is independent on opioid receptors.

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