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Sildenafil produces antinociception and increases morphine antinociception in the formalin test

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Abstract

The antinociceptive activity of an inhibitor of phosphodiesterase 5 alone or combined with morphine was assessed in the formalin test. Local administration of 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo [3,4-d]pyrimidin-5-yl)phenylsulfonyl]-4-methyl piperazine (sildenafil, inhibitor of phosphodiesterase 5) produced a dose-dependent antinociceptive effect in the second phase of the formalin test in female Wistar rats. In contrast, morphine produced antinociception in both phases. Sildenafil significantly increased the morphine-induced antinociception. The antinociception produced by the drugs alone or combined was due to a local action, as its administration in the contralateral paw was ineffective. Pretreatment of the paws with N^G-L-nitro-arginine methyl ester (L-NAME, nitric oxide (NO) synthesis inhibitor), 1*H*-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, guanylyl cyclase inhibitor) or naloxone blocked the effect of the combination. Results suggest that opioid receptors, NO and cyclic GMP are relevant in the combination-induced antinociception. In conclusion, sildenafil produced antinociception and increased that produced by morphine, probably through the inhibition of cyclic GMP degradation.

Keywords: Morphine; Sildenafil; cGMP; N^G-L-nitro-arginine methyl ester (L-NAME); ODQ (1*H*-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one); Naloxone

1. Introduction

Experimental evidence indicates a critical role of Ca^{2+} and cyclic AMP in the sensitization of the primary sensory neuron (Ferreira and Nakamura, 1979; Taiwo and Levine, 1991). This evidence is in line with the observation that prostanoid receptors are coupled with adenylyl cyclase (Smith et al., 1998) to produce cyclic AMP. Moreover, the possibility of such a role is supported by the fact that intraplantar administration of cyclic AMP analogues or non-specific phosphodiesterase inhibitors enhances prostaglandin E_2-induced mechanical hyperalgesia (Ferreira and Nakamura, 1979; Taiwo and Levine, 1991; Ouseph et al., 1995).

On the other hand, it has been proposed that cyclic GMP is involved in antinociception. This proposal was based on the observation that local administration of L-arginine produces antinociception in rats with carrageenin-induced hyperalgesia, the effect being blocked by nitric oxide (NO) inhibitors and methylene blue (a soluble guanylyl cyclase inhibitor) (Duarte et al., 1990). In prostaglandin- and carrageenin-induced hyperalgesia, the local administration of opiates or non-enzymatic NO donors also produces antinociception. While pretreatment of the rats' paws with methylene blue inhibits the action of morphine and the NO donor, the NO synthase inhibitor only inhibited opiate analgesia (Ferreira et al., 1991). The intraplantar injection of dibutyryl–cyclic GMP caused antinociception (Ferreira and Nakamura, 1979). Therefore, the neuronal balance of cyclic AMP and cyclic GMP concentrations seems to be very important for the functional up- or down-regulation of the nociceptor (Ferreira and Nakamura, 1979; Duarte et al., 1990; Cunha et al., 1999).

Intracellular cyclic GMP concentrations are regulated by the action of guanylyl cyclases and by the rate of degradation by cyclic GMP-specific phosphodiesterases (Beavo, 1995; Pyne et al., 1996). NO activates soluble guanylyl cyclase, which in turn catalyzes the formation of cyclic GMP from GTP, whereas cyclic GMP-specific phosphodiesterases catalyze the hydrolysis of cyclic GMP

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to GMP ending signal transduction (Pyne et al., 1996). Phosphodiesterase 5, phosphodiesterase 6 and phosphodiesterase 9 are specific for cyclic GMP, however, the phosphodiesterase 5 isoenzyme seems to be the most relevant enzyme in cyclic GMP inactivation in cells (Beavo, 1995; Pyne et al., 1996).

Sildenafil (Viagra®) is a novel inhibitor of the cyclic GMP-specific phosphodiesterase 5 (Terrett et al., 1996), which has been shown to be effective in the clinical management of erectile dysfunction (Boolell et al., 1996). Since sildenafil increases the intracellular concentrations of cyclic GMP, we hypothesized that sildenafil will indeed produced antinociception and/or increase that produced by morphine. The purpose of the present study was to investigate the peripheral effect of sildenafil (a specific inhibitor of phosphodiesterase 5) alone and combined with morphine in the formalin test.

2. Material and methods

2.1. Animals

Female Wistar rats aged 6–7 weeks (weight range, 120–160 g) from our own breeding facilities were used in this study. Female animals were used based on our observations that formalin injection produces the same pattern of flinching in either sex (unpublished observation). The stage of the estrous cycle was not determined in our experiments. The rats had free access to food and drinking water before the experiment. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (IASP, 1983). Additionally, the study was approved by the local Animal Care Committee.

2.2. Measurement of antinociceptive activity

Antinociception was assessed with the formalin test. Rats were placed in a open Plexiglas observation chamber for 30 min to allow them to accommodate to their surroundings, then they were removed for formalin administration. The rats were injected in the plantar surface of the right hind paw (intraplantar, i.pl.) with 50 μl of dilute formalin (1%), using a 30-gauge needle. The animal was then returned to the chamber for observation. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. The rats were observed for nociceptive behavior immediately after formalin injection. Nociceptive behavior was quantified as the number 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 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 killed in a CO_2 chamber.

2.3. Drugs

Morphine hydrochloride was obtained from Secretraría de Salud (Mexico City). Sildenafil citrate (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-*H*-pyrazolo [3,4-d]pyrimidin-5-yl)phenyl sulfonyl]-4-methyl-piperazine) was a gift of Laboratorios Proquigama (Mexico City). N^G-L-nitro-arginine methyl ester (L-NAME), N^G-D-nitro-arginine methyl ester (D-NAME), 1*H*-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and naloxone hydrochloride were purchased from Research Biochemical International (Natick, MA, USA).

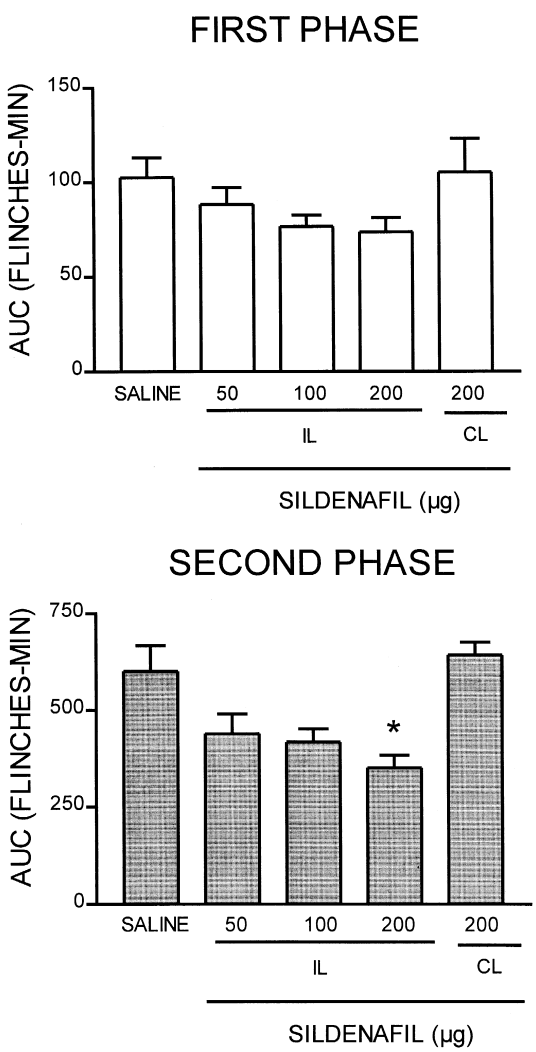

Fig. 1. Local antinociceptive effect of sildenafil in the formalin test. Rats were pretreated with an i.pl. injection of saline or sildenafil into either the right (ipsilateral, IL) or left (contralateral, CL) paw, before formalin injection. Data are expressed as the area-under-the-number-of-flinches-against time curve (AUC). Bars are the means ± S.E.M. for six to eight animals. * Significantly different from saline ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

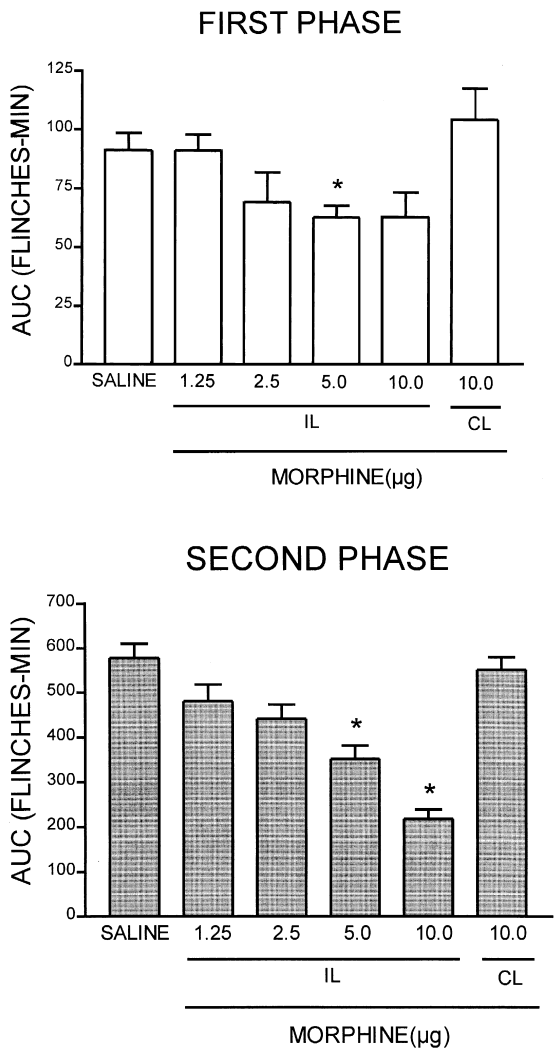

Fig. 2. Local antinociceptive effect of morphine in the formalin test. Rats were pretreated with an i.pl. injection of saline or morphine into either the right (ipsilateral, IL) or left (contralateral, CL) paw, before formalin injection. Data are expressed as the area-under-the-number-of-flinches-against time curve (AUC). Bars are the means ± S.E.M. for six to eight animals. * Significantly different from saline ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

2.4. Study design

The rats received saline and increasing doses of either sildenafil (50, 100 and 200 µg, i.pl.) or morphine (1.25, 2.5, 5 and 10 µg, i.pl.) 20 min before formalin injection. To determine if sildenafil was able to increase the effect of morphine, a non-effective dose of both drugs was used. To assess whether the effect of sildenafil on morphine-induced antinociception was mediated by the local increase in cyclic GMP or opiate receptors, the effect of L-NAME and ODQ as well as of naloxone on the antinociceptive effect was assessed. To assess if the effect was due to a local action, formalin was administered in one paw and the tested drug in the contralateral paw. Rats in all groups were tested for any possible behavioral side-effects like reduction in righting, stepping, corneal and pinna reflex and catalepsy.

2.5. Data analysis and statistics

All results are presented as means ± S.E.M. for six to eight animals per group. Curves were made for number of flinches against time. The area-under-the-number-of-flinches-against-time curves (AUC) for both the first and second phase was calculated by the trapezoidal rule. Anal-

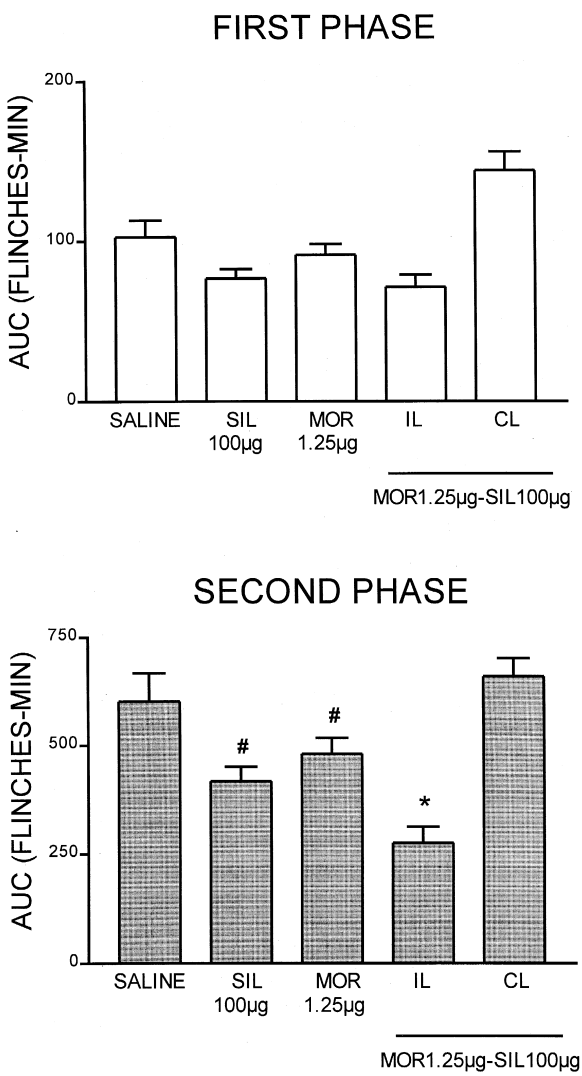

Fig. 3. Peripheral antinociceptive effect of the morphine–sildenafil combination in the formalin test. Bars corresponding to sildenafil or morphine alone were taken from data of Figs. 1 and 2. Rats were pretreated with saline or the sildenafil–morphine combination (MOR1.25–SIL100 µg) into either the 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 means ± S.E.M. for six to eight animals. * Significantly different from saline and either drug alone ($P < 0.05$), # significantly different from the morphine–sildenafil group ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

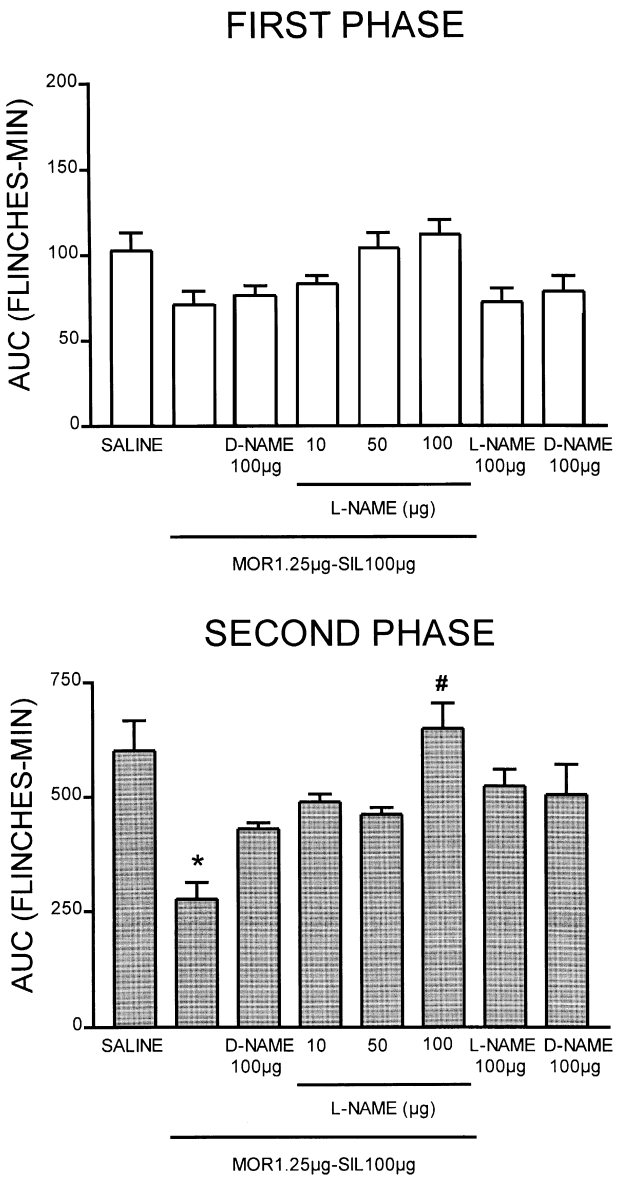

Fig. 4. Effect of L-NAME on the peripheral antinociception produced by morphine–sildenafil combination. Rats were pretreated with an i.pl. injection of L-NAME or D-NAME and the morphine–sildenafil combination into the right paw. Data are expressed as the area-under-the-number-of-flinches against time curve (AUC). Bars are the means ± S.E.M. for six to eight animals. * Significantly different from the saline group ($P < 0.05$) and #significantly different from the morphine–sildenafil group ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

ysis of variance followed by Tukey's test was used to test differences between treatments. A $P < 0.05$ was considered significant.

3. Results

3.1. Antinociceptive effect of sildenafil and morphine alone

Ipsilateral, but not contralateral, administration of sildenafil produced a dose-dependent reduction in flinching in the second phase, but not in phase 1, otherwise observed with 1% formalin injection (Fig. 1). Ipsilateral, but not contralateral, morphine injection produced a dose-dependent antinociception in both the first and second phase (Fig. 2). No side-effects were observed in either control or treated group.

3.2. Antinociception of the combination sildenafil–morphine

In order to test the effect of sildenafil on morphine-induced antinociception, a non-effective dose of sildenafil (100 µg, i.pl.) was combined with a non-effective dose of

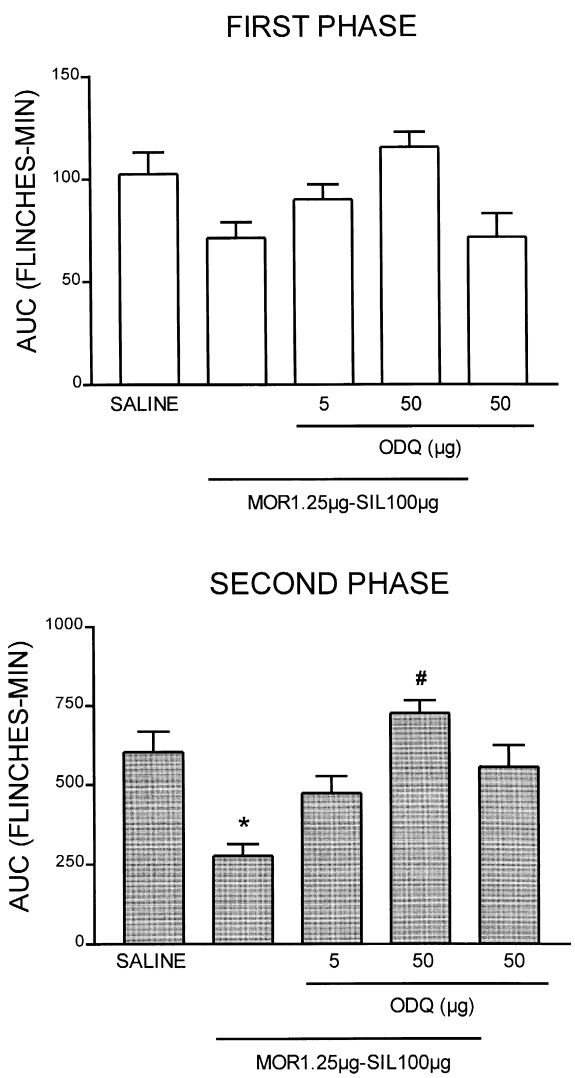

Fig. 5. Effect of ODQ on the peripheral antinociception produced by the morphine–sildenafil combination. Rats were pretreated with an i.pl. injection of ODQ and the morphine–sildenafil combination into the right paw. Data are expressed as the area-under-the-number-of-flinches against time curve (AUC). Bars are the means ± S.E.M. for six to eight animals. * Significantly different from the saline group ($P < 0.05$) and #significantly different from the morphine–sildenafil group ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

morphine (1.25 μg, i.pl.). Doses were selected based on their lack of significant effect in the formalin test. The local co-administration of sildenafil and morphine produced a reduction in the number of flinches (Fig. 3) compared to either drug alone ($P < 0.05$). The antinociceptive action of the combination was due to a local effect, as administration of the combination to the contralateral paw did not produce any effect (Fig. 3). No side-effects were observed with the combination.

3.3. Effect of L-NAME, ODQ and naloxone on the antinociception produced by the combination sildenafil–morphine

The NO synthesis inhibitor, L-NAME (100 μg, i.pl.), did not produce any antinociceptive effect by itself. However, L-NAME completely reversed, at the highest dose tested (100 μg), the antinociception produced by the sildenafil–morphine combination (Fig. 4). Administration of the inactive isomer of L-NAME, D-NAME, was not able to reverse the antinociception produced by the sildenafil–morphine combination. In addition, pretreatment of the paw with L-NAME or D-NAME alone did not significantly modify the effect of morphine (1.25 μg) alone.

The guanylyl cyclase inhibitor, ODQ (50 μg, i.pl.), or the opioid receptor antagonist naloxone (0.1 mg/kg, i.p.), alone, did not produce any antinociceptive effect. In contrast, pretreatment of the paw with ODQ (Fig. 5) or naloxone (not shown), but not saline, completely antagonized the antinociception produced by the sildenafil–morphine combination. Pretreatment of the paw with either ODQ or naloxone did not modify the effect of morphine (1.25 μg) alone.

4. Discussion

4.1. Antinociceptive action of sildenafil

Phosphodiesterase 5, 6 and 9 are specific for degradation of cyclic GMP. However, since phosphodiesterase 6 is only expressed in retina and phosphodiesterase 9 is not sensitive to sildenafil, it was assumed that phosphodiesterase 5 is the predominant enzyme responsible for cyclic GMP hydrolysis (Beavo, 1995; Moreland et al., 1999) in our study. Sildenafil is a potent, selective and reversible phosphodiesterase 5 inhibitor (Terrett et al., 1996; Moreland et al., 1999) that blocks cyclic GMP hydrolysis ($K_i = 3$ nM). Based on previous observations (Duarte et al., 1990, 1992; Granados-Soto et al., 1997), we hypothesized that sildenafil treatment will produce antinociception. At low doses (50–100 μg/paw), sildenafil was not able to reduce the number of flinches produced by formalin. In contrast, a greater dose (200 μg/paw) of the phosphodiesterase 5 inhibitor significantly decreased the second phase, but not the first phase, of the formalin-induced nociception. The antinociceptive effect of sildenafil was due to a local action, as the contralateral administration of the drug was ineffective to reduce flinching. This result suggests major participation of peripheral phosphodiesterase 5 in the second phase of the formalin test. This suggestion is in line with the evidence that phosphodiesterase 5 is expressed in several tissues (Beavo, 1995; Stacey et al., 1998; Moreland et al., 1999). Moreover, these data indicate that the inhibition of phosphodiesterase 5 and therefore the accumulation of cyclic GMP is enough to produce antinociception. To our knowledge, this is the first report of an antinociceptive effect of selective phosphodiesterase 5 inhibitors. It has been reported recently that zaprinast, another phosphodiesterase 5 inhibitor, has no effect on prostaglandin E_2-induced hyperalgesia (Cunha et al., 1999). The differences could be due to the different models of pain used, since, with a pure inflammatory stimulus like prostaglandin E_2, the action of some drugs may not produce activation of the arginine-NO-cyclic GMP pathway. This pathway, however, seems to be stimulated by the drugs administered with an inflammatory stimulus like formalin (this study), carrageenin or uric acid (Tonussi and Ferreira, 1994; Granados-Soto et al., 1995).

4.2. Antinociceptive action of morphine

Morphine is known to cause a local antinociceptive effect against hyperalgesia induced by pure hyperalgesic substances like prostaglandin E_2, noradrenaline, dopamine, 5-hydroxytriptamine (Ferreira and Nakamura, 1979; Taiwo and Levine, 1991) or inflammatory stimulus like carrageenin (Stein et al., 1989, 1993; Levine and Taiwo, 1989; Nagasaka et al., 1996) or formalin (Abbot, 1988; Hong and Abbot, 1995; Granados-Soto et al., 1997). In our study, we observed that the ipsilateral, but not contralateral, administration of morphine produced dose-related antinociception in the formalin test. These results confirm the antinociceptive efficacy of local morphine, supporting a significant participation of peripheral opioid receptors in the antinociceptive effect of this drug in several types of pain (Abbot, 1988; Millan, 1986; Stein et al., 1989, 1993; Levine and Taiwo, 1989; Taiwo and Levine, 1991; Duarte et al., 1992; Hong and Abbot, 1995; Nagasaka et al., 1996).

4.3. Potentiation of morphine antinociception by sildenafil

A non-effective dose of sildenafil (100 μg) increased the activity of a low dose of morphine (1.25 μg). The increase in the effect was greater than that observed with the components alone. The effect of the combination was due to a local action, as the contralateral administration of the morphine–sildenafil combination did not produce any effect. These results agree with previous observations on the ability of selective (1-[3-chloroanilino]-4-phenyl-

phthalazine, MY5445) and non-selective (caffeine) phosphodiesterase inhibitors to increase the antinociception produced by some non-steroidal anti-inflammatory drugs and morphine (Ferreira et al., 1991; Duarte et al., 1992; López-Muñoz et al., 1996; Aguirre-Bañuelos et al., 1999). However, the present results demonstrate for the first time that an interaction between sildenafil and morphine occurs at the local level, and hence involves a peripheral mechanism of action.

4.4. Effect of L-NAME, ODQ and naloxone on the antinociception produced by the combination sildenafil–morphine

In order to examine other possible mechanisms to explain the morphine–sildenafil interaction, the participation of the NO-cyclic GMP pathway was assessed. The involvement of the NO-cyclic GMP pathway was suspected because local administration of either NO synthesis or soluble guanylyl cyclase inhibitors has been reported to block the antinociception produced by morphine (Ferreira et al., 1991; Granados-Soto et al., 1997) and by some non-steroidal anti-inflammatory drugs (Duarte et al., 1992; Tonussi and Ferreira, 1994; Granados-Soto et al., 1995, 1997; Lorenzetti and Ferreira, 1996). In our case, pretreatment of the paw with L-NAME, but not D-NAME, blocked in a dose-dependent way the antinociception produced by the sildenafil–morphine combination. This suggests that the morphine–sildenafil interaction could be due to the local release of NO. In addition, pretreatment of the paws with ODQ, a soluble guanylyl cyclase inhibitor (Moro et al., 1996), also blocked in a dose-dependent way the antinociception produced by the sildenafil–morphine combination. Together these data suggest major participation of the NO-cyclic GMP pathway in the peripheral antinociception produced by the morphine–sildenafil combination. We suggest that cyclic GMP could come from the activation of the NO-cyclic GMP pathway by morphine and from the inhibition of cyclic GMP degradation by sildenafil. This suggestion is based on the fact that NO and cyclic GMP inhibitors were able to reverse dose dependently the effect of morphine alone (Duarte et al., 1992; Granados-Soto et al., 1997).

The opioid receptor antagonist, naloxone (0.1 mg/kg, i.p.), alone did not produce any effect, but completely blocked the antinociceptive effect of the combination, which suggests that local activation of opioid receptors is important in the potentiation of the morphine effect by sildenafil. Naloxone did not modify the effect of morphine (1.25 μg) or sildenafil (100 μg) alone (data not shown). Sildenafil does not act at opiate receptors, therefore the fact that naloxone blocked the combination-induced antinociception suggests that peripheral opioid receptors have a significant participation in the interaction. We suggest that activation of opioid receptors by morphine produces an increase in NO, which in turn increases cyclic GMP to produce antinociception. As mentioned previously, this suggestion is based on the fact that NO and cyclic GMP inhibitors are able to reverse the effect of local (Duarte et al., 1992; Granados-Soto et al., 1997) and spinal morphine (Pataki and Telegdy, 1998). When sildenafil is co-administered with morphine, it selectively prevents the cyclic GMP degradation, leading to accumulation of cyclic GMP and an increase in antinociception.

There are several observations indicating that the NO-cyclic GMP pathway plays a hyperalgesic rather than an antinociceptive role. It has been reported that either intraplantar, systemic or spinal administration of L-NAME, but not D-NAME, produces dose-dependent antinociception in the second phase of the formalin test (Haley et al., 1992; Malmberg and Yaksh, 1993; Aley et al., 1998). A nociceptive or inflammatory role of the NO-cyclic GMP pathway has been described for bradykinin, substance P and carrageenin (Kawabata et al., 1994). A possible explanation for these conflicting observations could be that the role of this pathway varies among the groups of primary sensory neurons mobilized by different types and intensities of nociceptive stimuli as proposed previously (Granados-Soto et al., 1997; Cunha et al., 1999). However, it is likely that other factors could play a significant role in the differences observed.

The current study provided evidence for a peripheral interaction between a phosphodiesterase 5 inhibitor (sildenafil) and morphine. This interaction suggests the possibility of using this combination in the management of inflammatory pain states generated after tissue injury. The rationale for the co-administration of a phosphodiesterase 5 inhibitor and an opioid agonist would be to diminish the doses and side effects otherwise observed after the doses necessary to produce a given therapeutic effect. This is particularly important as the local administration of analgesic drugs could be useful to produce antinociception with a low incidence of side-effects. Despite the relatively rare use of single drug therapy in the treatment of pain, there are relatively few studies aimed at identifying the formal interactions, both for wanted and unwanted effects, of analgesic drug combinations. Therefore, peripheral use of analgesics alone or combined may have a potential in the treatment of post-injury pain.

In summary, local administration of sildenafil and morphine produced peripheral antinociception in the formalin test. In addition, sildenafil increased the action of morphine and this effect was antagonized by naloxone, L-NAME and ODQ, suggesting that activation of opioid receptors, the local release of NO and cyclic GMP accumulation play an important role in the interaction.

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