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Analgesic effects of morphine and loperamide in the rat formalin test: Interactions with NMDA receptor antagonists

Natalja Sevostianova [a], Wojciech Danysz [a,*], Anton Y. Bespalov [b,1]

[a] *Merz Pharmaceuticals GmbH, Eckenheimer Landstrasse 100, 60318 Frankfurt am Main, Germany*
[b] *Pavlov Medical University, 6/8 Leo Tolstoy Street, St. Petersburg 197089, Russia*

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Abstract

To reveal peripheral components of opiate analgesia, effects of loperamide, opioid agonist which does not penetrate the blood–brain barrier, were examined in formalin and acute thermal pain tests in comparison with morphine. Formalin administration induces pain behaviour such licking/biting of injected paw expressed as two phases. The first phase is caused by C-fibre activation due to peripheral stimulation, the second phase attributed to ongoing input from peripheral site, leading to spinal hyperexcitability, which is dependent on *N*-methyl-D-aspartate (NMDA) receptor activation. Loperamide (3–10 mg/kg) and morphine (6 mg/kg) reduced formalin-induced nociceptive behaviours and these effects were reversed by naloxone methiodide (0.03–10 mg/kg), opioid receptor antagonist which poorly penetrates the blood–brain barrier. Loperamide action was enhanced only by centrally active NMDA receptor antagonists memantine (3 mg/kg) and CGP 37849 (3 mg/kg), but not by NMDA/glycine$_B$ receptor antagonists showing weak or no central nervous system (CNS) activity. Present results suggest that central NMDA receptor blockade may be necessary to enhance analgesia induced through peripheral opioid mechanisms in formalin-evoked nociception.

Keywords: Pain; Opiate; Formalin test; Hargreaves test; Quaternary naloxone; Post-treatment

1. Introduction

Opiates may produce antinociception via interaction with opioid receptors located in the central and peripheral nervous systems. The importance of central sites of action has been repeatedly confirmed by antinociceptive effects of central administration of opiates in models of acute pain such as hot plate and tail-flick tests (Yaksh, 1999). Many studies in animals have also demonstrated prominent antinociceptive effects of opiates in models of persistent pain with components of tissue inflammation (Joris et al., 1987). In addition, it has been shown that these anti-inflammatory effects are mainly due to activation of peripheral opioid receptors (Stein et al., 1989), which are up-regulated during inflammation and activated by endogenous opioid peptides produced by immune cells that migrated into injured tissues (Przewlocki et al., 1992; Stein et al., 1989).

The majority of previous studies investigated the role of peripheral opioid receptors by local application of opiates at small, systemically inactive doses (Obara et al., 2004; Stein et al., 1989). However, with increased blood flow secondary to inflammation, drugs may be absorbed and it cannot be excluded that it leads to systemically mediated side effects. Another, more direct approach to demonstrate the importance of peripheral opioid receptors in antinociceptive effects of opiates under conditions of inflammation is to use opioid receptor ligands, which do not penetrate the blood–brain barrier. For this purpose, the present studies examined the effects of systemic (subcutaneous) administration of peripherally acting opioid receptor agonist, loperamide, in models of inflammatory hyperalgesia (formalin test) and acute thermal pain (Hargreaves test) in comparison with those of morphine. In order to reveal

* Corresponding author. Tel.: +49 69 150 3564; fax: +49 69 596 2150.
E-mail address: wojciech.danysz@merz.de (W. Danysz).
[1] Current address: Abbott GmbH & Co. KG, P.O. Box 21 08 05, Ludwigshafen 67008, Germany.

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the peripheral components in the effects of morphine and loperamide, these drugs were administered alone and in combination with naloxone methiodide, an opioid receptor antagonist that poorly penetrates into the brain (Russell et al., 1982).

Formalin test is a widely accepted model of prolonged noxious stimulation, which is based on the assessment of behaviors induced by subcutaneous administration of aqueous formaldehyde solution (formalin) into the animal's paw (Dubuisson and Dennis, 1977). Over the period of 1 h, formalin-induced behaviours such as licking and biting of the injected paw are expressed as two clear-cut phases. The first phase is caused predominantly by C-fibre activation due to the peripheral stimulation (Martindale et al., 2001; McCall et al., 1996) and is thought to reflect acute pain state. The second phase has been attributed to ongoing afferent input from peripheral site (Pitcher and Henry, 2002) that leads to the development of spinal cord hyperexcitability, which is dependent on *N*-methyl-D-aspartate (NMDA) receptor activation (Coderre et al., 1990; Coderre and Melzack, 1992), and is commonly referred to as the "tonic" pain phase (Coderre and Yashpal, 1994). Thus, the formalin test allows to assess simultaneously acute and tonic pain states within the same subject.

Peripherally active opioid receptor agonists, which have restricted access to the central nervous system (CNS), might be useful in some cases in clinical practice, since they may be devoid of central side effects typical for morphine-like opiates (e.g. respiratory depression, abuse). Similarly, the risks of adverse side effects could be reduced by combining morphine-like opioid agonists with drugs that preferentially enhance their peripheral antinociceptive activity. In the formalin-induced inflammatory pain state, the afferent C-fibre barrage causes centrally amplified response involving activation of NMDA receptors (Haley et al., 1990). Accordingly, NMDA receptor antagonists enhance opiate analgesia in persistent pain models when they are given preemptively (Bernardi et al., 1996; Nishiyama, 2000). Formalin induces primary afferent activation and this results in glutamate release from primary afferent fibres (Davidson et al., 1997). Also it has been shown that excitatory amino acid receptors are present on sensory axons (Carlton et al., 1998) and they are up-regulated following inflammation (Coggeshall and Carlton, 1999; Carlton and Coggeshall, 2002). The increasing number of sensory axons containing ionotropic glutamate receptors may be a contributing factor to peripheral sensitization (Carlton and Coggeshall, 1999). Such observations suggest that NMDA receptors on cutaneous axons can be manipulated to reduce pain of peripheral origin. Thus, the aim of the present study was to investigate contribution of peripheral and central NMDA receptors to antinociceptive effects produced by stimulation of only peripheral (loperamide) or peripheral and central (morphine) opioid receptors. In line with this aim, it was examined whether different NMDA receptor antagonists with varying degrees of penetration through the blood–brain barrier enhanced and/or prolonged the antinociceptive effects of morphine and loperamide in the model of formalin-induced nociception in rats.

2. Materials and methods

2.1. Subjects

Adult male drug- and experimentally naive Sprague–Dawley rats (200–300 g; Janvier, France) were housed in groups of four with food and water available ad libitum and alternating 12 h/12 h day–night cycle (lights on at 07:00) for at least 6 days before the experiments were started. Colony room temperature and humidity were maintained at 20±1 °C and 60±3%, respectively. All experiments were conducted during the light period of a day–night cycle. The study was approved by the Ethical Committee, Regirungspräesidium Darmstadt, Hessen and were performed in accordance with the recommendations and policies of the U.S. National Institutes of Health Guidelines for the Use of Animals. Each animal was used only once.

2.2. Drugs

Two-percent formaldehyde was made from 1 part formalin (~36.6%; formalin, Fluka, Taufkirchen, Germany) and 17.3 parts of saline. Morphine sulphate (opioid receptor agonist; Sigma, Deisenhofen, Germany), naloxone methiodide (opioid receptor antagonist, which does not penetrate the blood–brain barrier, Sigma, Deisenhofen, Germany), CGP 37849 (D,L-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid, competitive NMDA receptor antagonist, Tocris Cookson Ltd., UK), memantine (HCl, 1-amino-3,5-dimethyladamantane, uncompetitive NMDA receptor antagonist, MERZ Pharmaceuticals, Frankfurt/M, Germany), MDL 105,519 ((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid, glycine$_B$ site antagonist, does not penetrate the blood–brain barrier (OpackaJuffry et al., 1998), Sigma, Deisenhofen, Germany) were dissolved in physiological saline. MRZ 2/596 (8-chloro-1,4-dioxo-1,2,3,4-tetrahydropyridazino (4,5-b) quinoline choline salt, glycine$_B$ site antagonist, does not penetrate the blood–brain barrier (Parsons et al., 1997), MERZ Pharmaceuticals, Frankfurt/M, Germany) was dissolved in distilled water. The vehicle for loperamide hydrochloride (opioid receptor agonist, which does not penetrate blood–brain barrier, Tocris Cookson Ltd., UK) was 20% water solution of 2-hydroxypropyl-beta-cyclodextrin (Fluka, Taufkirchen, Germany). Opioid ligands were administered s.c. NMDA receptor antagonists were injected i.p. All substances were injected in volume of 1 ml/kg.

2.3. Procedures

2.3.1. Formalin test

Rats were placed individually in an open Plexiglas chamber (bowl-like cage 40×35 cm) with a mirror angled at 45° positioned behind to allow an unobstructed view of the paws by the observer. The animals were habituated to the observation chamber for 30 min prior to the experimental sessions. Formalin (50 μl) was injected s.c. into the plantar surface of the rat hind paw (left or right, counterbalanced across each treatment group) using a 27-gauge needle. After injection, rats were

immediately returned to the observation chamber and the formalin-induced behaviours were recorded by a trained observer continuously for 60 min. Formalin injection produced characteristic behaviours consisting of flinching and licking/biting of the injected paw. Everyday at least one animal from each group was tested.

The first set of experiments focused on the role of peripheral opioid receptors in formalin-induced behaviours. Centrally and peripherally acting opioid receptor agonists, morphine (6 mg/kg) and loperamide (1, 3, 6, 10 mg/kg), respectively, were administered systemically (s.c.) 30 min before the injection of 2% formaldehyde. Naloxone methiodide, an opioid receptor antagonist that does not penetrate the blood–brain barrier, was administered s.c. immediately before the injection of formalin.

In the second set of experiments, the acute interactions between opioid receptor agonists and NMDA receptor antagonists were investigated. Morphine (3 mg/kg) was administered 30 min prior to formalin injection while memantine (5 mg/kg) or saline were given either 30 min before (i.e., together with morphine) or 6 min after formalin injection (i.e., right after the first phase of formalin-evoked behaviours). To evaluate the effects of peripherally and centrally active NMDA receptor antagonists on the antinociceptive effects of loperamide, memantine (1, 3 mg/kg), CGP 37849 (3 mg/kg), MRZ 2/596 (5 mg/kg), MDL 105,519 (1 mg/kg) or their vehicles were co-administered with loperamide (1 mg/kg) 30 min before the formalin application.

2.3.2. Hargreaves test

The hind paw thermal nociceptive threshold was assessed with a heat thermal stimulator, manufactured by Dr. G. Ozaki at the University of California (San Diego, USA). Four rats from different groups were placed in the clear plastic chambers (10 × 26 × 10 cm) on a glass floor and allowed to acclimatise to their environment for 30 min before testing. The glass surface temperature was maintained at 30 ± 01 °C by feedback control. The radiant heat source consisted of a high intensity projector lamp bulb (8 V, 50 W) located 1 cm below the glass floor and projecting through a small aperture in the top of a movable holder. During the test trials, the heat source was positioned manually directly beneath the heel portion of the plantar surface of hind paw, which was in contact with the glass. A switch was used to activate the radiant heat source. A photoelectric cell aimed at the aperture detects light reflected from the paw and the lamp and the electronic clock were turned off when paw movement interrupted the reflect light.

To assess the nociceptive response to thermal stimuli, the baseline levels for right and left hind paw were determined for each animal. Then rats were injected with drugs/vehicle and immediately returned to the chambers. The tests commenced 30 min later by activation of the stimulus, which initiated a timing circuit. The time interval between the application of the light and the hind paw withdrawal response was defined as the paw withdrawal latency. In the absence of a response, the stimulation was automatically terminated at 40 s to avoid tissue injury, and that time was assigned as the response latency. Baseline response latencies averaged approximately 7–8 s.

2.3.3. Rotarod test

The rotarod apparatus (Accelerating Model, Ugo Basile, Biological Research Apparatus, Varese, Italy) was used to measure motor coordination and balance. Rats received one trial per day for 2 consecutive days. Each rat was placed in its section and the rotor accelerated from 4 to 40 revolutions per minute in a period of 5 min. The latency to fall off the rotarod within this time period was recorded. The test was performed on the third day, where each animal was tested twice, and the mean latency to fall off the rotarod was recorded and used in subsequent analysis.

Two administration regimens were chosen corresponding to the tests of acute interaction between morphine and NMDA receptor channel blocker memantine in the formalin test. In the first experiment ($n = 8$ per group), to investigate motor impairing effects of acute morphine with memantine pretreatment, morphine (3 mg/kg)/saline, memantine (5 mg/kg)/saline alone or their combination were injected 60 min before the rotarod test. Pre-test injection time of 60 min corresponded to the period between the drug(s) injection and the second phase peak of formalin-induced response. In the second experiment ($n = 8$ per group), to control for the motor effects of memantine given immediately after the first phase of formalin-induced responding (i.e., 6 min post-formalin), injection of memantine 5 mg/kg or saline was given 36 min after the administration of morphine 3 mg/kg or saline. Rotarod test was conducted 24 min after the injection of memantine.

To assess the motor impairment from combination of loperamide (1 mg/kg, s.c.) with CGP 37849 (3 mg/kg, i.p.), both drugs were injected 30 min before the rotarod test ($n = 8$ per group).

2.4. Data analysis

In case of formalin model, custom-made behavioural scoring software was used to calculate durations (in seconds) of recorded behaviours per each 6-min interval of the 60-min observation period. The 6-min interval was chosen based on earlier report on the time-course of the first (0–6 min) and second (12–60 min) phases of the formalin-induced facial grooming (Eisenberg et al., 1996). Duration of licking/biting the injected paw were analysed by analysis of variance (ANOVA) using SigmaStat software (version 3.0, SPSS, Chicago, IL, USA). Statistical analyses were run separately for phase I (0–6 min) and phase II (12–60 min). The data are presented as mean ± standard error of the mean (S.E.M.). The Holm-Sidak's post hoc test was applied for between-group comparisons. Similarly, data from Hargreaves and rotarod tests were analysed by two-way ANOVA followed by Holm-Sidak's test.

3. Results

Injection of formalin into the rats' hind paws induced typical biphasic behaviours of licking/biting and flinching/shaking observed between 0–6 min (first phase) and 12–60 min (second phase) with nearly no responses recorded between 6 and 12 min.

Loperamide (1–10 mg/kg) given systemically (s.c.) 30 min before the formalin injection produced a dose-dependent antinociceptive effects (Fig. 1A). Statistically significant inhibition of the second phase of formalin-induced behaviours was obtained after administration of loperamide at a dose of 3 and 10 mg/kg ($F(3,29)=13.30$, $P<0.001$), whereas the inhibition of the first phase was observed only at the highest dose of 10 mg/kg ($F(3,29)=8.19$, $P<0.001$). Naloxone methiodide (1–10 mg/kg), injected immediately before formalin, reversed inhibitory effects of loperamide 6 mg/kg (Fig 1B; first phase—$F(5,44)=4.8$, $P<0.001$; second phase—$F(5,44)=15.98$, $P<0.001$).

In the Hargreaves test, administration of loperamide (10 mg/kg) 30 min before the application of the noxious heat stimulation did not affect the paw withdrawal latency (Fig. 2). At the same dose level, morphine significantly prolonged the response latencies ($F(3,28)=16.41$, $P<0.001$).

Morphine was also effective in the formalin test (Fig. 3). Systemic administration of morphine (6 mg/kg) 30 min before the injection of formalin significantly inhibited both first and second phases of formalin-induced paw licking and biting (F

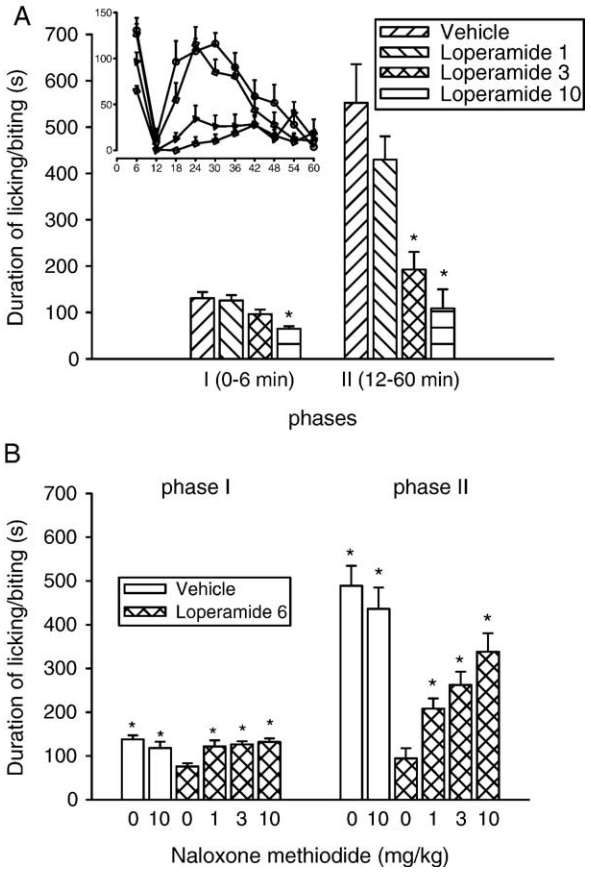

Fig. 1. Effects of loperamide on formalin-induced behaviours. Loperamide (1–10 mg/kg) or its vehicle (20% cyclodextrin) was given s.c. 30 min prior to the injection of formalin (panel A, $n=8$–9). Naloxone methiodide (1, 3, 10 mg/kg, s.c.) or saline was injected right before the formalin test (panel B, $n=8$–9). Data are presented as mean±S.E.M. duration (in seconds) of the paw licking and biting cumulatively for first and second phases of formalin-induced responding. Asterisks denote significant differences from groups treated with vehicle (panel A, $P<0.001$, Holm-Sidak's test) and loperamide alone (panel B, $P<0.001$, Holm-Sidak's test).

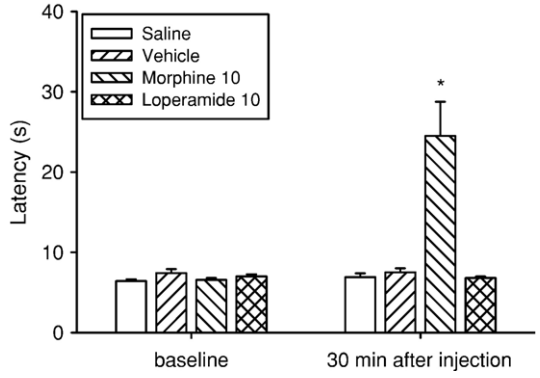

Fig. 2. Effects of morphine and loperamide in Hargreaves test of acute pain. Tests were performed before (baseline) and 30 min after the drug injections. Data are presented as mean±S.E.M. paw withdrawal latencies (s). Asterisks denote significant differences from group treated with morphine ($P<0.001$, Holm-Sidak's test, $n=8$).

(1,34)=145.9, $P<0.001$ and $F(1,34)=61.61$, $P<0.001$, respectively). Naloxone methiodide (0.01–10 mg/kg) reversed the analgesic effects of morphine in a dose–response manner. One-way ANOVA confirmed significant main effect of naloxone methiodide treatment on second phase ($F(8,89)=8.13$, $P<0.001$). Post hoc between-group comparisons indicated that naloxone methiodide (0.3 mg/kg and higher) antagonized the effects of morphine on the second phase, but not on the first phase.

Effects of morphine in the formalin test were enhanced by NMDA receptor channel blocker memantine (Fig. 4). Two-way ANOVA revealed significant main effects of treatment with morphine ($F(1,57)=16.67$, $P<0.001$) and memantine ($F(2,57)=6.91$, $P=0.002$) irrespectively whether memantine was injected before or after the first phase of formalin-induced responding. However, post hoc analysis demonstrated significant suppression of the second phase in animals treated only by

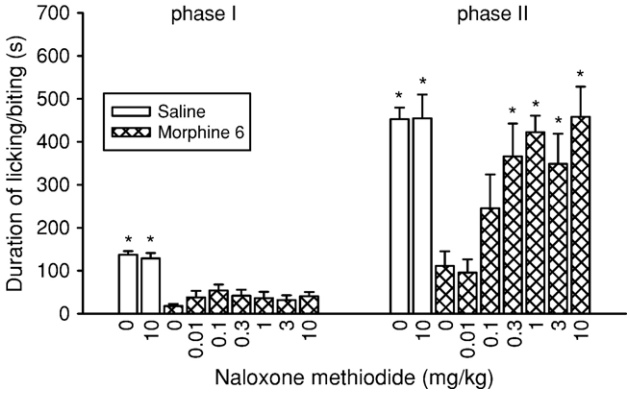

Fig. 3. Effects of naloxone methiodide on morphine-induced suppression of formalin behaviours. Rats were pretreated with morphine (6 mg/kg, s.c.) or saline 30 min before the formalin test. Naloxone methiodide (0.01, 0.1, 0.3, 1, 3, 10, s.c.) or saline was injected right before the formalin test. Data are presented as mean±S.E.M. duration (in seconds) of the paw licking and biting cumulatively for first and second phases of formalin-induced responding. Asterisks denote significant differences from group treated with morphine alone ($P<0.001$, Holm-Sidak's test, $n=8$).

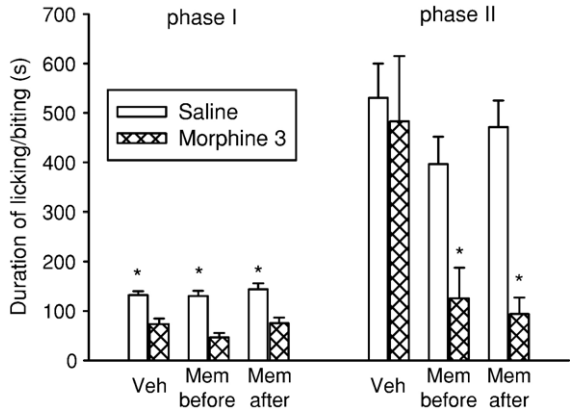

Fig. 4. Effects of combined administration of NMDA receptor channel blocker and morphine on formalin-induced behaviours. Rats were pretreated with morphine (3 mg/kg) or saline 30 min before the formalin test. Memantine (Mem, 5 mg/kg) or vehicle (Veh, saline) was given i.p. 30 min before or 6 min after the injection of formalin. Data are presented as mean±S.E.M. duration (in seconds) of the paw licking and biting cumulatively for first and second phases of formalin-induced responding. Asterisks denote significant differences from group treated with morphine alone ($P<0.001$, Holm-Sidak's test, $n=8$–13).

combination of morphine with memantine in comparison with all other groups.

Data from the rotarod test indicated that memantine–morphine interactions in the formalin test were not due to motor impairment induced by the combination of these agents. In one set of experiments, rats were injected with saline (latency to fall off rotarod—234.4±19.9 s), morphine alone (174.6±23.9 s), memantine alone (257.9±21.8 s) or with their combination (169.1±22.3 s) 60 min before the rotarod test. In another set of experiments aimed to parallel the design of formalin test studies when memantine was injected after the termination of the first phase responding, memantine/saline was given 36 min after the administration of morphine/saline and the rotarod test was run 24 min after the injection of memantine/saline. ANOVA revealed no main effects of memantine for groups treated with memantine alone (246.4±21.4 s) or for combination of memantine with morphine (184.6±17.3 s).

CGP 37849 (3 mg/kg) and memantine (1 and 3 mg/kg) enhanced the effects of loperamide (1 mg/kg) in the formalin test (Fig. 5). Two-way ANOVA revealed significant effects of loperamide (first phase: $F(1,54)=7.44$, $P=0.009$; second phase: $F(1,54)=40,35$, $P<0.001$) and memantine treatment (first phase: $F(2,54)=4.22$, $P=0.02$; second phase: $F(2,54)=3,56$, $P=0.035$, Fig. 5A). However, post hoc comparisons showed a significant difference only for combination of loperamide 1 mg/kg with memantine at dose 3 mg/kg during the first 3 intervals of the second phase (12–30 min) compared with the group that received loperamide only. To exclude the possibility that memantine changes antinociceptive effect of loperamide in the formalin test by inhibition of its metabolism and subsequent increasing the concentration of loperamide in the central compartment, the acute Hargreaves pain test was performed. The results did not reveal differences in paw withdrawal latency values between control (8.1±0.6 s), and, loperamide alone (9.0±0.5), memantine (8.8±0.5) and their combination (9.4±0.7 s), respectively.

Two-way ANOVA also revealed significant main effects of CGP 37849 3 mg/kg on first phase ($F(1,32)=4.92$, $P=0.034$), and second phase ($F(1,32)=10.55$, $P=0.003$), whereas loperamide 1 mg/kg alone did not affect formalin-induced behaviours (Fig. 5B). There was no statistically significant interaction between CGP 37949 and loperamide. Post hoc analysis demonstrated that CGP 37849 3 mg/kg significantly inhibited the manifestation of second phase versus its control group and also showed significant effects for treatment with combination of loperamide and CGP 37849 in comparison to group treated with loperamide alone.

Data from rotarod test revealed motor deficits in groups, which received injections of competitive NMDA-receptor antagonist CGP 37849 3 mg/kg. There was no difference in the mean latencies to fall off the rotarod for groups treated with vehicle/saline or loperamide/saline (225.4±27.2 s and 222.9±24.1, respectively), whereas rats, which were injected with

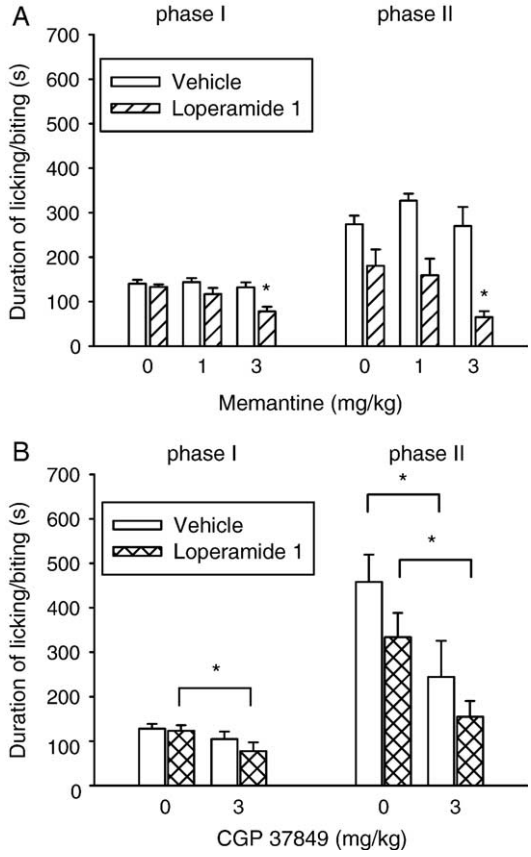

Fig. 5. Effects of combined administration of central active NMDA receptor channel blocker or competitive antagonist and loperamide on formalin-induced behaviours. Memantine (1, 3 mg/kg, panel A, $n=10$) or CGP 37849 (3 mg/kg, panel B, $n=9$) or saline was given i.p. together with loperamide (1 mg/kg, s.c.) or its vehicle (20% cyclodextrin) 30 min before the formalin injection. Data are presented as mean±S.E.M. duration (in seconds) of the paw licking and biting cumulatively for first (0–6 min) and second phases (12–30 min and 12–60 min for panels A and B, respectively) of formalin-induced responding. Asterisks denote significant differences from group treated with loperamide alone (panel A, $P<0.001$; panel B, $P<0.05$, Holm-Sidak's test) and vehicle (panel B, $P<0.05$, Holm-Sidak's test).

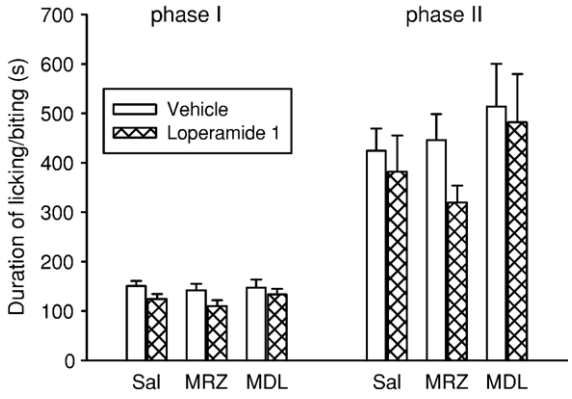

Fig. 6. Effects of peripheral restricted antagonists of the glycine site of the NMDA receptor on antinociceptive action of loperamide. Loperamide (1 mg/kg, s.c.) or its vehicle (20% cyclodextrin) and NMDA receptor antagonists which do not penetrate the blood–brain barrier (MRZ 2/596 and MDL 105,519 at 5 mg/kg and 1 mg/kg, respectively, i.p.) were given 30 min prior to the injection of formalin. Data are presented as mean±S.E.M. duration (in seconds) of the paw licking and biting cumulatively for first and second phases of formalin-induced responding (n=8–12).

CGP 37849 3 mg/kg alone and also in combination with loperamide 1 mg/kg, showed the reduced latencies (129.4 ±30.4 and 137.6±44.1 s, respectively). Two-way ANOVA confirmed significant motor impairing effects of CGP 37849 3 mg/kg ($F(1,28)=7.85$, $P<0.009$).

The glycine$_B$ site NMDA receptor antagonists that do not penetrate the blood–brain barrier, MDL 105,519 (1 mg/kg) and MRZ 2/596 (5 mg/kg), did not modify the effects of loperamide in the formalin test (Fig. 6).

4. Discussion

The main finding of the present studies is that under conditions of peripheral inflammation such as after injection of formalin into the plantar surface, peripheral opioid receptors appear to play an important role in the analgesic effects of opiates. Loperamide, which unlike morphine does not penetrate the blood–brain barrier (Heykants et al., 1974; Schinkel et al., 1996), reduced the formalin-induced nociceptive behaviours and these effects were reversed by systemically administered naloxone methiodide, opioid antagonist having weak penetration to the brain (Russell et al., 1982). These data correspond to previous results where the antinociceptive effects of loperamide were observed in different models of chemical-induced inflammatory pain and constant thermal hyperalgesia after local or systemic routes of administration (DeHaven-Hudkins et al., 1999; Nozaki-Taguchi and Yaksh, 1999; Shannon and Lutz, 2002). In addition, naloxone methiodide antagonized the effects of morphine in the formalin test. It should be stressed that naloxone methiodide reversed only the effects of morphine on second phase, but did not change the effects of morphine on the first phase of formalin-evoked pain behaviour. Furthermore, although loperamide significantly inhibited the second phase of formalin response, the suppression of the first phase of acute pain had been observed only at the highest dose. Loperamide also failed to produce antinociceptive effects in the model of acute thermal pain (Hargreaves test), confirming previous data in the tail-flick test (Niemegeers et al., 1979; Wuster and Herz, 1978). These results are generally consistent with the view that analgesic effects of opiates on acute pain are primarily mediated through receptors located in the central nervous system (McNally, 1999; Yaksh and Rudy, 1978).

The mechanisms of the second phase of formalin response appear to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Coderre et al., 1990; Coderre and Melzack, 1992). Considerable debates still persist concerning peripheral versus central mechanisms underlying the second phase of the nociceptive response in the formalin test in rats. There were several studies that clearly suggested that the second phase may be attributed to peripheral component, they emphasized the essential role of afferent input during the second phase from peripheral site in generating and maintaining second phase of nociceptive score in formalin test (Abbadie et al., 1997; Dickenson and Sullivan, 1987; Pitcher and Henry, 2002). Also, chemical inflammatory mediators liberated locally by formalin injection may contribute to continuous activation of nociceptors throughout the second phase (Sawynok, 2003). During inflammation the new opioid receptors are formed in the dorsal root ganglion cells and transported into the fine afferent fibres (Stein, 1995). Taken together, data from the present study provided further evidence that the peripheral components are sufficient for accounting for the antinociceptive effects of opiates in the formalin test.

The activation of NMDA receptors is important for the generation of inflammation-evoked hyperexcitability of spinal cord neurons (Coderre and Yashpal, 1994; Dickenson, 1997; Herrero et al., 2000). Previous studies revealed that systemically applied antagonists of NMDA receptors attenuated nociceptive response scores during the formalin test (Eisenberg et al., 1993; Vaccarino et al., 1993). It was demonstrated both in laboratory animals and in clinical studies that NMDA receptor antagonists potentiate opiate analgesic effects (Bespalov et al., 1998; Price et al., 2000; Unlugenc et al., 2002). Furthermore, recent studies indicate that NMDA-dependent pain facilitatory mechanisms can be triggered by opiates and it expresses as hyperalgesic state (Celerier et al., 1999, 2000). Consistent with these observations, the present findings demonstrated that NMDA receptor channel blocker memantine given either before or after the first phase was able to enhance morphine's inhibitory effects on the second phase of formalin-induced nociceptive behaviour. Data from the rotarod test showed that such enhancement of morphine antinociceptive effect by memantine was not due motor impairment. It is noteworthy that NMDA receptor antagonists alone produce antinociceptive effects in formalin test only when given before (Coderre and Melzack, 1992; Eisenberg et al., 1993; Haley et al., 1990), but not after first phase (Vaccarino et al., 1993; Yamamoto and Yaksh, 1992). However, enhancement of morphine action in the present study was seen in either case. This suggests that NMDA receptor related mechanisms for antinociceptive action and enhancement of morphine effects are different.

In formalin-induced nociception, NMDA-type glutamate receptors are involved in the cascade of events, which take place in the spinal cord, leading to the central sensitization. Furthermore, NMDA receptors have been identified on thin, unmyelinated nociceptive fibres in the skin (Carlton et al., 1995; Coggeshall and Carlton, 1998) and the number of sensory axons containing ionotropic glutamate receptors increases during inflammation, which may be a contributing factor to peripheral sensitization (Carlton and Coggeshall, 1999; Liu et al., 2002). In the present studies, the antinociceptive effects of loperamide, opiate which does not penetrate into the brain, in formalin-induced pain behaviour were enhanced only by the central acting memantine and CGP 37849, channel blocker and competitive antagonist of the NMDA receptor complex, respectively. The dose of CGP 37849 3 mg/kg was selected from previous dose–response studies in rats (Hunter and Singh, 1994; Singh et al., 1996), whereas in mice significant inhibition of formalin-induced pain behaviour had been observed only at dose 10 mg/kg (Berrino et al., 2003). However, the data obtained with the combination of loperamide and CGP 37849 should be treated with caution, because animals pretreated with CGP 37849 at dose 3 mg/kg may be deficient in motoric performance as suggested by the results of the rotarod test. Antagonists of NMDA receptors MDL 105,519 and MRZ 2/596, at the doses devoid of CNS activity according to the previous studies (OpackaJuffry et al., 1998; Danysz et al., 2005), did not affect the loperamide analgesic action.

These results are in agreement with the hypothesis on the possible interactions between the NMDA system and the peripheral opioid system, where opiates acting peripherally decrease the release of excitatory amino acids at the central level and NMDA receptor antagonists block the central hyperexcitability (Christensen et al., 1998; Martinez et al., 2002). The safety profile of loperamide has been established over many years since it penetrates the blood–brain barrier very poorly due to extrusion by P-glycoprotein from the brain endothelial cells (Schinkel et al., 1996). Thus, it potentially cannot be excluded that memantine, as an inhibitor of cytochrome *P*450 (CYP) 2B6 (Micuda et al., 2004), increases the amount of loperamide in plasma. The high concentration of loperamide in plasma could lead to oversaturation of P-glycoprotein and consequently to augmentation of the central concentration. However it is more plausible that analgesic effect of co-administered loperamide with memantine has pharmacodynamic nature because loperamide is mostly metabolized by CYP3A (Gimenez et al., 2004). Furthermore, present results in Hargreaves test demonstrated that co-administration with memantine did not conduce to analgesic effects of loperamide in model of acute pain. Such lack of antinociceptive effects provides indirect evidence for peripheral origin of loperamide action. Thus, opiates seem to affect the afferent input from peripheral site whereas the antagonists of NMDA receptors modulate elevated discharge of spinal nociceptive dorsal horn neurons and such interaction expressed as an additive suppressive action on manifestation of second phase of formalin-induced nociceptive behaviour.

In conclusion, peripheral opioid receptors clearly play a role in inflammation-induced nociception and this provides basis for therapeutic use of opiates that have limited ability to penetrate the blood–brain barrier and therefore are less likely to produce CNS side effects. Moreover, it can be suggested that these antinociceptive effects may be enhanced only by NMDA receptor antagonists penetrating to the CNS.

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