Full Paper

Involvement of Peripheral Mechanism in the Verapamil-Induced Potentiation of Morphine Analgesia in Mice

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Abstract. Morphine's analgesic actions are thought to be mediated through both the central and peripheral nervous systems. L-type calcium channel blockers have been reported to potentiate the analgesic effects of morphine, but the locus of this interaction is not known. In this experiment, we examined the site of verapamil-induced potentiation of morphine analgesia in mice using the quaternary opioid receptor antagonist naloxone-methiodide (NLX-M). Subcutaneous injections of morphine increased locomotor activity and serum corticosterone level, which are mediated by the central nervous system. These central effects were not antagonized by 0.1 mg/kg of NLX-M, whereas this dose of NLX-M partially antagonized the analgesic effect of morphine. Treatment with verapamil potentiated morphine analgesia in a dose-dependent manner. The verapamil-induced potentiation of morphine analgesia was abolished by pretreatment with NLX-M (0.1 and 1 mg/kg). These findings suggest that peripheral mechanisms partially contribute to morphine analgesia and mediate the potentiation of morphine analgesia by verapamil.

Keywords: morphine, peripheral analgesia, calcium channel blocker, verapamil, naloxone-methiodide

Introduction

Morphine, a mu opioid receptor agonist, is the current standard of care for the treatment of severe pain. Mu opioid agonists mediate their actions by binding and activating receptors in both central and peripheral nervous systems. Peripheral mechanisms of morphine's analgesic effect have been reported, and their importance is becoming increasingly appreciated (1–8).

It is well known that L-type calcium channel blockers (CCBs) potentiate morphine analgesia (9–13). Intracerebroventricular (14, 15) or intrathecal (16, 17) injection of L-type CCBs potentiated morphine analgesia, indicating that the enhancement of morphine analgesia could be mediated by the central nervous

system (CNS) when the CCBs were administered centrally. However, it was demonstrated in rhesus monkeys that systemic administration of the L-type CCB, diltiazem, potentiated morphine analgesia but did not enhance morphine's centrally-mediated respiratory depressant effect (18). This finding raised the possibility that when administered systemically, L-type CCBs may potentiate morphine analgesia through the peripheral nervous system.

The quaternary opioid receptor antagonist naloxone-methiodide (NLX-M) is thought not to cross the blood-brain barrier (BBB) readily when it is injected systemically; it has been used as a research tool to distinguish between central and peripheral action of opioids (6, 19).

In this experiment, we tried 1) to confirm a dose of NLX-M that did not antagonize the central effects of morphine, 2) to clarify a peripheral component of morphine analgesia, and 3) to characterize the involve-

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ment of peripheral mechanisms in L-type CCBs-induced potentiation of morphine analgesia.

Materials and Methods

Animals

Male ICR mice (27–33 g) were obtained from SLC (Osaka). The animals were housed at a temperature of 23–24°C with a 12 h light-dark cycle (light on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum.

The present study was conducted in accordance with the Declaration of Helsinki and with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by The Japanese Pharmacological Society.

Locomotor activity

The locomotor activity of mice was measured by MK-ANIMEX-AUTO (Muromachi Kikai Co., Ltd., Tokyo). The activity monitor was composed of an infrared ray sensor placed over an open-top box (24-cm-wide × 36-cm-long × 29-cm-high). The sensor detected the movement of animals on the basis of released infrared rays associated with their temperature. Mice were habituated to individual locomotion counter chamber for 105 min prior to morphine administration. Following morphine administration, counts of locomotor activity were collected in 15-min intervals for 120 min. NLX-M or naloxone (NLX) was administered 15 min before morphine administration. A time-count curve was constructed and total activity counts over 120 min was used as the indicator of locomotor activity.

Serum corticosterone (SCS) level

Blood samples for SCS assay were obtained by decapitation. Blood was collected into test tubes 60 min after morphine administration, and serum was separated by centrifugation (2000 × *g* for 20 min at 4°C). The serum samples were stored at –20°C until the fluorometric assay of SCS level according to the method of Zenker and Bernstein (20).

Morphine analgesia

The analgesic effect of morphine was measured by the tail-pinch method (21). A flattened clip (approximately 6-mm-wide) was placed at the base of the tail. The pressure produced by the clip on the tail was adjusted to 500 g. A nociceptive response was indicated by the time (latency) required for the mouse to respond to this pressure by vocalizing or biting at the clip. The clip was never applied for longer than 15 s. The percent maximum possible effect (%MPE) was calculated using the following formula: %MPE = 100 × (each

latency – baseline latency) / (15 – baseline latency).

Verapamil and NLX-M (or NLX) were administered 30 and 15 min before morphine administration, respectively. Following morphine administration, analgesia was measured every 15 min for 120 min. A time-action curve was constructed and area under the curve (AUC) was used as the indicator of the analgesic effect of morphine.

Drugs

The drugs used and their suppliers were as follows: morphine hydrochloride (Takeda Chemical Industries, Ltd., Osaka) and naloxone-methiodide (NLX-M), naloxone hydrochloride (NLX), and (±)-verapamil hydrochloride (Sigma, St. Louis, MO, USA). All the drugs were dissolved in saline. Verapamil and the opioid antagonists were administered 15 and 30 min before morphine administration, respectively.

Statistical analysis

Data are presented as means with S.E.M. In the experiments measuring the time course (analgesia and locomotor activity), the statistical evaluations were performed using two-way analysis of variance followed by the Bonferroni test. In the experiment evaluating morphine-induced increase in the SCS level, the statistical significance was assessed with one-way analysis of variance followed by the Dunnett test. In the experiment evaluating the effects of NLX and NLX-M, the statistical significance was assessed with one-way analysis of variance followed by the Student-Newmann-Keuls test. The differences were regarded as significant when *P* values were less than 0.05.

Results

Administration of morphine (2–16 mg/kg, s.c.) increased locomotor activity in a dose-dependent manner. The total activity (± S.E.M.) averaged for 6 mice over 120 min was 2 mg/kg, 2523.8 (±576.6); 4 mg/kg, 1756.3 (±138.4); 8 mg/kg, 6848.0 (±1825.9); and 16 mg/kg, 21176.2 (±5277.4).

To determine the dose of NLX-M that did not antagonize the central effects of morphine, we measured the effect of NLX-M (0.01–1 mg/kg, s.c.) on morphine-induced hyperlocomotion. In this antagonist study, we used 10 mg/kg of morphine, since this dose of morphine produced sufficient hyperlocomotion and was necessary for the antagonist study of SCS (the morphine dose of the two antagonist studies determining the dose of NLX-M should be the same). Subcutaneous administration of 10 mg/kg of morphine produced a significant increase in locomotor activity (Fig. 1). The administra-

Fig. 1. Effects of naloxone-methiodide (NLX-M) and naloxone (NLX) on morphine-induced hyperlocomotion. A: Time-course of locomotor activity. Each point represents the mean activity counts for 15 min with S.E.M. of 6 or 7 mice. B: Total locomotor activity. Each column represents the mean activity for 120 min with S.E.M. of 6 or 7 mice. Mice received NLX-M (NM; 0.01, 0.1, and 1 mg/kg, s.c.), NLX (N; 0.01, 0.1, and 1 mg/kg, s.c.), or saline (SAL, s.c.) 15 min before morphine (MOR; 10 mg/kg, s.c.) or saline (sal) administration. The statistical significance was assessed with one-way analysis of variance followed by the Student-Newmann-Keuls test. F-value is 4.9. * $P < 0.05$, ** $P < 0.01$, vs SAL + MOR 10.

tion of NLX (0.1 and 1 mg/kg, s.c.) prior to morphine antagonized the morphine-induced hyperlocomotion. In contrast, the administration of NLX-M (0.1 and 1 mg/kg, s.c.) had no effect on the morphine-induced hyperlocomotion, indicating that these doses did not have obvious central opioid receptor antagonist effects.

We examined the effect of NLX-M (0.1 and 1 mg/kg, s.c.) on morphine-induced increases in SCS level. Morphine increased SCS level in a dose-dependent manner, and this effect reached a plateau at 20 mg/kg of morphine (Fig. 2A). We used 10 mg/kg of morphine in the antagonist study, because this dose of morphine produced sufficient increase in SCS level and the

Fig. 2. Effects of naloxone-methiodide (NLX-M) and naloxone (NLX) on morphine-induced increase in serum corticosterone (SCS) level. A: Dose-dependent effects of morphine on SCS level. Each column represents the mean SCS level with S.E.M. of 6 mice. B: Effects of NLX-M and NLX on morphine (10 mg/kg, s.c.)-induced increase in SCS level. Each column represents the mean SCS level with S.E.M. of 5 mice. Mice received NLX-M (0.1 and 1 mg/kg, s.c.), NLX (0.1 and 1 mg/kg, s.c.), or saline (SAL, s.c.) 15 min before morphine (MOR, s.c.) injection. Blood samples were collected 60 min after morphine administration. The statistical significance was assessed with one-way analysis of variance followed by the Dunnett test (A) and by the Student-Newmann-Keuls test (B). F-values are 62.8 (A) and 11.3 (B), respectively. * $P < 0.05$, ** $P < 0.01$, vs SAL.

SCS level did not reach the plateau. The administration of NLX (0.1 and 1 mg/kg, s.c.) prior to morphine (10 mg/kg, s.c.) antagonized the morphine-induced increase in SCS levels (Fig. 2B). NLX-M at 0.1 mg/kg did not influence the morphine-induced increases in SCS level, whereas NLX-M at 1 mg/kg partially antagonized this effect of morphine (Fig. 2B), indicating that with this measure, NLX-M at the dose of 0.1 mg/kg has little CNS opioid antagonist activity.

Subcutaneous administration of morphine produced

Fig. 3. Effects of naloxone-methiodide (NLX-M) and naloxone (NLX) on morphine analgesia. A: Time-course of morphine analgesia. Each point represents the mean analgesic effect with S.E.M. of 6–13 mice. B: Morphine analgesia presented by area under the curve (AUC; %MPE $\times$ h). Each column represents the mean with S.E.M. of 6–13 mice. Mice received NLX-M (NM; 0.01, 0.1, and 1 mg/kg, s.c.), NLX (N; 0.01, 0.1, and 1 mg/kg, s.c.), or saline (SAL, s.c.) 15 min before morphine (MOR; 7 mg/kg, s.c.) administration. The statistical significance was assessed with one-way analysis of variance followed by the Student-Newmann-Keuls test. F-value is 17.7. $**P < 0.01$, vs SAL.

dose-dependent analgesia (AUC of 5 mice, %MPE $\times$ h: 1 mg/kg, 10.6 ± 4.1 ; 2 mg/kg, 15.1 ± 2.5 ; 4 mg/kg, 43.0 ± 4.1 ; 8 mg/kg, 77.2 ± 14.5). Saline treatment did not show any analgesic effect (data not shown), as we reported previously (13). We used 7 mg/kg of morphine in the antagonist study because if higher doses of morphine were used, almost all mice reached the cut-off time, and analgesic potency was not reflected precisely in AUC.

Administration of 7 mg/kg of morphine produced a robust analgesic effect (Fig. 3). A dose of 0.01 mg/kg NLX-M or NLX had no effect on this morphine-induced analgesia (Fig. 3B). Pretreatment with 0.1 and 1 mg/kg

Fig. 4. Effects of naloxone-methiodide (NLX-M) on verapamil-induced potentiation of morphine analgesia. Morphine analgesia is shown as the area under the curve (AUC). Each column represents the mean with S.E.M. of 10 mice. Mice were given verapamil (VER; 20 and 30 mg/kg, s.c.) or saline (sal, s.c.) 30 min before morphine (MOR; 4 mg/kg, i.p.) administration. NLX-M (0.1 and 1 mg/kg, s.c.) or saline (SAL, s.c.) were given 15 min before morphine. The statistical significance was assessed with one-way analysis of variance followed by the Student-Newmann-Keuls test. F-values are 10.2 (SAL), 0.6 (NLX-M 0.1), and 0.8 (NLX 0.1). $*P < 0.05$, $**P < 0.01$, vs SAL + Sal + MOR 4. $##P < 0.01$, vs NLX-M 0.1 + Sal + MOR 4. N.S., not significant.

of NLX-M produced a similar, moderate decrease in the analgesic effect of morphine. On the other hand, these doses of NLX markedly antagonized morphine analgesia (Fig. 3B), again indicating that NLX-M is less potent than NLX perhaps because of limited access of NLX-M to the CNS.

The administration of verapamil (20 and 30 mg/kg, s.c.) prior to morphine potentiated the analgesic effect of morphine (4 mg/kg, i.p.) in a dose-dependent manner (Fig. 4). Although NLX-M (0.1 mg/kg, s.c.) and NLX (0.1 mg/kg, s.c.) significantly decreased morphine analgesia, the potency of NLX-M was lower than that of NLX. The potentiating effects of both doses of verapamil on morphine analgesia were abolished by the treatment with both NLX-M and NLX.

Discussion

The present study, using the quaternary opioid antagonist NLX-M, which distinguishes between the central and peripheral actions of opioids (6, 19), demonstrated that the L-type CCB verapamil potentiates morphine analgesia through a peripheral mechanism.

We first determined the dose of NLX-M that did not antagonize the central effects of morphine. For this purpose, we observed morphine-induced increases in locomotor activity and SCS levels in mice. It is widely

recognized that morphine-induced hyperlocomotion is the result of opioid actions on the CNS, particularly the mesolimbic dopaminergic system (22–24). Some reports have demonstrated that morphine-induced increase in SCS levels is also mediated by the central nervous system. For example, increase in plasma corticosterone levels was produced by intraventricular (25) or intrahypothalamic injection (26) of morphine, and the effect of morphine on the hypothalamic-pituitary-adrenal (HPA) axis was absent in hypothalamic lesioned (27) and hypophysectomized (28) rats. In vitro studies also demonstrated that morphine stimulated secretion of corticotropin-releasing factor (CRF) in the isolated hypothalamus but not of adrenocorticotrophic hormone (ACTH) in the pituitary segments (29). Moreover, this effect of morphine was antagonized by naloxone (29). These findings suggest that systemically injected morphine activates the HPA axis through binding to opioid receptors in the hypothalamus, and consequent CRF release.

The present study showed that NLX (0.1 and 1 mg/kg, s.c.), which readily crosses the BBB, antagonized both morphine-induced hyperlocomotion and SCS increases. On the other hand, NLX-M (0.1 and 1 mg/kg, s.c.) had no effect on morphine-induced hyperlocomotion. NLX-M at 0.1 mg/kg also had no effect on morphine-induced increases in SCS level, although moderate antagonism of morphine's effects on SCS were shown by this larger dose of NLX-M. Although the reason why 1 mg/kg of NLX-M antagonized morphine-induced increase in SCS level but not hyperlocomotion is not clear, there may be some differences in the tightness of the BBB in the different brain regions that are related to hyperlocomotion and CRF release. Nevertheless, taken together, these results indicate that subcutaneous administration of 0.1 mg/kg of NLX-M does not antagonize the central effects of morphine.

Unlike the locomotor activity and the SCS levels, morphine analgesia was significantly attenuated by administration of both 0.1 and 1.0 mg/kg of NLX-M. Taken with the previous results, this finding supports the notion that the smaller dose of NLX-M is likely to have antagonized selectively peripherally mediated morphine analgesia, with the remaining amount of analgesia being mediated centrally. This is in agreement with earlier findings that morphine acts on both central and peripheral sites to produce analgesia (5, 8, 30).

The potentiating effect of L-type CCBs on morphine analgesia has been demonstrated in rodents (9–13). Similar to these previous studies, we also confirmed that verapamil potentiated morphine analgesia without any antinociceptive effect alone (31). Interestingly, the potentiating effect of verapamil on morphine analgesia

was abolished by treatment with 0.1 mg/kg of NLX-M, the dose of NLX-M that is restricted to peripheral opioid receptors. This result implies that verapamil potentiates selectively peripherally mediated morphine analgesia.

Although pharmacological mechanisms for the potentiating effect of verapamil on peripherally mediated morphine analgesia are unclear, it is possible that verapamil enhances peripheral morphine-induced attenuation of primary afferent activity through the blockade of peripheral Ca^{2+} channels. Moreover, our previous studies (13, 31) reported that L-type CCBs (diltiazem, nimodipine, and verapamil) markedly increased morphine levels in the serum but not in the brain while potentiating morphine-induced analgesia. Therefore, in addition to pharmacological mechanisms, there may also be pharmacokinetic mechanisms (i.e., verapamil increases morphine concentration at peripheral opioid receptors) involved in the potentiating effect of verapamil on peripherally-mediated morphine analgesia.

In conclusion, the present results suggest that peripheral opioid receptors may play an important role in the systemically administered morphine analgesia. Moreover, our results strongly suggest that the L-type CCB verapamil potentiates morphine analgesia through peripheral mechanisms, at least measured by the tail-pinch test. These results may extend the possibility of the clinical use of morphine in combination with L-type CCBs for pain relief.

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