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Antinociceptive mechanism of the spirocyclopiperazinium compound LXM-10 in mice and rats

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ABSTRACT

Drugs typically used to treat pain are limited by their undesirable side effects, which has prompted a search for mechanistically different analgesic agents. We report the antinociception effect of the spirocyclopiperazinium compound LXM-10 via activation of peripheral α7 nicotinic and muscarinic acetylcholine receptors in mice. This effect was attenuated by hexamethonium, atropine methylnitrate, methyllycaconitine citrate, tropicamide, bicuculline, and phaclofen. Competition receptor-binding assays in vitro showed that LXM-10 binds with high affinity α7 nAchR and with low affinity M4 receptors. Our findings show that the antinociception signaling pathway of LXM-10 underlies activation of peripheral α7 nicotinic and possibly of M4 muscarinic receptors, which activate $GABA_A$ and $GABA_B$ receptors, resulting in antinociceptive effects without obvious side effects.

1. Introduction

Most pharmacologic agents used in pain management can be divided into 2 broad categories: those that bind to opioid receptors and those that inhibit cyclooxygenase. Both categories have undesirable side effects associated with their use, which has prompted a search for mechanistically different analgesic agents (Kingery, 1997; Przewlocki and Przewlocka, 2001). Nicotinic acetylcholinergic mechanisms seem to play a role in analgesia (Millan, 2002). It has been reported that nicotine and other agonists of nicotinic acetylcholine receptors (nAChRs), such as epibatidine derivatives, have an antinociceptive effect by acting on nAChRs in the central nervous system (Hama et al., 2001). Nicotine and epibatidine are powerful antinociceptive agents in animals, but they have unique shortcomings, such as ataxia, seizures, and hypothermia (Badio and Daly, 1994), which might be related to non-selective binding of nAChRs. For an analgesic to avoid these side effects, it must bind selectively to the subpopulation of nAChRs involved with antinociception. In addition, the muscarinic acetylcholine receptors (mAChRs) have emerged as a viable target in the treatment of pain. Recently, peripheral mAChRs have been shown to modulate nociceptive processing as well as neurogenic inflammatory responses (Dussor et al., 2004); however, because the precise subtype of mAChR that mediates analgesia has not yet been identified and located, the mechanisms of this modulation are not well understood.

LXM-10 (2,4-dimethyl-9-β-phenylethyl-3-oxo-6, 9-diazaspiro [5.5] undecane chloride, Fig. 1) is a monospirocyclopiperazinium salt, which has difficulty to cross the blood-brain barrier and therefore it should be devoid of central effects. Previous studies have shown that LXM-10 produces antinociception in models of nociception in mice without obvious side effects. It is probable that this effect was achieved by activating peripheral neuronal nAChRs and mAChRs (Yue et al., 2007), but it is not clear why LXM-10 does not produce the typical side effects of muscarinic or nicotinic agonists. The purpose of the study was to explore the underlying mechanisms of antinociception by LXM-10 and to determine the subtype of nAChR and mAChR that it may involve.

2. Materials and methods

2.1. Animals

We obtained both sexes of C57/BL6 mice, weighing 20–22 g, and male Wistar rats, weighing 170–230 g, from the Department of Laboratory Animal Science of Peking University. Mice were used randomly in each set of experiments, and the number of male and female mice was equal in each group. All experiments were carried out according to the International Association for the Study of Pain ethical guidelines (Zimmermann, 1983) and approved by the Institutional Animal Care and Use Committee of Peking University. All behavioral measurements were performed in awake, unrestrained, age-matched adult mice of both sexes and were conducted in a blinded manner.

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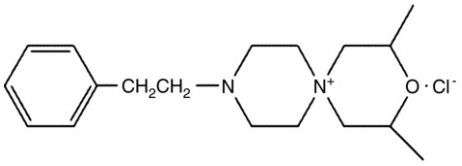

Fig. 1. The chemical structure of the spirocyclopiperazinium compound LXM-10.

2.2. Drugs and administration

We used the following drugs: morphine hydrochloride (Neuroscience Research Institute of Peking University, Beijing, PR. China), hexamethonium chloride, atropine methylnitrate, methyllycaconitine citrate, tropicamide, bicuculline, phaclofen, aspirin, nicotine, bovine serum albumin (BSA), polyethyleneimine and atropine (Sigma Chemical Co., St. Louis, MO, USA), [^{3}H] Methyllycaconitine ([^{3}H]MLA, 100 Ci/mmol, American Radiolabeled Chemicals, Inc.), [N-methyl-^{3}H] scopolamine methyl chloride ([^{3}H] NMS, 3.03 TBq/mmol), Liquid Scintillation Cocktails (PerkinElmer Life Sciences, Boston, MA), Modified Lowry Protein Assay Kit (Beijing Applygen Technologies Inc. PR. China), and Tris–HCl (Beijing Topbio Science and Technology Co., Ltd. PR. China). LXM-10 was synthesized by Runtao Li and Qi Sun. The doses of drug and receptor antagonists were selected on the basis of previous reports and our preliminary studies (Beirith et al. 1998; Decker et al. 1998; Boccia et al. 2001). All drugs were dissolved in distilled water immediately before use. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml/kg by subcutaneous injection (s.c.) for LXM-10 and morphine hydrochloride; intraperitoneal injection (i.p.) for hexamethonium chloride, atropine methylnitrate, methyllycaconitine citrate, tropicamide, bicuculline, and phaclofen; or intragastric injection (i.g.) for aspirin.

2.3. Hot-plate test

The hot-plate test was performed as described by O'Callaghan and Holtzman (1975). The time from when the animal was placed on the hot plate until it first licked its hind paws, shook, or jumped off the surface was recorded as response latency. We first placed mice on the metal surface (which was maintained at 54 °C) to obtain baseline response latencies before drug administration. The hot-plate response latencies were measured 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 h after administering LXM-10 (20, 10, or 5 μmol/kg, s.c.), morphine (18 μmol/kg, s.c.), or vehicle. In order to exclude hyposensitive or hypersensitive mice, we eliminated all animals with baseline latency shorter than 5 s or longer than 20 s. We used the cut-off time of 20 s to prevent tissue damage. Antinociception was quantified according to the maximal possible effect (MPE), which was calculated as MPE (%)>[(response latency − baseline latency)/(cut-off time − baseline latency)]×100.

2.4. Formalin test

The formalin test was conducted according to methods published previously (Bhave et al., 2001). We administered LXM-10 (20, 10, or 5 μmol/kg, s.c.), morphine (18 μmol/kg, s.c.), aspirin (2778 μmol/kg, i.g.), or vehicle to each mouse 1 h before injecting 20 μl of 1% formalin subcutaneously into the right forepaw. The time mice spent licking or biting their injected paws was recorded after formalin injection. The total time spent lifting or licking in the first phase (phase I, 0–5 min) and second phase (phase II, 10–60 min) was recorded. The percentage of inhibition of phase I or phase II was determined for each experimental group by using the following formula: inhibition (%)>(time spent lifting or licking in control group − time spent lifting or licking in experiment group)/time spent lifting or licking in control group×100.

2.5. Investigation of the antinociceptive mechanisms of LXM-10

To address some of the antinociceptive mechanisms of LXM-10, we pretreated some mice with different receptor antagonists in the hot-plate test and formalin test.

2.5.1. Effect of receptor antagonists on LXM-10-induced antinociception in the hot-plate test

In the hot-plate test, we pretreated mice with hexamethonium (a peripheral neuronal nAChR antagonist, 15 μmol/kg, i.p.), atropine methylnitrate (a peripheral mAChR antagonist, 15 μmol/kg, i.p.), methyllycaconitine citrate (an α7 nAChR antagonist, 3, 0.3, or 0.03 μmol/kg, i.p.), tropicamide (an M4 muscarinic receptor antagonist, 3, 0.3, or 0.03 μmol/kg, i.p.), bicuculline (a $GABA_A$ receptor antagonist, 8, 0.8, or 0.08 μmol/kg, i.p.), or phaclofen (a $GABA_B$ receptor antagonist, 40, 4, or 0.4 μmol/kg, i.p.). After 15 min, the animals received the injection of LXM-10 (20 μmol/kg, s.c.) or vehicle. Other animals were pretreated with vehicle as control, and after 15 min the animals received the injection of LXM-10 (20 μmol/kg, s.c.) or vehicle. The hot-plate response latencies were measured at 1, 2 and 3 h after administering LXM-10 or vehicle.

2.5.2. Effect of receptor antagonists on LXM-10-induced antinociception in the formalin test

In the formalin test, we pretreated mice with methyllycaconitine citrate (3, 0.3, or 0.03 μmol/kg, i.p.), tropicamide (3, 0.3, or 0.03 μmol/kg, i.p.), bicuculline (8, 0.8, or 0.08 μmol/kg, i.p.), or phaclofen (40, 4, or 0.4 μmol/kg, i.p.). After 15 min, the animals received the injection of LXM-10 (20 μmol/kg, s.c.) or vehicle. Other animals were pretreated with vehicle as control, and after 15 min the animals received the injection of LXM-10 (20 μmol/kg, s.c.) or vehicle. One hour later, all animals were injected 20 μl of 1% formalin subcutaneously into the right forepaw.

2.6. Receptor-binding assays

2.6.1. Brain membrane preparations

We killed 12 male Wistar rats by cervical dislocation. Hippocampus and corpus striatum fractions of brain were homogenized in 10 volumes of ice-cold 0.32 M sucrose and centrifuged at 1000×g for 10 min. The supernatant was then centrifuged in 50 mM Tris–HCl (pH 7.4) at 20,000×g for 30 min. This procedure was repeated, and the resulting pellet was suspended in the buffer and stored at −20 °C for the binding assay. Protein concentrations were measured by the modified Lowry protein assay according to the manufacturer's instructions.

2.6.2. [^{3}H] MLA binding assay

Competition binding assays with [^{3}H] MLA were done according to previous reports (Davies et al., 1999). Briefly, hippocampus membranes (containing 0.4 mg of protein) were incubated in borosilicate glass tubes with 2 nM [^{3}H] MLA (100 Ci/mmol) in a final volume of 0.5 ml for 2 h at 4 °C, in the absence or presence of increasing concentrations of LXM-10 from 1.37×10^{-9} to 1.00×10^{-6} M. Non-specific binding was determined from tubes containing 1 mM nicotine. Incubation buffer consisted of phosphate buffer (20 mM Na_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4) supplemented with 0.1% (w/v) BSA. Incubation was completed by rapid filtration under vacuum through Whatman GF/B glass fibre filters (Whatman Intl. Ltd., Maidstone, UK) pre-soaked in 0.3% (v/v) polyethyleneimine overnight. Tubes and filters were washed rapidly three times with 3 ml ice-cold PBS. Filters were then dried and counted on liquid scintillation spectrometry (Tri-Carb 2100TR, PerkinElmer, USA).

2.6.3. [^{3}H] NMS binding assay

The binding assay for mAChR was performed using [^{3}H] NMS as previously described (Ehlert and Tran, 1990). Corpus striatum

membranes (containing 0.2 mg of protein) were incubated in tubes with 0.5 nM [^{3}H] NMS (60 Ci/mmol) in a final volume of 1 ml for 1 h at 25 °C. Competitive binding was determined in the presence of increasing concentrations of LXM-10 from 5.00×10^{-6} to 1.60×10^{-4} M. Non-specific binding was defined in the presence of 10 μM atropine. Incubation buffer consisted of Tris–HCl buffer (50 mM, pH 7.4). The remainder of the procedure was done as in the MLA binding assay.

2.7. Statistical analysis

Each group contained 10 mice. All data were analyzed by using repeated-measures analysis of variance. Differences were considered significant at $P<0.05$. The IC_{50} value for LXM-10 from competition binding assay was determined by nonlinear regression analysis with SPSS16.0 software.

3. Results

3.1. Antinociceptive activity of LXM-10

As shown in Fig. 2A, mice administered LXM-10 (20, 10, or 5 μmol/kg, s.c.) had delayed latency times compared with the vehicle group in the hot-plate test ($P<0.01$), and the effect was both dose- and time-dependent. The antinociceptive effect of LXM-10 began at 1.0 h, peaked at 2.0 h, and persisted 3.0 h after s.c. administration.

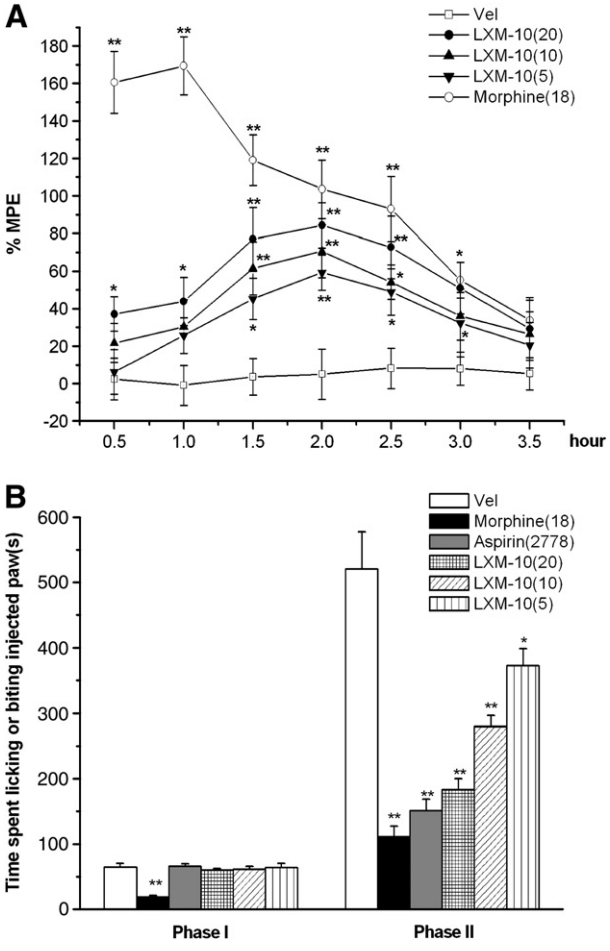

Fig. 2. Antinociceptive effect of LXM-10. The mice were administered LXM-10 (20, 10, or 5 μmol/kg, s.c.), aspirin (2778 μmol/kg, i.g.), morphine (18 μmol/kg, s.c.), or vehicle (Vel). (A) Antinociceptive effect of LXM-10 in the hot-plate test. LXM-10 increased the % MPEs in a dose-dependent and time-dependent manner. (B) Antinociceptive effect of LXM-10 in the formalin test. LXM-10 reduced the time mice spent licking or biting their injected paws in a dose-dependent and time-dependent manner. All data are mean ± S.E.M. of 10 mice per group. (*$P<0.05$, **$P<0.01$ compared with vehicle group).

LXM-10 showed a significant increase in MPE compared with vehicle in the hot-plate test. Peak MPEs were 84%, 71%, and 59%, respectively, at the progressively lower doses of LXM-10. Consistent with its effects on the hot-plate test, LXM-10 (20, 10, or 5 μmol/kg, s.c.) markedly reduced the time spent licking or biting the injected paw in the formalin test (Fig. 2B), compared with the vehicle group in phase II ($P<0.01$; Fig. 2B), with the inhibitions of 65%, 46%, and 28% respectively. However, LXM-10 did not reduce nociception during phase I (Fig. 2B).

3.2. Potential antinociceptive mechanisms of LXM-10

3.2.1. Involvement of peripheral nicotinic acetylcholine and muscarinic acetylcholine system

As shown in Fig. 3, when mice were predosed with hexamethonium (15 μmol/kg, s.c.), the MPEs of LXM-10 (20 μmol/kg, s.c.) were inhibited to −6%, −1% and −4% at 1.0 h, 2.0 h and 3.0 h after LXM-10 administration. We found that the effect of LXM-10 (20 μmol/kg, s.c.) was also blocked by atropine methylnitrate (15 μmol/kg, s.c.), to 6%, 4%, 0.2% at 1.0 h, 2.0 h and 3.0 h after LXM-10 administration. These findings show that the antinociceptive effect of LXM-10 may be related to peripheral nAChRs or mAChRs.

3.2.2. Involvement of α7 nicotinic acetylcholine system

As shown in Fig. 4, after pretreatment with methyllycaconitine citrate (3 or 0.3 μmol/kg, i.p), the antinociceptive effect of LXM-10 was attenuated in both the hot-plate test and formalin test. MPEs were reduced to 12% and 33% at the 2nd hour in the hot-plate test (Fig. 4A), and the inhibitions were attenuated to 12% and 38% in the formalin test (Fig. 4B) at the 3 and 0.3 μmol/kg doses of methyllycaconitine citrate, respectively. The lowest dose of methyllycaconitine citrate (0.03 μmol/kg, i.p.) had no effect in either model. These findings indicate that LXM-10 activates α7 nAChR, resulting in antinociception.

3.2.3. Involvement of M4 muscarinic acetylcholine system

Tropicamide, a slightly selective antagonist of M4 receptors, attenuated the effect of LXM-10 in the hot-plate test and reduced the MPE to 14% and 43% at 3 and 0.3 μmol/kg, respectively at the 2nd hour (Fig. 5A). Also in the formalin test, tropicamide attenuated the effect of LXM-10, which reduced the response to the algesic agent only by 12% and 37% (Fig. 5B). Tropicamide had no effect on LXM-10 activity at the lowest dose (0.03 μmol/kg, i.p.) in either model.

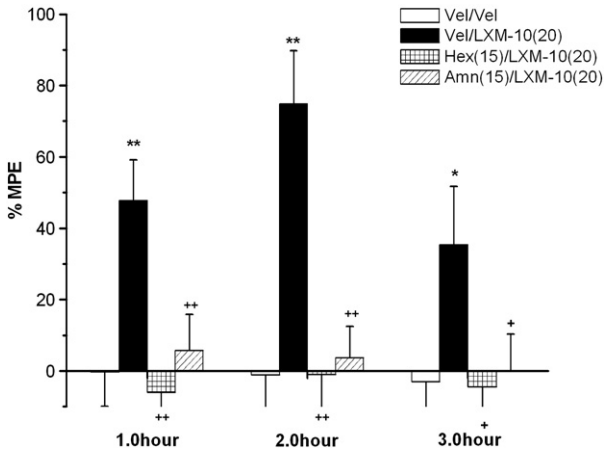

Fig. 3. Reversal of LXM-10-induced antinociception by hexamethonium (Hex) and atropine methylnitrate (Amn) in the hot-plate test. The mice were pretreated with Hex (15 μmol/kg, i.p.), Amn (15 μmol/kg, i.p.), or vehicle before administration of LXM-10 (20 μmol/kg, s.c.) or vehicle. All data are mean ± S.E.M. of 10 mice per group (*$P<0.05$, **$P<0.01$ vs Vel/Vel group; $^{+}P<0.05$, $^{++}P<0.01$ vs Vel/LXM-10 group at the same time).

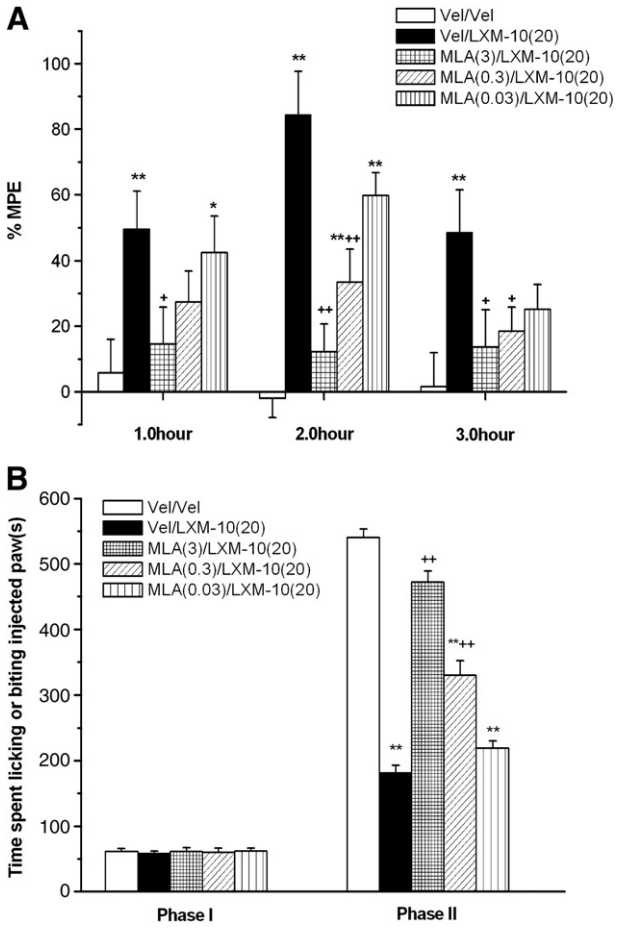

Fig. 4. Effect of methyllycaconitine citrate (MLA) on LXM-10-induced antinociception. The mice were pretreated with methyllycaconitine citrate (3, 0.3 or 0.03 μmol/kg, i.p.) or vehicle before administration of LXM-10 (20 μmol/kg, s.c.) or vehicle. (A) Effect of methyllycaconitine citrate on LXM-10-induced antinociception in the hot-plate test. Methyllycaconitine citrate blocked the LXM-10-induced MPE increase at doses of 3 and 0.3 μmol/kg, i.p. (B) Effect of methyllycaconitine citrate on LXM-10-induced antinociception in the formalin test. Methyllycaconitine citrate blocked the LXM-10-induced decrease in the time spent licking or biting at doses of 3 and 0.3 μmol/kg, i.p. All data are mean ± S.E.M. of 10 mice per group (*$P<0.05$, **$P<0.01$ vs Vel/Vel group; $^{+}P<0.05$, $^{++}P<0.01$ vs Vel/LXM-10 group at the same time).

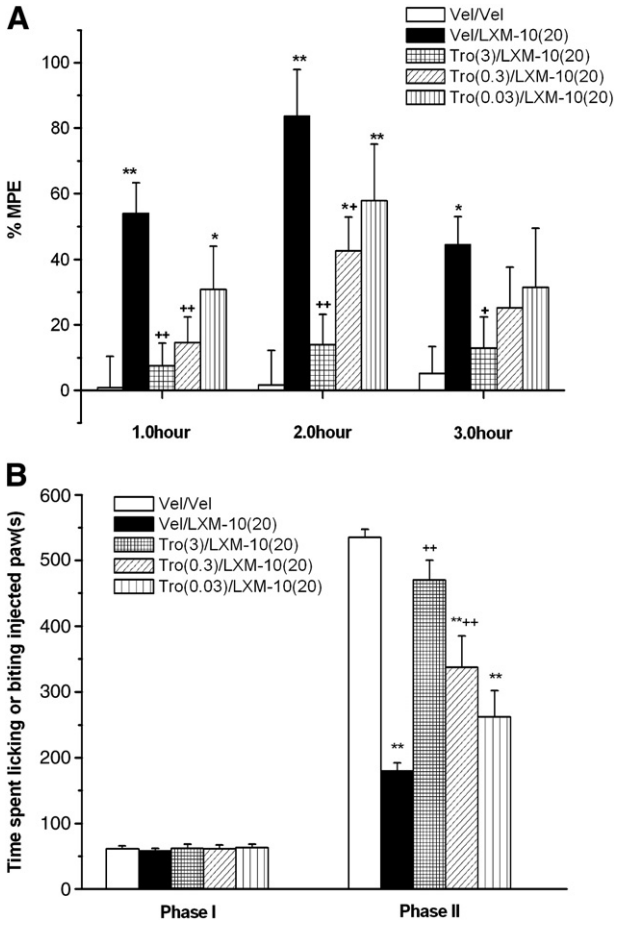

Fig. 5. Effect of tropicamide (Tro) on LXM-10-induced antinociception. The mice were pretreated with Tro (3, 0.3, or 0.03 μmol/kg, i.p.) or vehicle before administration of LXM-10 (20 μmol/kg, s.c.) or vehicle. (A) Tro blocked the antinociception effects of LXM-10 in the hot-plate test. The LXM-10-induced increase in MPE was blocked by Tro at doses of 3 and 0.3 μmol/kg, i.p. (B) Tro blocked the antinociception effects of LXM-10 in the formalin test. The LXM-10-induced decrease in time spent licking or biting was blocked by Tro at doses of 3 and 0.3 μmol/kg, i.p. All data are mean ± S.E.M. of 10 mice per group (*$P<0.05$, **$P<0.01$ vs Vel/Vel group; $^{+}P<0.05$, $^{++}P<0.01$ vs Vel/LXM-10 group at the same time).

3.2.4. Blocking GABA receptors inhibits thermal and inflammatory pain

Bicuculline, a $GABA_A$ receptor antagonist, markedly attenuated the antinociceptive effect of LXM-10; at doses of 8 and 0.8 μmol/kg, i.p., MPEs were reduced to 22% and 41% at the 2nd hour in the hot-plate test (Fig. 6A), and the inhibition was reduced to 12% and 34% during phase II of the formalin test (Fig. 6B). However, no effect was observed at the dose of 0.08 μmol/kg, i.p. in either test. At the same time, this effect was blocked by phaclofen, a $GABA_B$ receptor antagonist, which reduced MPEs to 10% and 46% at the 2nd hour in the hot-plate test (Fig. 7A) and inhibitions to15% and 31% in the formalin test (Fig. 7B) at doses of 40 and 4 μmol/kg, respectively. The effect of LXM-10 was not blocked at the lowest dose of phaclofen (0.4 μmol/kg, i.p.) in either model. These results indicate that $GABA_A$ and $GABA_B$ receptors are activated in the antinociception signaling pathway of LXM-10.

3.2.5. Receptor-binding assays

The competition binding assay in vitro showed that LXM-10 displaces [^{3}H] MLA binding in the hippocampus membranes of rat brains in a concentration-dependent fashion; the IC_{50} was 1.57×10^{-7} M (Fig. 8A). In the same way, LXM-10 also displaced [^{3}H] NMS binding from rat corpus striatum fractions, with an IC_{50} of 5.02×10^{-5} M (Fig. 8B).

4. Discussion

Our results show that LXM-10 produces significant antinociceptive effects that are blocked fully by hexamethonium or atropine methylnitrate, showing that the antinociceptive effect of LXM-10 is likely related to peripheral nAChR or mAChR. LXM-10 did not affect behavior or body temperature, nor did it produce significant changes in animals' gross behavior or polysialia at the minimal lethal dose (1367.10 μmol/kg, s.c.) in acute toxicity tests (Yue et al., 2007). The median lethal dose (LD_{50}) of LMX-10 was 1573.0 μmol/kg, s.c. (95% confidence interval 1509.0–1641.8 μmol/kg), which suggests that LXM-10 does not produce the typical side effects of muscarinic or nicotinic agonists. LXM-10 produces antinociception without side effects, possibly by binding selectively to subtypes of the nAChR and mAChR.

The antinociception of nAChR is mainly attributed to α4β2 subtype (Jones and Dunlop, 2007). Receptor-binding assays showed that LXM-10 does not affect this subtype (Sun et al., 2007). Activation of peripheral or central α7 nAChR appears to be involved in the antinociception (Wang et al., 2005; Hamurtekin and Gurun, 2006; Haberberger et al., 2007). Reports show that cytidinediphosphocholine mediates the antinociceptive responses by activating central α7 nAChR

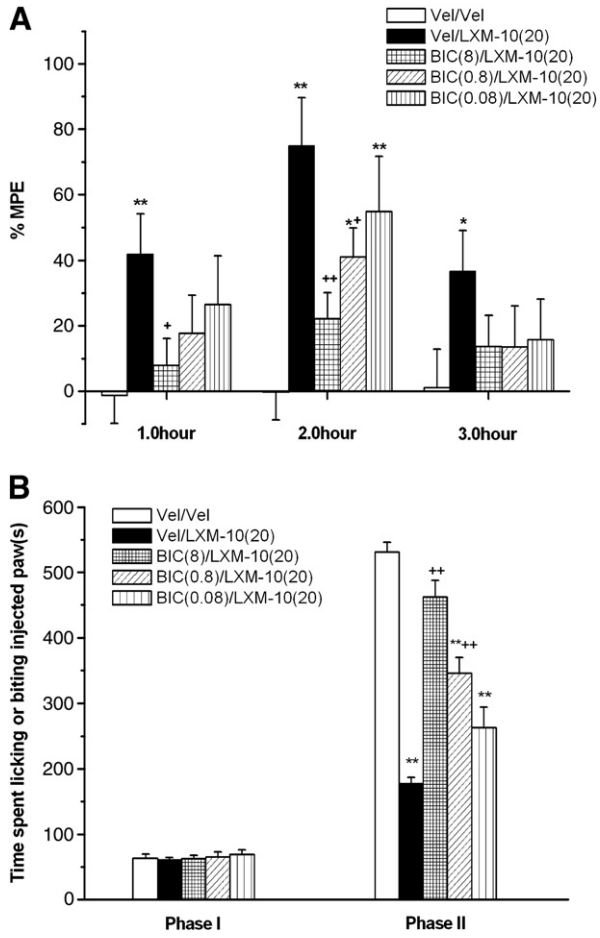

Fig. 6. Effect of bicuculline (BIC) on LXM-10-induced antinociception. The mice were pretreated with BIC (8, 0.8, or 0.08 μmol/kg, i.p.) or vehicle before administration of LXM-10 (20 μmol/kg, s.c.) or vehicle. (A) BIC reversed the antinociception caused by LXM-10 in the hot-plate test. The increase in MPE caused by LXM-10 was reduced by BIC at doses of 8 and 0.8 μmol/kg, i.p. (B) BIC reversed the antinociception caused by LXM-10 in the formalin test. The decrease in time spent licking or biting caused by LXM-10 was blocked by BIC at doses of 8 and 0.8 μmol/kg, i.p. All data are mean ± S.E.M. of 10 mice per group (*P<0.05, **P<0.01 vs Vel/Vel group; ^{+}P<0.05, ^{++}P<0.01 vs Vel/LXM-10 group at the same time).

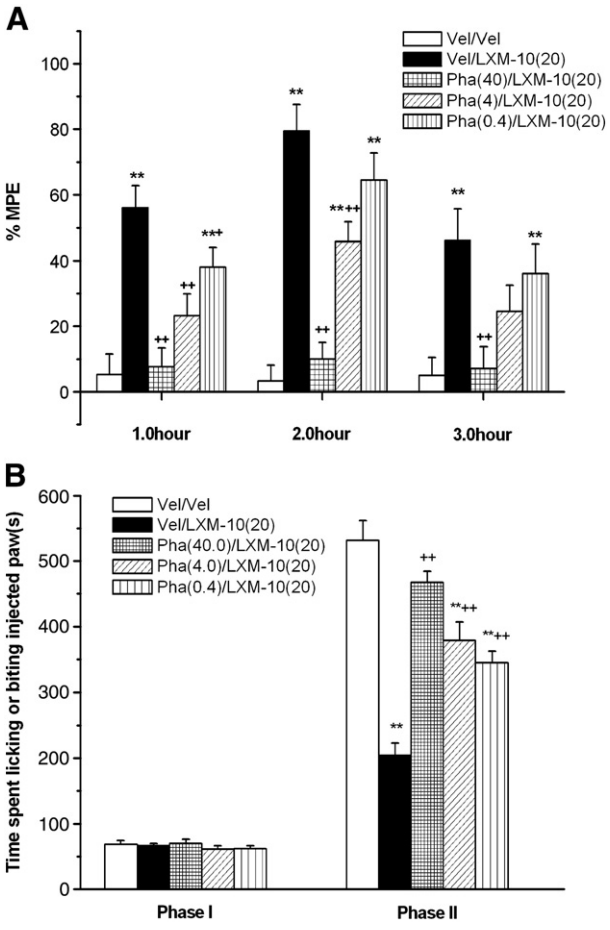

Fig. 7. Effect of phaclofen (Pha) on LXM-10-induced antinociception. The mice were pretreated with Pha (40, 4, or 0.4 μmol/kg, i.p.) or vehicle before administration of LXM-10 (20 μmol/kg, s.c.) or vehicle. (A) Effect of Pha on LXM-10-induced antinociception in the hot-plate test. The increase in MPE caused by LXM-10 was reduced by Pha at doses of 40 and 4 μmol/kg, i.p. (B) Effect of Pha on LXM-10-induced antinociception in the formalin test. The decrease in time spent licking or biting caused by LXM-10 was reduced by Pha at doses of 40 and 4 μmol/kg, i.p. All data are mean ± S.E.M. of 10 mice per group (*P<0.05, **P<0.01 vs Vel/Vel group; ^{+}P<0.05, ^{++}P<0.01 vs Vel/LXM-10 group at the same time).

(Hamurtekin and Gurun, 2006), whereas the α7 nAChR agonist GTS-21 did not produce antinociception (Damaj et al., 2000), which suggests α7 nAChR may not be the only subtype involved in pain modulation. In our experiments, MLA significantly reduced the antinociceptive effect of LXM-10 in a dose-dependent manner in both models, indicating that α7 nAChR activation was likely to be involved in antinociception of LXM-10.

The analgesic effects of muscarinic receptor agonists depend not only on central systems but also on peripheral systems, in which M2 and M4 subtypes play an important role (Mulugeta et al., 2003; Wess et al., 2003; Dussor et al., 2004). It is well-known that peripheral mAChRs mediate other physiologic processes, for example, the M2 receptor mediates atrial negative chronotropism and the M3 receptor stimulates smooth muscle contraction and glandular secretion (Caulfield, 1993). The M4 receptor does not seem to regulate critical physiologic functions (Caulfield, 1993; Duttaroy et al., 2002). LXM-10 did not affect heart rate in mice, nor was glandular secretion affected, so we propose that the antinociception of LXM-10 may be related to the M4 receptor. We found that tropicamide reduced the antinociceptive effect of LXM-10 in the hot-plate and formalin tests.

Further, we used [^{3}H] MLA and [^{3}H] NMS to investigate the specific binding of LXM-10 at α7 nAChR or M4 receptors in the binding assays. In [^{3}H] NMS binding assay, we chose corpus striatum in which mostly the M1 and M4 receptors are enriched (Nathanson, 2008). [^{3}H] NMS is mAChR non-selective radioligand, which bound both M1 receptors and M4 receptors. Binding assays showed LXM-10 have high specificity to bind at α7 nAChR or M4 receptors. Our studies indicated activation of peripheral α7 nAChR and M4 mAChR, may be related to antinociception of LMX-10.

However, we do not know what signaling pathway is involved. Reports indicate $GABA_A$ receptors are involved in pain states, and activation of nAChR or mAChR may increase the release of GABA (Maggi et al., 2001). The antinociceptive effects produced by mAChR agonists are likely due to their combined effects of increasing inhibitory GABA release and decreasing excitatory glutamate release (Li et al., 2002; Zhang et al., 2005; Zhang et al., 2007). In addition, activation of α7 nAChR may directly increase the release of GABA through a presynaptic site (Maggi et al., 2001). We found that the antinociceptive effect of LXM-10 was significantly reduced by bicuculline, which indicates that the $GABA_A$ receptor may be involved in the antinociception signaling pathway of LXM-10.

The $GABA_B$ receptor is a G protein-coupled receptor, which influences presynaptic neurotransmitter release and causes postsynaptic inhibition of excitatory neurotransmission. $GABA_B$ agonists were shown to be antinociceptive (Sawynok, 1987; Patel et al., 2001). Activation of α7 nAChR may regulate GABA release, and stimulation of

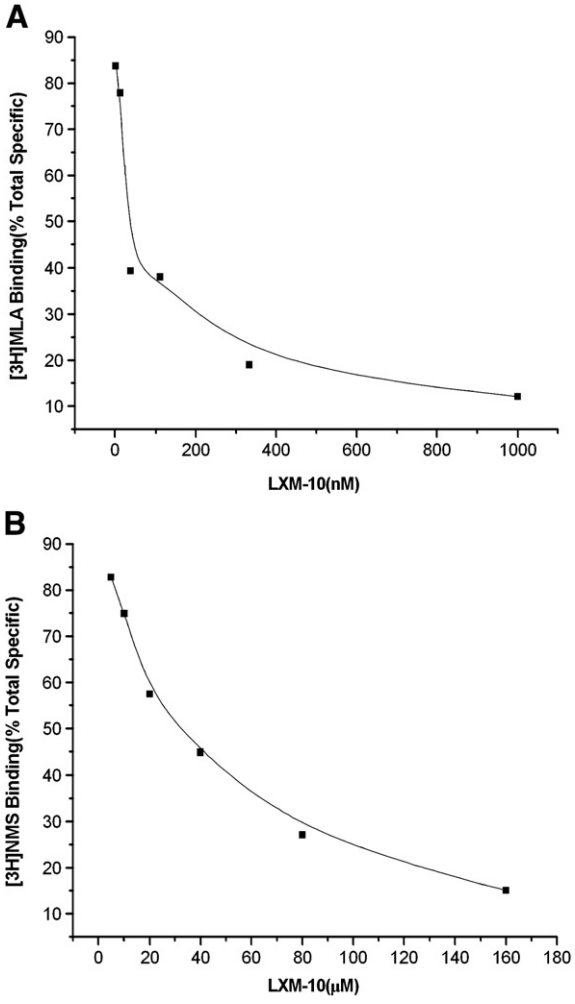

Fig. 8. (A) Inhibition curves of the binding of [^{3}H]MLA to rats hippocampus membranes by LXM-10 in receptor-binding assays. The concentration of LXM-10 was in the range 1.4–1000 nM. (B) Inhibition curves of the binding of [^{3}H] NMS to rats corpus striatum membranes by LXM-10 in receptor-binding assays. The concentration of LXM-10 was in the range 5–160 μM.

mAChR leads to increased GABA release (Gray et al., 1996; Vogt and Regehr, 2001; Hamurtekin and Gurun, 2006). Our results showed that the $GABA_B$ receptor may be also involved in the antinociception signaling pathway of LXM-10.

In summary, we found that LXM-10 produces antinociceptive effects without side effects, by binding and activating peripheral α7 nAChR and, possibly, M4 mAChR, which trigger the activation of GABA receptors, resulting in antinociceptive effects. These findings may pave the way toward the development of novel analgesic drugs with reduced side effects.

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