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Antinociception of spirocyclopiperazinium salt compound LXM-10-M targeting α7 nicotinic receptor and M4 muscarinic receptor and inhibiting CaMKIIα/CREB/CGRP signaling pathway in mice

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Chemical compounds studied in this article:
Methyllycaconitine (PubChem CID: 494471)
Tropicamide (PubChem CID: 5593)
Methoctramine (PubChem CID: 107759)
Aspirin (PubChem CID: 2244)
Morphine (PubChem CID: 5288826)
Formalin (PubChem CID: 712)
Acetic acid (PubChem CID:176)

ABSTRACT

The present study was designed to investigate the antinociception of spirocyclopiperazinium salt compound LXM-10-M (2,4-dimethyl-9-β-m-hydroxyphenylethyl-3-oxo-6, 9-diazaspiro [5.5] undecane chloride) in thermal and chemical pain models, and further to explore the molecular target and potential signal pathway. We assessed the antinociception of LXM-10-M in hot-plate test, formalin test and acetic acid writhing test in mice. The possible changes of calcium/calmodulin-dependent protein kinase IIα (CaMKIIα)/cAMP response element-binding protein (CREB)/calcitonin gene related peptide (CGRP) signaling pathway were detected by Western Blot in mice. Administration of LXM-10-M produced significant antinociception in hot-plate test, formalin test and acetic acid writhing test in mice, with no obvious toxicity. The antinociceptive effects were blocked by pretreatment with methyllycaconitine citrate (MLA, α7 nicotinic receptor antagonist) or tropicamide (TRO, M4 muscarinic receptor antagonist). Western blot analysis showed that the upregulations of p-CaMKIIα, p-CREB and CGRP in the spinal cord were reduced by LXM-10-M in chemical pain model in mice, and the effects were blocked by MLA or TRO pretreatment. This is the first paper to report that LXM-10-M exerted significant antinociception, which may be attributed to the activation of α7 nicotinic receptor and M4 muscarinic receptor and thereby triggering the inhibition of CaMKIIα/CREB/CGRP signaling pathway in mice.

1. Introduction

Pain can cause a series of pathological symptoms and psychological changes (Ripamonti and Bandieri, 2009; Silva et al., 2010), which severely affect the quality of people's life. Therefore, it is very urgent to search for novel drugs with greater potency and fewer side effects. Numerous novel approaches to pain relief are under investigation. Nicotinic acetylcholine receptors as promising targets for analgesics are being developed. It has been studied that α7 nicotinic receptor is involved in many important functions such as neuroprotection and antinociception (Wang et al., 2005; Egea et al., 2007). On the other hand, the development of selective M4 muscarinic receptor agonists as novel analgesic agents is an attractive therapeutic goal (Wess et al., 2007; Martino et al., 2011). However, there is currently no analgesic via activation of α7 nicotinic receptor and M4 muscarinic receptor used in clinic, and it remains unclear what the mechanisms underlying the contribution of α7 nicotinic receptor and M4 muscarinic receptor to analgesia are.

Spirocyclopeperazinium salt compound LXM-10-M (2,4-dimethyl-9-β-m-hydroxyphenylethyl-3-oxo-6, 9-diazaspiro [5.5] undecane chloride) (Fig. 1) is the metabolite derived from LXM-10 which has been proved to have obvious antinociceptive effect without significant effect on motor performance, spontaneous activity and body temperature, and the antinociceptive mechanism underlies activating peripheral α7 nicotinic receptor and M4 muscarinic receptor (Yue et al., 2007; Xiong et al., 2010). The present study is to evaluate the antinociception of LXM-10-M and further to explore the possible targets and signaling pathway.

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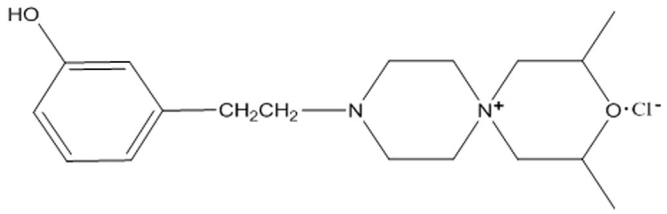

Fig. 1. The chemical structure of the spirocyclopiperazinium compound LXM-10-M.

2. Materials and methods

2.1. Animals

ICR mice (20–22 g) of both sexes were purchased from the Department of Laboratory Animal Science of Peking University. The mice were used randomly in each set of experiments, and the number of male and female mice was equal in each group. They were housed in a room maintained at 22 ± 0.5 °C and relative humidity of $55 \pm 5\%$ with an alternating 12 h light-dark cycle, and were provided with food and water ad libitum. All of the experiments were approved by the Institutional Animal Care and Use Committee of Peking University, and complied with the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.2. Drugs and reagents

We used the following drugs: methyllycaconitine citrate (MLA), tropicamide (TRO), methoctramine (Metho), aspirin (Sigma Chemical Co., St. Louis, MO, USA), morphine (Neuroscience Research Institute of Peking University, Beijing, PR. China), LXM-10-M was synthesized by Runtao Li and Qi Sun (Fig. 1). All of the drugs were dissolved in distilled water or normal saline immediately before use and administered in a volume of 10 ml/kg by intragastric (i.g.), subcutaneous (s.c.) or intraperitoneal (i.p.) injection.

2.3. Antinociceptive tests

2.3.1. Hot-plate test

In the experiment, mice were placed into a transparent cylinder on the heated surface (55 ± 0.5 °C), and the reaction time from the placement to the first response (licking or biting their hind paws or jumping) was recorded as an index of pain threshold (Sulaiman et al., 2008). Only mice that showed nociceptive responses between 5 and 20 s were selected for the experiment. Baseline latency was considered as the mean reaction time measured by three times with 30 min intervals. 20 s was defined as cut off time in order to avoid possible damage to the paws. Mice received LXM-10-M (0.4, 0.2, 0.1 mg/kg, i.g.), morphine (5 mg/kg, s.c.), or vehicle (distilled water, i.g.) respectively, with volume of 10 ml/kg. The latency was recorded at the time of 30 min before administration as baseline latency and 1 h, 2 h, 3 h, 4 h after administration. The percentage of Pain Threshold Elevated Rate (PTE%) was calculated using the following formula:

$$\text{PTE}(\%) = 100 \times (\text{latency of experiment} - \text{latency of vehicle}) / \text{latency of vehicle}$$

2.3.2. Formalin test

Mice were pretreated with LXM-10-M (0.4, 0.2, 0.1 mg/kg, i.g.), aspirin (300 mg/kg, i.g.) or vehicle (distilled water, i.g.) for 1.5 h before injection of 2.5% formalin (20 μl, in normal saline, s.c.) in the right hind paw (Seo et al., 2008). Mice were observed for 60 min during each 5 min interval after the injection of formalin, and the time spent licking and biting the injected paw was measured as an indicator of pain response. The first 5 min after formalin injection is considered as the first phase (neurogenic pain response) and the period from 10 min to 60 min as the second phase (inflammatory pain response). The percentage of inhibition was calculated using the following formula:

$$\text{Inhibition}(\%) = 100 \times (\text{time of vehicle} - \text{time of experiment}) / \text{time of vehicle}$$

2.3.3. Acetic acid writhing test

Mice were pretreated with LXM-10-M (0.4, 0.2, 0.1 mg/kg, i.g.), aspirin (300 mg/kg, i.g.) or vehicle (distilled water, i.g.) for 1.5 h before injection of 0.6% acetic acid solution (10 ml/kg, i.p.). The number of abdominal constrictions was recorded from 5 min to 20 min after acetic acid injection (Galeotti et al., 2008). Writhing response is equal to abdominal constriction. The percentage of inhibition was expressed by following formula:

$$\text{Inhibition}(\%) = 100 \times (\text{number of vehicle} - \text{number of experiment}) / \text{number of vehicle}$$

2.4. Potential antinociceptive mechanisms

2.4.1. Receptor blocking test involving α7 nicotinic receptor, M4 and M2 muscarinic receptor.

We pretreated mice with MLA (3 μmol/kg, i.p.), TRO (3 μmol/kg, i.p.), Metho (3 μmol/kg, i.p.) or vehicle (normal saline, i.p.) respectively. After 15 min, the mice received LXM-10-M (0.4 mg/kg, i.g.) or vehicle (i.g.). The hot-plate test, formalin test and acetic acid writhing test were carried out as above methods. In preliminary experiment, we tested the doses of antagonists (0.03, 0.3, 3 μmol/kg, i.p.), and the highest dose blocked the antinociception completely (Xiong et al., 2010).

2.4.2. Western blot analysis

Grouping based on above, the animals were killed at 10 min after injection of formalin or acetic acid followed by harvest of ipsilateral or bilateral spinal cord lumbar segments (L4–L6) in the formalin test or acetic acid writhing test respectively. The spinal tissues were weighed and quickly homogenized in nine volumes of ice-cold RIPA lysis buffer supplemented with 1% protease inhibitors and 1% phosphatase inhibitors (Bejing Applygen Technologies Inc. PR China) in glass homogenizers. The tissue serum was collected to centrifuge at 12,000*g* for 5 min. The supernatant was collected to measure the protein concentration by the Bicinchoninic acid (BCA) protein assay kit (Bejing Applygen Technologies Inc. PR China) according to the manufacturer's instructions, and then boiled in sodium dodecyl sulfated (SDS) sample buffer for 5 min. Equal amounts of protein were separated using 10–15% sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in Tris-Buffered Saline with Tween-20 (TBST) for 1.5 h at room temperature, and then incubated with primary antibodies including anti- calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) antibody, anti-phospho-CaMKIIα (Thr286) antibody, anti-calcitonin gene related peptide (CGRP) antibody (Abcam, Cambridge, USA), anti-cAMP response element-binding protein(CREB) antibody, anti-phospho-CREB (Ser133) antibody (Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin monoclonal antibody (Beijing Zhongshan Golden Bridge Biotechnology Co Ltd. PR China) (diluted in 5% skim milk in TBST, 1:1000–1:5000) overnight at 4 °C. The membrane was washed three times with TBST and then incubated