Pharmacological Studies on Lappaconitine: Possible Interaction with Endogenous Noradrenergic and Serotonergic Pathways to Induce Antinociception

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ABSTRACT — Systemic and intracerebroventricular (i.c.v.) injections of lappaconitine (LA) produced a dose-dependent inhibition of the response to thermal stimulation in sham-operated mice as assayed by the tail-immersion test. After spinal transection, the antinociceptive potencies of s.c.- or i.c.v.-administered LA were markedly reduced. Antinociception induced by systemically administered LA was clearly reduced by pretreatment with 6-hydroxydopamine or 5,7-dihydroxytryptamine through the i.c.v. and intrathecal (i.t.) routes. When LA was administered by i.c.v.-injection, the LA-induced antinociception was reduced by pretreatment with timolol, a β-adrenergic antagonist, and ketanserin, a 5-HT2 antagonist. Administration of LA by the i.t. route resulted in a significant antinociceptive activity, which was also reduced by pretreatment with phenoxybenzamine, an α-adrenergic antagonist, and mianserin, a 5-HT1 antagonist. The results of these studies suggest that the central noradrenergic and serotonergic systems may be involved in the antinociception of systemically administered LA, and these pathways are mediated by β-adrenoceptors and 5-HT2 receptors in the brain and α-adrenoceptors and 5-HT1 receptors in the spinal cord.

In a previous paper, we demonstrated that lappaconitine (LA) had naloxone-resistant analgesic effects (1). Furthermore, we reported that a supraspinal-spinal interaction was important for the production of the antinociceptive action of systemically administered LA (2) and that LA acted at the supraspinal level to inhibit nociceptive transmission or to block the spinal action of nociceptive neurotransmitters via the descending pathways (3).

It is well-known that the descending inhibitory systems play an important role in pain modulation and analgesia, and central noradrenergic and serotonergic pathways, particularly bulbospinal pathways, may modulate the transmission in these systems (4, 5).

The purpose of the present study was to examine the role of central noradrenergic and serotonergic systems in the antinociceptive action of LA.

MATERIALS AND METHODS

Animals

Male mice of the Std::ddY strain, weighing 20 to 30 g, were used. Mice were maintained in a temperature- and humidity-controlled room (22–23°C, 50–60%) and allowed free access to food and water.
Drugs and treatment

Lappaconitine hydrobromide (LA, Showa Yakuhin Kako), desipramine hydrochloride (DMI, Sigma) and timolol maleate (TIM, Banyu) were dissolved in saline or artificial cerebrospinal fluid (ACSF). 6-Hydroxydopamine hydrobromide (6-OHDA, Aldrich) and 5,7-dihydroxytryptamine creatinine sulfate (5,7-DHT, Sigma) were dissolved in saline containing 0.2 mg/ml ascorbic acid. Mianserin hydrochloride (MIA, Research Biochemicals, Inc.) and ketanserin tartrate (KET, Research Biochemicals, Inc.) were dissolved in distilled water, and phenoxybenzamine hydrochloride (PBZ, Nacalai Tesque) was dissolved in acidic saline. The doses are given in terms of the salt.

Mice were injected with either 6-OHDA (50 μg/mouse) or 5,7-DHT (80 μg/mouse), i.c.v. and with either 6-OHDA (20 μg/mouse) or 5,7-DHT (20 μg/mouse), intrathecally (i.t.). The mice were injected with either saline or DMI (25 mg/kg, intraperitoneally [i.p.]) 30 min before receiving the vehicle or 6-OHDA, to compare the effects of 6-OHDA-induced depletion of brain catecholamines (NA + dopamine) and the specific depletion of dopamine via the combined use of DMI and 6-OHDA (6). On the other hand, mice were pretreated i.p. with DMI, 25 mg/kg, 60 min before infusion of 5,7-DHT to prevent damage to catecholamine-containing neurons (7). Anti-nociceptive testing was performed 7 days after 6-OHDA or 5,7-DHT injection.

PBZ, used as an α-adrenergic receptor antagonist, was given i.p. at the dose of 10 mg/kg. TIM, used as a β-adrenergic receptor antagonist, was given s.c. at the dose of 3 mg/kg. MIA and KET, used as 5-HT1 and 5-HT2 receptor antagonists, respectively, were administered s.c. in doses of 3 mg/kg and 0.3 mg/kg, respectively. Pretreatment with PBZ, TIM, MIA, and KET was performed 45 min, 10 min, 35 min, and 50 min, respectively, before the administration of LA.

Procedures for i.c.v.- and i.t.-injection in mice

The procedure for i.c.v.-injection was adapted from the method of Haley and McCormick (8). Hamilton microsyringes bearing a 27-gauge needle with a stop at 3 mm from the needle tip were utilized for administration. The animals were gently restrained, and 5 μl of drug solution was administered into the lateral ventricle. The i.t.-injection procedure essentially followed the method described by Hylden and Wilcox (9). Lumbar puncture was performed using a 28-gauge needle connected to a Hamilton microsyringe. The needle was inserted between the L5 and L6 vertebrae, and drugs were delivered in a volume of 5 μl.

 Transection of the spinal cord

The spinal cord was mechanically transected at T9-T11 under pentobarbital sodium (50 mg/kg, i.p.) anesthesia. Sham-operated animals also received an incision under pentobarbital anesthesia. On the basis of the results obtained from the investigation of Spaulding et al. that mice recovered from spinalization within one day (10), animals were tested 24 hours after spinalization.

Measurement of analgesic activity

Antinociceptive activity was evaluated by the tail-immersion and hot-plate tests. The tail-immersion test described by Janssen et al. was used (11). Antinociceptive responses were determined in separate groups of mice using water at 48°C as the noxious stimulus. The latency to the first sign of a rapid tail-flick was taken as the endpoint. Prior to the spinal transection, mice not responding within 6 sec were eliminated from the experiment. The hot-plate test was carried out according to a previously described method (12). The nociceptive stimulus in the hot-plate was given from a platform maintained at 55°C. The baseline (control) latency for mice to jump or lick a hind paw was determined before LA administration and was found to be 1 to 6 sec. The post-drug latency was determined at 5, 15, 30, 45, and 60 min after administration of LA. Of these post-drug latencies, a maximum value was represented as a percentage of the max-
imum possible effect (% of MPE) factor according to the equation:

% of MPE = \frac{(\text{maximum post-drug latency} - \text{control latency})}{(\text{cut-off value} - \text{control latency})} \times 100

where the cut-off time was set at 15 sec in the tail-immersion test or 45 sec in the hot-plate test. The % of MPE was calculated for each mouse, using 15 to 20 mice per dose or time point. Furthermore, to study the influences of neurotoxins and antagonists, the antinociceptive activity for mice treated with neurotoxins or antagonists was expressed as a percentage of the % of MPE for the vehicle-treated group (% of control analgesia).

**Statistical analysis**

Significant differences were determined by Student’s t-test. The criterion of statistical significance was set up at P < 0.05. The results were expressed as means ± S.E.

**RESULTS**

**Antinociceptive effects of s. c. - or i. c. v. -administration of LA in spinalized mice**

The antinociceptive activity of s.c.- or i.c.v.-administered LA was initially evaluated in both the sham-operated and spinalized mice by the tail-immersion test (Table 1).

The control latency in the sham-operated group varied between 1.59 and 4.10 sec, while the control latency in the spinalized group ranged from 1.85 - 4.08 sec. The control latencies in both groups were not different. In spinalized mice, s.c.- and i.c.v.-administered LA-induced antinociception was markedly reduced as compared with that of the sham-operated mice. Particularly, i.c.v.-LA-induced antinociception was almost completely inhibited by spinalization.

**Effects of 6-OHDA or 5,7-DHT on the antinociceptive response to LA administered systemically**

In the hot-plate test, the pre-LA injection baseline value varied between 1.41 and 5.79 sec, and differences the vehicle and neurotoxin pretreated groups showed similar baseline values. Administration of LA (5 mg/kg) by the s.c. route produced a moderate antinociceptive effect in mice pretreated with vehicle through i.c.v. and i.t. routes (% of MPE: 58.0 ± 3.0 and 60.8 ± 3.9). The antinociceptive effect of LA was significantly reduced by pretreatment with 6-OHDA, 6-OHDA plus DMI, and 5,7-DHT plus DMI (Fig. 1); in mice pretreated i.c.v. with 6-OHDA, 6-OHDA plus DMI, and 5,7-DHT plus DMI, LA-induced antinociception was reduced to 35%, 34%, and 50%, respectively, as compared with those of the vehicle group (%

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose</th>
<th>Sham operated mice</th>
<th>Spinalized mice</th>
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<tbody>
<tr>
<td>s.c.</td>
<td>vehicle</td>
<td>14.3 ± 4.4</td>
<td>13.4 ± 3.1</td>
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<tr>
<td></td>
<td>2.5 mg/kg</td>
<td>21.9 ± 2.9</td>
<td>14.7 ± 1.5</td>
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<tr>
<td></td>
<td>5.0 mg/kg</td>
<td>45.1 ± 5.1</td>
<td>22.5 ± 2.9*</td>
</tr>
<tr>
<td></td>
<td>7.5 mg/kg</td>
<td>74.0 ± 5.5</td>
<td>44.0 ± 7.4**</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>vehicle</td>
<td>14.7 ± 2.1</td>
<td>12.0 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>800 ng/mouse</td>
<td>32.0 ± 3.0</td>
<td>11.0 ± 1.0* ***</td>
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<tr>
<td></td>
<td>1100 ng/mouse</td>
<td>52.6 ± 4.4</td>
<td>13.7 ± 1.7** ***</td>
</tr>
<tr>
<td></td>
<td>1500 ng/mouse</td>
<td>70.2 ± 4.5</td>
<td>11.8 ± 1.5** ***</td>
</tr>
</tbody>
</table>

The data are expressed as a percentage of the maximum possible effect, and values are means ± S.E. of 10–17 mice. Significant differences from sham-operated groups, *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t-test).
The baseline value varied between 1.64 and 5.57 sec, and there were no differences between the baseline values in the vehicle and antagonist pretreated groups. In the hot-plate test, i.c.v. and i.t. administration of LA (800 ng/mouse) produced moderate antinociceptive effects in mice pretreated with vehicle through i.c.v. and i.t. routes (% of MPE: 69.3 ± 5.5 and 63.9 ± 5.3). Pretreatment with TIM and KET, but not with PBZ and MIA, reduced the antinociceptive effect of i.c.v.-administered LA. The antinociceptive effects of LA in the TIM- and KET-pretreated groups were 34% and 67% of those in the vehicle group, respectively. Conversely, pretreatment with PBZ and MIA, but not with TIM and KET, resulted in a reduction of i.t.-LA-induced antinociception. The antinociceptive effects of LA in the PBZ- and MIA-pretreated groups were 57% and 41% of those in the vehicle group, respectively (Fig. 2).

**DISCUSSION**

Recently, we demonstrated that i.c.v. and i.t. injection of LA produces dose-dependent antinociception (13); and LA may act supraspinally to inhibit the spinal nociceptive transmission (3). In the present study, s.c.- and i.c.v.-administered LA-induced antinociception was markedly reduced after spinalization. This result indicates that a supraspinal descending mechanism plays a role in LA-induced antinociception.

After the intraventricular injection of 6-OHDA, there was a long lasting reduction in the brain concentrations of NA and dopamine; however, there was no significant effect on brain concentrations of 5-HT or γ-aminobutyric acid (14, 15). Breese and Traylor (16) reported that DMI inhibited depletion of NA produced by 6-OHDA, but did not alter depletion of dopamine. I.t. administration of 6-OHDA reduced the NA levels in the spinal cord (17). On the other hand, 5,7-DHT has a neurotoxic mode of action on 5-HT neurons, and it also has this mode of action, to a lesser extent, on NA neurons in the CNS (18–20).
Björklund et al. (7) reported that DMI effectively protected NA neurons against the neurotoxic actions of 5,7-DHT. Central NA and 5-HT systems consist of distinct ascending and descending projections and specific aspects of these projections are amenable to direct study by the microinjection of the neurotoxins 6-OHDA and 5,7-DHT into discrete brain regions to lesion ascending projections or into the spinal subarachnoid space to lesion descending projections (21). In the present study, i.c.v. and i.t. pretreatment with 6-OHDA, 6-OHDA plus DMI and 5,7-DHT plus DMI inhibited the antinociceptive action of systemically administered LA. These results suggest that ascending and descending monoaminergic projections may play a role in the action of LA.

The vast majority of studies examining the participation of NA and 5-HT neurons in pain modulation have focused on the spinal cord. Behavioral evidence suggesting that $\alpha_2$-adrenoceptors and 5-HT$_1$ receptors in the spinal cord modulate nociception, respectively, has been provided by the effects of intrathecally administered NA and 5-HT agonists and antagonists (22, 23). In the present study, the $\beta$-adrenoceptor antagonist TIM and the 5-HT$_2$ receptor antagonist KET attenuated the antinociceptive effect of i.c.v. LA. Intrathecal LA-induced antinociception was reduced by pretreatment with the $\alpha$-adrenoceptor antagonist PBZ and the 5-HT$_1$ receptor antagonist MIA. Currently available data suggest that the primary type of 5-HT receptor in the rat spinal cord is the 5-HT$_1$ receptor. No measurable specific binding of the 5-HT$_2$-selective antagonist $^3$H-ketanserin was observed in the spinal cord (24, 25). Recently, Zemlan et al. (26) have reported that 5-HT$_1$ and 5-HT$_2$ receptors are located in the spinal cord and cortex, respectively, and that these receptors participate in the increase or inhibition of pain and supraspinal narcotic analgesia. This classification of 5-HT receptor subtypes helps us to explain the differences between 5-HT$_1$ and 5-HT$_2$ receptor mediated-antinociception at the spinal and supraspinal levels. These results support our concept that spinal 5-HT$_2$ receptors are not involved in the antinociceptive action of i.t. LA. $\alpha$-Adrenergic agonists other than NA have been reported to produce antinociception when administered i.t. to mice and rats (27–34). It is equally conceivable that $\alpha$-adrenoceptors may mediate LA-induced antinociception at the spinal level. On the other hand, i.t. injection of a $\beta$-adrenergic agonist had only a weak effect on nociceptive behaviors (29). Murayama and Hikino (35) reported that the
antinociceptive action of mesaconitine, similar to LA, one of a series of diterpene alkaloids obtained from aconitine roots, was significantly potentiated by the $\beta$-adrenoceptor agonist isoproterenol, while it was suppressed by the $\beta$-adrenoceptor antagonist propranolol. However, no satisfactory explanation has yet been found for the $\beta$-adrenoceptor mediated-antinociception induced by LA at the supraspinal level.

The above evidence suggests that the central noradrenergic and serotonergic pathways, which are linked to $\alpha$-adrenoceptors and 5-HT receptors in the spinal cord and $\beta$-adrenoceptors and 5-HT$_2$ receptors in the brain, are involved in LA-induced antinociception in the mouse.

Acknowledgments
The authors wish to thank Prof. K. Kisara, Dr. S. Sakurada and Dr. T. Sakurada, Tohoku College of Pharmacy, for helpful discussions and advice. We also thank Mr. N. Ohkura for technical assistance.

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