Pharmacological Studies on Lappaconitine: Antinociception and Inhibition of the Spinal Action of Substance P and Somatostatin

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ABSTRACT—The pain response of mice to an injection of 0.5% formalin into the dorsal surface of a hindpaw is biphasic, with a first phase lasting for 5 min and a second phase lasting from 10 to 30 min post-injection. Intrathecal (i.t.) injection of [D-Pro2, D-Trp7-9]-substance P inhibited the first phase, and i.t. cysteamine inhibited the second phase. Lappaconitine (LA) and morphine (MOR) inhibited both phases equally in a dose-dependent manner. Diclofenac inhibited both phases, but the second phase was inhibited by lower doses. An i.t. injection of substance P (SP) or somatostatin (SOM) produced a characteristic behavioral response (scratching, biting, and licking). This behavioral response to SP and SOM was inhibited by s.c., intracerebroventricular (i.c.v.), or i.t. injection of MOR. In contrast, LA inhibited the SP and SOM-induced response when injected s.c. or i.c.v., but had no effect when injected intrathecally. These results indicate that LA may act supraspinally to inhibit the transmission of nociceptive information by SP and/or SOM.

Substance P (SP) and somatostatin (SOM) have been shown to be localized in the dorsal horn of the spinal cord (1, 2) and much evidence suggests a role for SP and SOM as possible mediators for pain transmission, as reviewed by Sweet (3) and Luttinger et al. (4).

The formalin test was originally described in rats and cats by Dubuisson and Dennis (5); the injection of formalin produced a biphasic pain response in rats. Takahashi et al. (6) and Hunskaar et al. (7) reported a slightly modified formalin test in mice and examined the effects of many narcotic analgesics, centrally acting non-narcotic analgesics, steroidal anti-inflammatory drugs, and non-steroidal anti-inflammatory drugs (NSAIDs) (8, 9). Based on these reports, it has been concluded that the biphasic response induced by the injection of formalin reflects the operation of different nociceptive mechanisms. The first phase is due to a direct effect on nociceptors, and the second phase may be due mainly to subsequent inflammation. Takagi and Kuraishi (10), and Kantner et al. (11) showed that SP and SOM were released from the dorsal horn in response to formalin injection, and Ohkubo et al. (12) showed that intrathecal (i.t.) injection of SP antagonist and SP antiserum inhibited only the first phase, while i.t. injection of SOM antagonist and SOM antiserum inhibited only the second phase. These facts indicate that SP is involved in the transmission of the first phase and that SOM is involved in the transmission of the second phase of formalin-
induced nociception. On the other hand, recent studies have demonstrated that i.t.-injected SP or SOM induces a characteristic behavioral response, i.e., biting or licking of the hindlegs and lower abdomen, a syndrome indicating nociceptive stimulation (13–16).

In previous papers, we demonstrated that lappaconitine (LA) has a centrally acting antinociceptive action (17); and after intracerebroventricular (i.c.v.), intracisternal, or i.t. injection, it produces dose-dependent antinociception (18). The present study was performed in order to investigate whether LA-induced antinociception involves modulation of the SP and SOM neuronal systems in the brain and spinal cord.

MATERIALS AND METHODS

Drugs
Lappaconitine hydrobromide (LA, Showa Yakuhin Kako), morphine hydrochloride (MOR, Takeda), diclofenac sodium (DF, Kyowa Hakko Kogyo), substance P (SP, Sigma), somatostatin (SOM, Sigma), [D-Pro²,D-Trp⁷,⁹]-substance P (DPDT-SP, Sigma), and cysteamine (CYS, Sigma) were dissolved in saline or artificial cerebrospinal fluid. The doses are given in terms of the salt.

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 were allowed free access to food and water. Each mouse was placed in an individual observation cage more than 30 min prior to the formalin, SP, or SOM injection.

Procedures for i.c.v. and i.t. injections in mice
The procedure for i.c.v. injection was essentially that described by Haley and McCormick (19). Hamilton microsyringes bearing a 27-gauge needle with a stop at 3 mm from the needle tip were used 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 (20). Lumbar puncture was performed using a 28-gauge needle with a Hamilton microsyringe. The needle was inserted between the L5 and L6 vertebrae, and drugs were delivered in a volume of 5 µl. The success rates of i.c.v. and i.t. injections were approximately 90 percent, as determined by dye injections in a separate group of animals.

Formalin-induced nociception
The method used was that described by Shibata et al. (9). Twenty-five µl of 0.5% formalin was injected into the right hind paw. Immediately following injections, mice were placed in the observation cages and their pain response was recorded for a period of 30 min. The summation of time (in seconds) spent in licking and biting of the injected paw during each 5 min block was measured as an indicator of the pain response. LA, MOR, and DF were administered subcutaneously 15 min prior to the formalin injection. DPDT-SP and CYS were injected i.t. 5 and 180 min prior to the formalin injection, and the results were converted to percent pain response with respect to the formalin injected control group.

SP- or SOM-induced behavioral response
The procedure for the behavioral test was based on that described by Takahashi et al. (21). Immediately following i.t. injection of SP or SOM, mice were placed in the observation cages, and the total time spent scratching, biting, and licking was measured with a stopwatch during the first period of 5 or 10 min post-injection. LA and MOR were administered s.c. and i.c.v. 15 and 10 min prior to the i.t. injection of SP or SOM, and LA and MOR were administered i.t. together with SP or SOM. The antinociceptive activities were evaluated in terms of the behavioral response and expressed as a percentage of that of the control group.

Statistical analysis
The significance of differences was deter-
RESULTS

Effects of LA, MOR and DF on the formalin-induced nociceptive response

The formalin-induced nociceptive response and the antinociceptive effects of LA, MOR and DF are shown in Figs. 1 and 2.

Two distinct periods of high response could be identified, the early phase lasting for the first 5 min and the delayed one lasting from 10 to 30 min after the injection of formalin. The peak period of the second phase was from 15 to 20 min. Hereafter, these two distinct periods (0–5 min, 10–30 min) are designated as the first and second phases, respectively.

Fig. 1. Time course of pain response induced by 0.5% formalin injection in mice. Each point represents the mean ± S.E. (n = 15).

Fig. 2. Effects of s.c. administration of lappaconitine (LA), morphine (MOR) and diclofenac (DF) on formalin-induced biphasic pain response. Data are expressed as the total duration of responses occurring in the first phase (0–5 min) or second phase (10–30 min). Each point represents the mean ± S.E. (n = 13–15). *P < 0.05, **P < 0.01, when compared to the formalin-injected control (Duncan's multiple range test).
the first phase and the second phase. LA (2.5–7.5 mg/kg, s.c.) and MOR (2.5–7.5 mg/kg, s.c.) dose-dependently inhibited both phases of the formalin-induced pain response. DF (100 mg/kg, s.c.) inhibited both phases, but the second phase was inhibited by lower doses (25–50 mg/kg, s.c.) than the first phase.

Effects of DPDT-SP and CYS on the formalin-induced nociceptive response

I.t. injection of DPDT-SP (1 µg) resulted in a reduced first phase response. When administered i.t., CYS (50 µg) inhibited only the second phase of the formalin-induced nociceptive response. Higher dosage of DPDT-SP (5 µg) or CYS (100 µg) caused a significant inhibition of both phases (Fig. 3).

Effects of LA and MOR on the SP-induced behavioral response

Immediately after i.t. injection, SP (0.05–2.0 nM) elicited a dose-related behavioral response which consisted of scratching, biting, and licking (Fig. 4). The peak period was from 2–3 min following i.t. injection of SP.

Figure 5 shows that s.c., i.c.v., or i.t. injection of MOR produced a dose-related inhibition of the SP (0.1 nM)-induced behavioral response. The s.c. injection of LA also resulted in a dose-dependent reduction of the SP-induced response. When given i.c.v. at large doses (125, 500, and 1000 ng), LA produced a significant inhibition of the SP-induced behavioral response. The i.t. injection of LA, even at large doses, had no significant effect.

Effects of LA and MOR on the SOM-induced behavioral response

During the first 10 min immediately after the i.t. injection, SOM (0.1–1.0 nM) elicited a dose-related behavioral response which was qualitatively similar to that induced by SP (Fig. 4). The SOM-induced behavioral response lasted slightly longer than that seen with SP. The peak period was from 5–10 min following i.t. injection of SOM.

As shown in Fig. 6, the response to SOM (0.5 nM) was dose-dependently inhibited by s.c., i.c.v., or i.t. injection of MOR. Injection of LA s.c. or i.c.v. inhibited the SOM-induced response, in contrast to the effect of LA injected i.t.

![Fig. 3. Effects of i.t. injection of [D-Pro²,D-Trp⁷⁵⁴]-substance P (DPDT-SP) and cysteamine (CYS) on the first phase (open columns; 0–5 min after the injection) and second phase (hatched columns, 10–30 min after the injection) pain response induced by formalin. Data are expressed as percentages of formalin-treated controls. Each value represents the mean ± S.E. (n = 12–16). **P < 0.01, when compared to the control (Duncan's multiple range test).](image-url)
DISCUSSION

The injection of formalin into the dorsal surface of a hindpaw in mice produced a biphasic pain response. Assessment of the effects of various analgesics, using the formalin-induced biphasic response, revealed that centrally acting analgesics such as narcotics inhibited the pain response equally at the first and second phases. In contrast, peripherally acting drugs such as aspirin, indomethacin, oxyphenbutazone, and dexamethasone inhibited only the second phase (8, 9). In the present study, the injection of LA inhibited both phases, as did MOR. According to this result, LA may be grouped as a centrally acting analgesic. The analgesic action of NSAIDs is generally considered to be related to inhibition of the enzyme cyclooxygenase and to involve a peripheral site of action.
Fig. 6. Effects of s.c., i.c.v., and i.t. administration of lappaconitine (open columns) and morphine (hatched columns) on 0.5 nM somatostatin-induced behavioral responses in mice. Data are expressed as percentages of the somatostatin-treated controls (dotted columns). Each point represents the mean ± S.E. (n = 12–15). *P < 0.05, **P < 0.01, when compared to the somatostatin-treated control (Duncan’s multiple range test).

However, recent studies have indicated a central, opioid receptor-mediated effect of the NSAIDs. Okuyama and Aihara (22, 23) demonstrated a possible central antinociceptive effect of NSAIDs in arthritic rats. A decreased pituitary and hypothalamic content of beta-endorphin in experimental animals have been shown to follow administration of the NSAID DF (24). Moreover, DF exerts a central, naloxone-reversible antinociceptive action in rats after noxious visceral stimuli, such as the writhing test, but not after somatosensory stimuli, such as the tail-flick test and hot-plate test (25). However, in the present formalin test, DF chiefly inhibited the second phase.

A number of studies have demonstrated a role for the neuropeptides SP and SOM as possible mediators of pain transmission. Recent evidence indicates that mechanical or thermal noxious stimuli specifically increase the release of immunoreactive SP or SOM, respectively, from the dorsal horn of the spinal cord of the rabbit (26). On the other hand, Ohkubo et al. (12) reported that SP and SOM act as neurotransmitters in the first and second phases of the formalin-induced nociceptive response, respectively. Moreover, in the present study, i.t. injection of DPDT-SP (1 μg/mouse), an SP antagonist (27, 28), or CYS (50 μg/mouse), an SOM depletor (29), inhibited the first phase or the second phase response, respectively. These observations, and the fact that LA inhibited the first and second phases of the formalin-induced nociceptive response, indicate that LA may have an inhibitory effect on the actions of SP and SOM.

SP and SOM administered i.t. elicit a characteristic behavioral response, consisting of scratching, biting, and licking, in mice and rats (14, 30); the behaviors elicited in response to i.t. SP and SOM have been shown to be due to central and not peripheral actions of the two peptides (15). These reports support a direct action of SP and SOM on ascending sensory neuronal circuitry. In the present study, many differences were observed in the behavioral responses to i.t. SP and SOM. First, the peaks of the responses to SP and SOM were obtained 2–3 min and 5–10 min after i.t. injection, respectively. Second, the durations of action of SP and SOM were 5 min and 15 min, respectively. Third, a lower potency of SOM, expressed as behavioral response/min, was seen in comparison to the effect of SP. These results may be explained by immunohistochemical studies, which indicate the presence of differences of distribution and localization between SP and SOM neurons. That is, SP terminals are densely distributed in lamina I and outer lamina II, and
SOM terminals are dispersed deeper into lamina II (1, 2, 31), and the SP- and SOM-containing neurons represent 15–20% and 5–10% of the total population of dorsal root ganglion cells, respectively (32).

In the present experiment, the behaviors induced by SP and SOM were reduced by s.c., i.c.v., or i.t. administration of MOR. However, the effect of i.t. MOR was weaker than that of i.c.v. MOR. Concerning the effect of i.t.-injected MOR on the SP-induced behavioral response, there have been contrasting findings: that i.t. MOR was effective in inhibiting the behavior induced by SP (16, 21, 33, 34), and that i.t. MOR did not inhibit the response to SP (35). Hylden and Wilcox (14) inferred that MOR may act at a supraspinal level to decrease the elicited behavior or to block the spinal action of SP via a descending neural system. On the other hand, the behaviors induced by exogenously applied SP and SOM were reduced by s.c.- and i.c.v.-administered LA, as found in the case of MOR, but LA administered i.t. was ineffective in inhibiting these behaviors. Thus, LA was considered to act at a supraspinal level to decrease the elicited behavior.

When injected i.t., LA showed dose-dependent antinociceptive activities against mechanical, heat, and chemical nociception (18). Furthermore, we observed that systemically administered LA was distributed to the brain and the spinal cord, and the supraspinal-spinal interaction is important in antinociceptive action (36). From these findings and from the present results, it is speculated that LA acts at supraspinal and spinal levels to inhibit nociceptive transmission or to block the spinal action of neurotransmitters.

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