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Analgesia-Producing Mechanism of Processed *Aconiti* Tuber: Role of Dynorphin, an Endogenous κ-Opioid Ligand, in the Rodent Spinal Cord

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ABSTRACT—The analgesia-producing mechanism of processed *Aconiti* tuber was examined using rodents whose nociceptive threshold was decreased by loading repeated cold stress (RCS). The antinociceptive effect of processed *Aconiti* tuber (0.3 g/kg, p.o.) in RCS-loaded mice was antagonized by pretreatment with a κ-opioid antagonist, nor-binaltorphimine (10 mg/kg, s.c.), and was abolished by an intrathecal injection of anti-dynorphin antiserum (5 μg). The *Aconiti* tuber-induced antinociception was inhibited by both dexamethasone (0.4 mg/kg, i.p.) and a dopamine D_2 antagonist, sulpiride (10 mg/kg, i.p.), in RCS-loaded mice, and it was eliminated by both an electric lesion of the hypothalamic arcuate nucleus (HARN) and a highly selective dopamine D_2 antagonist, eticlopride (0.05 μg), administered into the HARN in RCS-loaded rats. These results suggest that the analgesic effect of processed *Aconiti* tuber was produced via the stimulation of κ-opioid receptors by dynorphin released in the spinal cord. It was also shown that dopamine D_2 receptors in the HARN were involved in the expression of the analgesic activity of processed *Aconiti* tuber.

Keywords: Processed *Aconiti* tuber, Antinociception, Dynorphin, Spinal cord, Hypothalamic arcuate nucleus

The tuber of the species *Aconitum* is a crude drug that has been utilized from ancient times. Attempts to reduce this drug's toxicity have been made by immersion in salt solution or heating. It is well known that the analgesic effect of processed *Aconiti* tuber is caused by the pharmacological actions of the aconitine alkaloids (e.g., aconitine, mesaconitine and benzoylmesaconine) (1, 2). We have shown that the antinociceptive effect of processed *Aconiti* tuber is due to activation of a descending pain inhibitory system transmitted by both noradrenaline and serotonin in a study using rats whose nociceptive response was enhanced by a repeated cold stress (RCS) load (3). However, the mechanism underlying the activation of the descending pain inhibitory system by processed *Aconiti* tuber remains to be clarified.

There are several studies indicating that the processed *Aconiti* tuber-induced antinociception is caused by the aconitine alkaloids acting at the supraspinal level (4, 5). The effects of a topical injection of mesaconitine or benzoylmesaconine into a particular nucleus or the effects of intracerebroventricular (i.c.v.) administration must be carefully interpreted because the aconitine alkaloids, which are sodium channel openers as well, may cause a nonspecific depolarization of the neurons related to antinociception. We recently suggested that the antinociceptive effect of processed *Aconiti* tuber is expressed via non-μ-type opioid receptors in the spinal cord. This was based on our observation that an intrathecal (i.t.) injection of naloxone inhibited the antinociceptive activity of processed *Aconiti* tuber in normal mice, but 10- to 20-fold more naloxone was required for the inhibition of the antinociception elicited by processed *Aconiti* tuber than for the inhibition of the antinociception elicited by morphine (6).

We also observed that RCS-loaded animals expressing the increased antinociceptive effect of processed *Aconiti* tuber were hyposensitive to supraspinal μ-opioid receptor-mediated antinociception, while the opioid analgesia associated with intraspinal κ-opioid receptors was increased in those animals (Y. Omiya et al., submitted manuscript). In the present study, we tested the possibility that the analgesic effect of processed *Aconiti* tuber is expressed via κ-opioid receptors in the spinal cord. A study was also made to elucidate the pathway of the descending pain inhibitory system activated by this tuber.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats initially weighing 250–350 g (Charles River Japan, Inc., Kanagawa) and male ddY mice weighing 20–25 g (Japan SLC, Inc., Shizuoka) at the beginning of the experiment were used. Four animals were housed per cage under a 12-hr light and dark cycle (lights on between 7:00–19:00), and the animals had free access to food and water ad libitum throughout the experiments.

RCS load

The animals were exposed to a cold environment (4℃) from 16:30–10:00 and then alternately to 24℃ and 4℃ at 30-min intervals from 10:00 to 16:30. The RCS schedule started at 16:30 on day 0 and stopped at 10:00 on day 3. The nociception test was carried out during the period of 10:00–18:00 on day 3 (7).

Surgical preparation

Seven days prior to the experiments, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and mounted on a stereotaxic apparatus. The scalp was incised, and a 1-mm hole was drilled in the cranium dorsal to the target site. The lesioning electrode (0.4-mm diameter) was inserted into the hypothalamic arcuate nucleus (HARN) and attached to the cranium with dental cement. The coordinates for the placement of the lesioning electrode, derived from the atlas of Paxinos and Watson (8) were as follows: A −4.2, L 0.4, H −10.0.

Electric lesion of the HARN

The electric lesion (50 mA, 5 sec) of the HARN was made just after the first assessment of the antinociceptive effect of processed *Aconiti* tuber or U-50488H.

Microinjection into the HARN

Under 50 mg/kg pentobarbital anesthesia, a 0.55-mm metal guide cannula was inserted to just above the HARN. With a microsyringe, eticlopride in 0.5 μl of saline was applied just after the first assessment of the antinociceptive effect of processed *Aconiti* tuber through a 0.3-mm metal tube inserted into the guide cannula.

Identification of lesion and injection sites

After the experiments were finished, methylene blue was injected into the HARN. The stained site in the 15-μm coronal sections cut with a microtome were identified under a microscope.

Drugs

TSUMURA Shuchi-Bushi Powder N for Etical Dispensing (TJ-3022; Tsumura, Tokyo) used as processed *Aconiti* tuber in this study was obtained after the tuber of *Aconitum carmichaeli* DEBX. was treated in an autoclave at 105℃ for 50 min, dried, and reduced to powder (9). The processed *Aconiti* tuber was suspended in a 5% arabic gum solution. *trans*-(±)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U-50488H), β-funaltrexamine, nor-binaltorphimine and eticlopride (Research Biochemicals International, Natick, MA, USA); naltrindole and sulpiride (Sigma Chemical, St. Louis, MO, USA); and anti-dynorphin A (1–13) antiserum (Peninsula Laboratories, Belmont, CA, USA) were dissolved in saline. Anti-human IgG antiserum (Rockland, Gillbertsville, PA, USA) was used as the control for the anti-dynorphin A (1–13) antiserum. Dexamethasone (Sigma Chemical), dissolved in ethanol to the final concentration of 5%, was suspended in 5% arabic gum.

I.c.v. and i.t. injection

The i.c.v. and i.t. injections were carried out using a 10-μl Hamilton syringe according to the procedures of Haley and McCormick (10) and Hylden and Wilcox (11), respectively. The saline and drug solutions were administered in a volume of 5 μl for both injections.

Nociception test

Antinociceptive responses were evaluated by a tail-pressure test (Pressure analgesimeter; Ugo Basile, Milan, Italy) in the mice and a paw-pressure test (Analgesimeter; Unicom, Chiba) in the rats. The weight (g) at which animals struggled or withdrew was considered the nociceptive threshold. The antinociceptive activities were calculated as the area under the time exposure curve (AUC) by plotting the percent increase in the nociceptive threshold (100 × (post-drug nociceptive threshold / pre-drug nociceptive threshold) − 100) on the ordinate and the time interval (hr) on the abscissa.

Statistical analyses

The results are presented as means±S.E.M. The significance of differences was determined by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. For all cases, differences of $P<0.05$ were considered significant.

RESULTS

Effects of β-funaltrexamine, naltrindole, nor-binaltorphimine and anti-dynorphin A (1–13) antiserum on the antinociceptive activity of processed Aconiti tuber in RCS mice

The pretreatment of RCS-loaded mice with a highly selective κ-opioid antagonist, nor-binaltorphimine (10

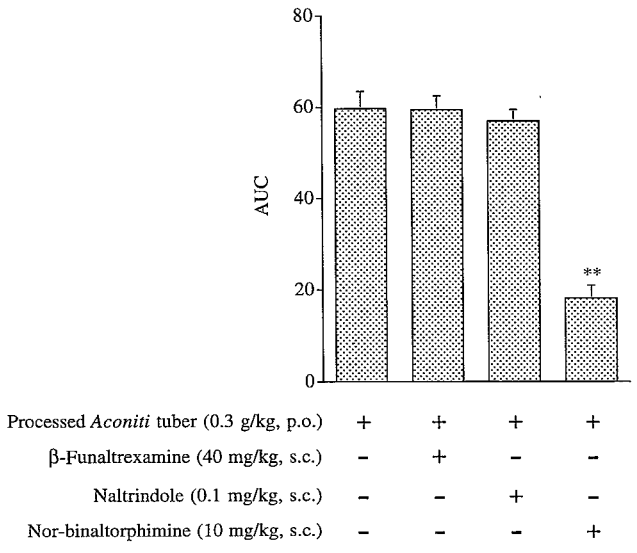

Fig. 1. Antagonistic effect of nor-binaltorphimine on processed *Aconiti* tuber-induced antinociception in RCS mice. β-Funaltrexamine or nor-binaltorphimine was given 24 or 2 hr prior to the processed *Aconiti* tuber administration, respectively. Naltrindole was injected 15 min after the processed *Aconiti* tuber administration. The antinociceptive activities were measured by the tail-pressure test every 0.5 hr after processed *Aconiti* tuber treatment for 3 hr and were expressed as the area under the curve (AUC). Each column represents the mean±S.E.M. of 8–18 animals. **P<0.01, compared with the saline-treated group (Dunnett's *t*-test).

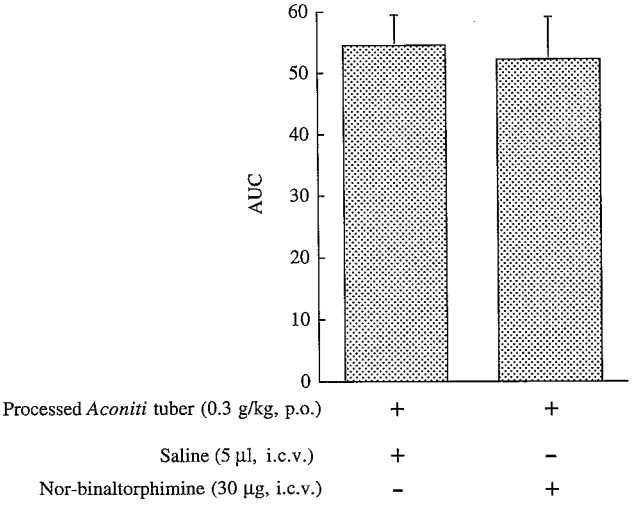

Fig. 3. Effect of nor-binaltorphimine injected intracerebroventricularly on processed *Aconiti* tuber-induced antinociception in RCS mice. Nor-binaltorphimine was given 2 hr prior to the processed *Aconiti* tuber administration. Each column represents the mean ±S.E.M. of 10 animals.

mg/kg, s.c.), reduced the antinociceptive activity of processed *Aconiti* tuber (0.3 g/kg, p.o.) by 68%. The μ-opioid antagonist β-funaltrexamine (40 mg/kg, s.c.) and the δ-opioid antagonist naltrindole (0.1 mg/kg, s.c.) had no effect (Fig. 1). The processed *Aconiti* tuber-induced antinociception was completely eliminated by pre-administered anti-dynorphin A (1–13) antiserum (5 μg, i.t.) (Fig. 2). An i.c.v. injection of nor-binaltorphimine (30 μg) did not affect the increase in the nociceptive threshold elicited by processed *Aconiti* tuber (Fig. 3).

Effects of dexamethasone and sulpiride on the antinociceptive activity of processed Aconiti tuber or U-50488H in RCS mice

The antinociceptive effect of processed *Aconiti* tuber (0.3 g/kg, p.o.) was inhibited by 82% by dexamethasone (0.4 mg/kg, i.p.) and by 74% by the dopamine D_2 antagonist sulpiride (10 mg/kg, s.c.) (Fig. 4). The antinociceptive activity of the κ-opioid agonist U-50488H administered subcutaneously or intrathecally was inhibited 61% by dexamethasone (0.4 mg/kg, i.p.) and 52% by sulpiride (10 mg/kg, s.c.), respectively (Fig. 5: A and B).

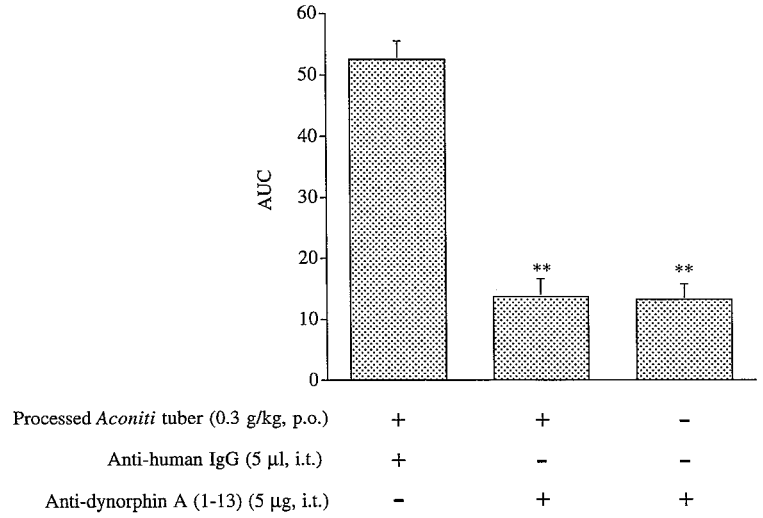

Fig. 2. Antagonistic effect of anti-dynorphin A (1–13) antiserum on processed *Aconiti* tuber-induced antinociception in RCS mice. Anti-dynorphin A (1–13) or anti-human IgG antiserum was injected just before the treatment with processed *Aconiti* tuber. The AUC values were obtained as described in the legend to Fig. 1. Each column represents the mean±S.E.M. of 8–9 animals. **P<0.01, compared with the anti-human IgG antiserum-treated group (Dunnett's *t*-test).

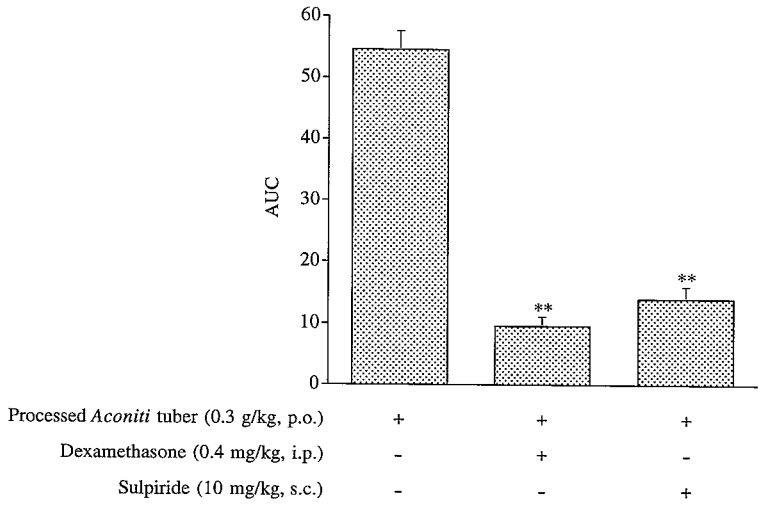

Fig. 4. Antagonistic effects of dexamethasone and sulpiride on processed *Aconiti* tuber-induced antinociception in RCS mice. Dexamethasone and sulpiride were injected 30 min before the treatment with processed *Aconiti* tuber. Each column represents the mean±S.E.M. of 8–9 animals. **P<0.01, compared with the vehicle-treated group (Dunnett's *t*-test).

Effects of electric lesion of the HARN on the antinociceptive activity of processed Aconiti tuber or U-50488H in RCS rats

The increases in the nociceptive threshold elicited by processed *Aconiti* tuber (0.3 g/kg, p.o.) or U-50488H (3 mg/kg, s.c.) were completely eliminated by the electric lesion of the HARN in the RCS-loaded rats. The electric lesion of the HARN itself did not affect the nociceptive threshold in these animals (Fig. 6: A and B).

Effect of topical administration of eticlopride into the HARN on processed Aconiti tuber-induced antinociception in RCS rats

The highly selective dopamine D_2 antagonist eticlopride (0.05 μg), topically administered into the HARN, did not affect the nociceptive threshold in the RCS-loaded rats, but completely abolished the processed *Aconiti* tuber (0.3 g/kg, p.o.)-induced antinociception (Fig. 7).

DISCUSSION

The present results suggest that the antinociceptive effect of processed *Aconiti* tuber is mediated by κ-opioid receptors. This idea is based on our observation that the processed *Aconiti* tuber-induced antinociception was eliminated by pretreatment with nor-binaltorphimine but not β-funaltrexamine or naltrindole. This result explains why the antinociceptive activity of processed *Aconiti*

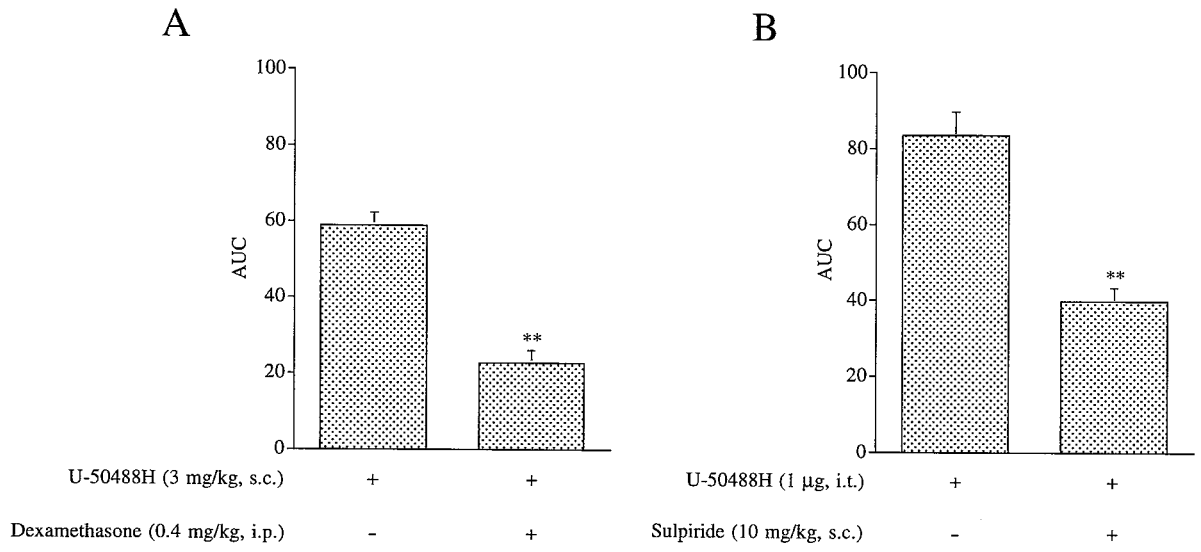

Fig. 5. Antagonistic effects of dexamethasone (A) and sulp iride (B) on U-50488H-induced antinociception in RCS mice. Dexamethasone and sulpiride were given 30 min prior to U-50488H administration. Each column represents the mean±S.E.M. of 8–10 animals. **P<0.01, compared with the vehicle-treated group (Student's *t*-test).

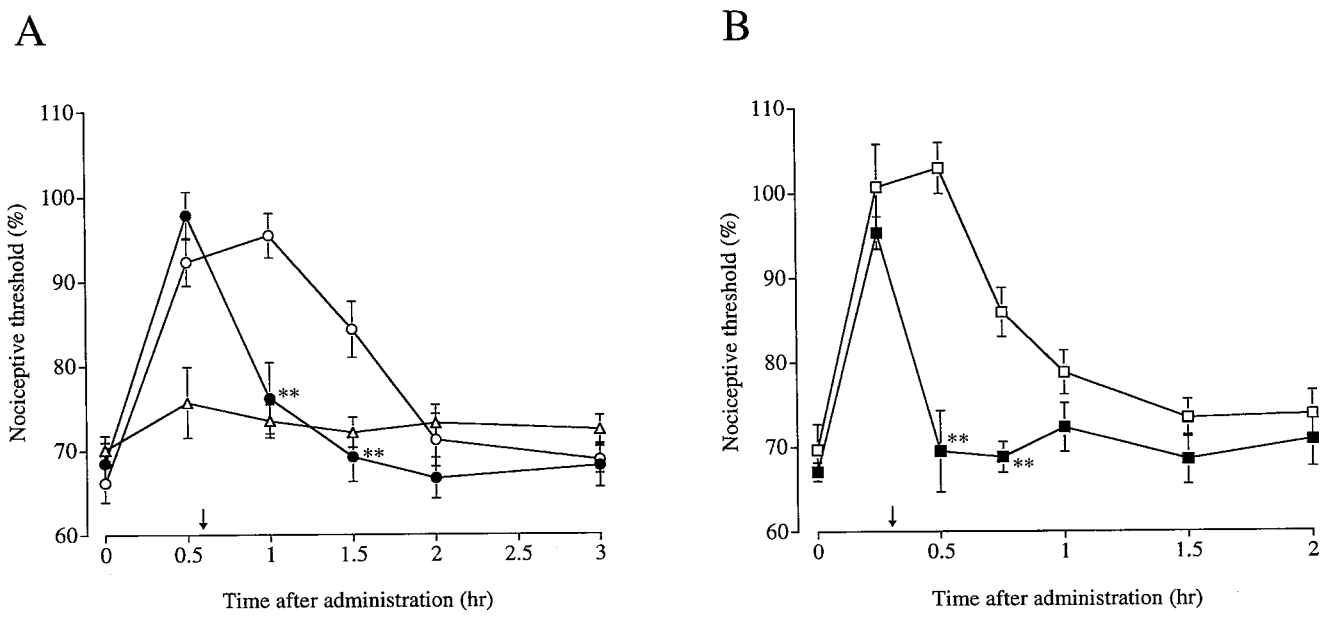

Fig. 6. Alteration of processed *Aconiti* tuber (A) or U-50488H (B)-induced antinociception by electric lesion of the HARN in RCS rats. The nociceptive threshold was evaluated by the paw-pressure test. A: ○: processed *Aconiti* tuber (0.3 g/kg, p.o.) + sham-operation, ●: processed *Aconiti* tuber + lesion of the HARN, △: vehicle (10 ml/kg, p.o.) + lesion of the HARN. B: □: U-50488H (3 mg/kg, s.c.) + sham-operation, ■: U-50488H + lesion of the HARN. The electric lesion of the HARN was performed just after the first assessment of the antinociceptive effect of processed *Aconiti* tuber or U-50488H (arrow). Each point represents the mean±S.E.M. of 5–9 animals. **P<0.01, compared with the sham-operated group (A: Dunnett's *t*-test, B: Student's *t*-test).

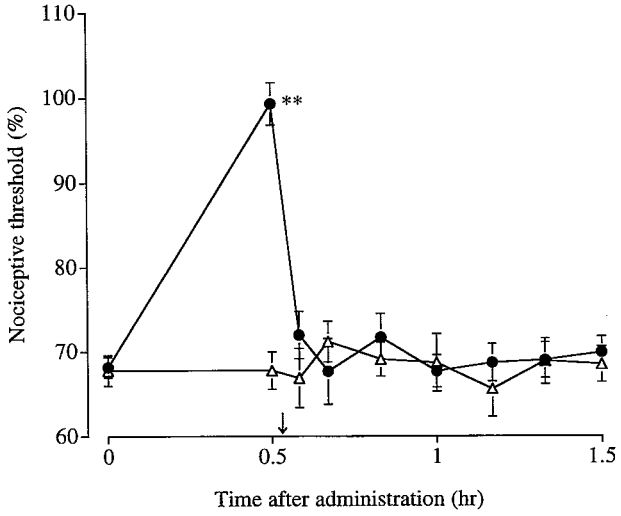

Fig. 7. Antagonism of processed *Aconiti* tuber-induced antinociception by microinjection of eticlopride in RCS rats. The nociceptive threshold was evaluated by the paw-pressure test. ●: processed *Aconiti* tuber (0.3 g/kg, p.o.)-treated group, △: vehicle (10 ml/kg, p.o.)-treated group. Eticlopride (0.05 μg) was applied into the HARN just after the first assessment of the antinociceptive effect of processed *Aconiti* tuber (arrow). Each point represents the mean±S.E.M. of 6–8 animals. **P<0.01, compared with the vehicle-treated group (Student's *t*-test).

tuber becomes prominent in RCS-loaded animals (3), which show an increase in opioid analgesia associated with spinal κ-opioid receptors (Y. Omiya et al., submitted manuscript).

We speculated that processed *Aconiti* tuber secondarily stimulates intraspinal κ-opioid receptors by promoting release of dynorphin since the i.t. administration of the antiserum against dynorphin (which is an endogenous κ-opioid ligand) eliminated the processed *Aconiti* tuber-induced antinociception. It has been reported that dynorphin inhibited N-type calcium channels in dorsal root ganglion neurons (12). However, it is unlikely that increased extracellular concentration of dynorphin in the spinal cord after processed *Aconiti* tuber administration directly inhibited the release of neurotransmitter substances (e.g., substance P, somatostatin) that transmit nociception in the spinal dorsal horn because the increase of nociceptive threshold elicited by processed *Aconiti* tuber was eliminated by both lesion of the HARN and the administration of eticlopride into the HARN. It has been demonstrated that the posterior HARN is the initial sector of the efferent pathway in the descending pain inhibitory system transmitted by both noradrenaline and serotonin (13). The activation of descending inhibitory α_2-noradrenergic and serotonergic systems has been suggested to induce antinociception by inhibiting the release of neurotransmitter substances from the nociceptive pri-

mary afferent terminals (14, 15). Thus, the results in this study indicate that the activation of the descending pain inhibitory system through the HARN after stimulation of intraspinal κ-opioid receptors by dynorphin is required for the expression of the antinociceptive activity of processed *Aconiti* tuber. The effect of U-50488H used for direct stimulation of κ-opioid receptors also disappeared after destruction of the HARN. This finding suggests that a pathway in which the HARN intervenes is important in the increase of nociceptive threshold following stimulation of κ-opioid receptors. Oral administration of processed *Aconiti* tuber that releases dynorphin in the spinal cord appears to have effects similar to those of intraspinal U-50488H injection.

Takeshige et al. (16, 17) reported that both dexamethasone and haloperidol administered into the posterior HARN inhibited nonacupuncture point stimulation-produced analgesia (NAA) in which the release of dynorphin in the spinal cord was involved. They suggested that this NAA was expressed via dopaminergic transmission in the HARN, which is under the positive control of adrenocorticotropic hormone (17). The present finding that U-50488H-induced antinociception was inhibited by dexamethasone is consistent with the findings of a previous study (16). Thus, the inhibitory effects of dexamethasone and sulpiride on the increase of the nociceptive threshold elicited by processed *Aconiti* tuber or U-50488H administration could be due to an inhibition of dopaminergic transmission in the HARN. In addition, our observation that eticlopride administered in the HARN eliminated the antinociceptive effect of processed *Aconiti* tuber suggested that the dopamine D_2 receptors in the HARN were strongly involved in the processed *Aconiti* tuber-induced antinociception. We suspect that an activation of spinal κ-opioid receptors after processed *Aconiti* tuber or U-50488H administration induces stimulation of the dopamine D_2 receptors in the HARN and thereby causes an antinociceptive effect via activation of the descending pain inhibitory system, as is the case with NAA. In RCS-loaded animals, activation of dopaminergic transmissions in the HARN by stimulation of spinal κ-opioid receptors may more readily occur.

The lack of effect of the i.c.v. injection of nor-binaltorphimine suggested that the antinociceptive effect of processed *Aconiti* tuber must be caused by dynorphin released in spinal sites, but not in supraspinal sites. As yet, however, it is not known why the supraspinal κ-opioid receptors would not contribute to the expression of the processed *Aconiti* tuber-induced antinociception. It is necessary to perform further studies on the selectivity of the site of promotion of dynorphin release by processed *Aconiti* tuber. On the other hand, it has been reported that the content of dynorphin was increased in the spinal cord in several animal models that were hyperalgesic due to inflammation or peripheral neuropathy (18, 19). We also observed that increased antinociception via intraspinal κ-opioid receptors was stimulated not only in RCS-loaded mice, but also in diabetic mice expressing a lowered nociceptive threshold; the antinociceptive activity of processed *Aconiti* tuber was also increased in these mice (Y. Suzuki et al., submitted manuscript). These results suggest the existence of diseases in which the analgesic effect of processed *Aconiti* tuber can be enhanced.

In conclusion, our results suggest that the antinociceptive effect of processed *Aconiti* tuber is produced by stimulating κ-opioid receptors via dynorphin release in the spinal cord and subsequently activating the dopamine D_2 receptors in the HARN. Our results indicate the possibility that the analgesic effect of processed *Aconiti* tuber is enhanced in pathologic conditions in which the dynorphin content in the spinal cord is increased or the opioid analgesia associated with κ-opioid receptors in the spinal cord is activated.

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