## Implication of the Descending Dynorphinergic Neuron Projecting to the Spinal Cord in the (+)-Matrine- and (+)-Allomatrine-Induced Antinociceptive Effects

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We previously reported that either (+)-matrine (matridin-15-one) or (+)-allomatrine (the C-6 epimer of matrine)-induced antinociceptive effect was attenuated by s.c. pretreatment with a k-opioid receptor (KOR) antagonist nor-binaltorphimine (nor-BNI), indicating the critical role of KORs in antinociceptive effects induced by these alkaloids. In the present study, we found that i.c.v. administration of either (+)-matrine- or (+)-allomatrine induced antinociceptive effects in the mouse tail-flick and warm-plate test, whereas these alkaloids when given spinally failed to induce antinociception. In the guanosine-5'-O-(3-[<sup>35</sup>S]thio)trisphosphate ([<sup>35</sup>S]GTP $\gamma$ S) binding assay, we demonstrated that neither (+)-matrine nor (+)-allomatrine produced the stimulation of [<sup>35</sup>S]GTP<sub>γ</sub>S binding in the membranes of the spinal cord, indicating that (+)-matrine- and (+)-allomatrine-induced supraspinal antinociceptive actions was not due to a direct stimulation of KORs by these alkaloids. Therefore, we next investigated the involvement of dynorphin A (1-17) release at the spinal or supraspinal site in (+)matrine- or (+)-allomatrine-induced antinociception. The i.c.v. pretreatment with an antiserum against dynorphin A (1-17) could not affect the antinociceptive effect induced by s.c. treatment of (+)-matrine. In contrast, the s.c.-administered (+)-matrine- and (+)-allomatrine-induced antinociceptive effect was significantly attenuated by i.t. pretreatment of an antiserum against dynorphin A (1-17). The present data suggest that either (+)-matrine or (+)-allomatrine when given i.c.v. may stimulate the descending dynorphinergic neuron, resulting in the stimulation of KORs in the spinal cord, and this phenomenon in turn produces the antinociception in mice.

Key words  $\kappa$ -opioid receptor; dynorphin A (1-17); antinociceptive effect; mouse; pain controlling system

Apparent subpopulations of  $\kappa$ -opioid receptor (KOR) have been detected in central nervous system of rodents, nonhuman primates and human based on radioligand binding studies.<sup>1,2)</sup> KOR agonists may have pharmacotherapeutic potential in the treatment of pain or hyperalgesia and in the management of psychostimulant addiction.<sup>3)</sup> However, the clinical application of selective KOR agonists, such as ethylketocyclazocine and U-50488, has been limited by the occurrence of characteristic undesirable effects, *e.g.* aversion, sedation and dysphoria.

(+)-Matrine (matridin-15-one) and its C-6 epimer, (+)-allomatrine, are the typical lupine alkaloid along with lupinine, sparteine and cytisine (Fig. 1). These alkaloids occur in many leguminous plants, especially in the genus *Sophora* and are one of the main basic constituents in both *Sophora flavescens* and *S. tonkinensis*.<sup>4,5)</sup> Dry roots of these plants have been used as important Chinese drugs 'Ku-shen' and 'Shan-dougen' respectively, for the treatment of fever and as analgesics.

We previously demonstrated that (+)-matrine, when given





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s.c., induced a dose-dependent antinociceptive effect, which was identical to that of pentazocine, in the acetic acid-induced abdominal contraction test.<sup>6)</sup> (+)-Allomatrine also showed its antinociceptive property with one-third the potency of that of (+)-matrine.<sup>7)</sup> It should be noted that either (+)-matrine- or (+)-allomatrine-induced antinociceptive effect was markedly inhibited by s.c.-pretreatment with a selective KOR antagonist nor-binaltorphimine (nor-BNI), indicating the critical role of KOR in antinociceptive effects of these alkaloids.<sup>6,7)</sup> These findings pointed out the possibility that either (+)-matrine or (+)-allomatrine, which possess the distinct chemical structure from conventional KOR agonists, may be candidate for the effective option for the pain control in the clinical. It is, therefore, worthwhile to further ascertain the antinociceptive properties of (+)-matrine and (+)-allomatrine. Thus, the goal of the present study was to investigate the possible involvement of either supraspinal or spinal KORs in antinociceptive effects induced by (+)-matrine or (+)-allomatrine.

## MATERIALS AND METHODS

The isolation of (+)-matrine or (+)-allomatrine was performed according to the following method. Dry plant material was cut into small chips and extracted with 75% MeOH several times at room temperature. The aqueous concentrate was acidified with 10% HCl to pH 3 and the resulting precipitate was filtered off. The filtrate was extracted three times with  $CH_2Cl_2$ . The aqueous layer was made strongly alkaline with  $K_2CO_3$  and extracted with  $CH_2Cl_2$  three times. The  $CH_2Cl_2$  extracts were combined, dried over  $K_2CO_3$  and concentrated *in vacuo* to give crude base. The crude alkaloid mixture was separated by silica gel column chromatography and then purified by aluminum oxide column chromatography or preparative HPLC to give (+)-matrine and (+)-allomatrine.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animal used in the following experiments.

Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan), weighting about 25—28 g at the beginning of experiments, were housed in groups of 8—12 in a temperature-controlled room. The animals were housed at a room temperature of  $22\pm1$  °C with a 12 h light–dark cycle (light on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum.

Antinociception was determined by the tail-flick and warm-plate test. In the tail-flick test, the intensity of the heat stimulus was adjusted so that the animal flicked its tail after 3-5 s. The inhibition of this tail-flick response was expressed as a percentage of the maximum possible effect, which was calculated as  $[(T_1-T_0)/(T_2-T_0)] \times 100$ , where  $T_0$ and  $T_1$  were the tail-flick latencies before and after the injection of (+)-matrine or (+)-allomatrine and  $T_2$  was the cut-off time (set at 10 s) to avoid injury to the tail. In the warm-plate test, the antinociceptive response was evaluated by recording the latency to paw licking or tapping in the warm-plate test, where the metal plate was thermostatically controlled at  $51\pm0.5$  °C. To prevent tissue damage, we established a 30 s cut-off time. Each animal served as its own control, and the latency to responses was measured both before and after drug administration. Antinociception was calculated as percentage of the maximum possible effect (% Antinociception) according to the following formula; % Antinociception=(test  $latency-pre-drug latency)/(cut-off time-pre-drug latency) \times$ 100. The pre-drug latency, measured at 30 min intervals, was the mean of two values for each animal.

Guanosine-5'-*O*-(3-[<sup>35</sup>S]thio)triphosphate  $([^{35}S]GTP\gamma S)$ binding assay was performed according to the following method. For the membrane preparation, the cerebellum was removed from guinea pig, and rapidly transferred to a tube filled with an ice-cold buffer. The membrane homogenate  $(3-8 \mu g \text{ protein/assay})$  was prepared as described<sup>8)</sup> and incubated at 25 °C for 2 h in 1 ml of assay buffer with various concentrations of (+)-matrine or (+)-allomatrine (0.1-10) $\mu$ M), 30  $\mu$ M guanosine-5'-diphosphate (GDP) and 50 pM guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate ([<sup>35</sup>S]GTP $\gamma$ S: specific activity, 1000 Ci/mmol; Japan Radioisotope Association, Tokyo, Japan). The reaction was terminated by the filtration using Whatman GF/B glass filters. The filters were washed three times and then transferred to scintillationcounting vials containing tissue solubilizer (Soluene-350; Packard Instrument Co., Meriden, CT) and scintillation cocktail (Hionic Fluor; Packard Instrument Co.). The radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of  $10 \,\mu\text{M}$  unlabeled GTP $\gamma$ S.

The drugs used in the present study were acetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and nor-BNI (Tocris Cookson, Ballwin, MO, U.S.A.). Nor-BNI (3 mg/kg) was administered s.c. 4 h before test compound injection. All drugs were dissolved in saline. Antiserum against dynorphin A (1-17) was diluted 1 : 100 in 0.9% sterile saline.

The data are expressed as the mean with S.E.M. The statistical significance of differences between the groups was assessed two-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn multiple comparison test.

## **RESULTS AND DISCUSSION**

At first, we evaluated the antinociceptive effects induced by i.t. and i.c.v. administration of (+)-matrine or (+)-allomatrine, respectively. In either the mouse tail-flick or warmplate test, i.c.v.-administered these alkaloids (100 nmol/ mouse) markedly produced antinociceptive effects, reaching maximal antinociceptive responses at 10 min after the injection (Figs. 2A, B). On the contrary, i.t. administration of either (+)-matrine (100 nmol/mouse) or (+)-allomatrine (100 nmol/mouse) failed to induced antinociception in the tailflick and warm-plate test (Figs. 3A, B). In the present study, the saline when given either i.c.v. or i.t. did not produce any changes in the latency to each responses in the tail-flick and warm-plate test (data not shown). We previously demonstrated that both s.c. (+)-matrine- or (+)-allomatrine-induced antinociceptive effects were markedly suppressed by s.c.-pretreatment with a KOR antagonist nor-BNI.<sup>6,7)</sup> Taken together, these findings indicate the possibility that supraspinal KOR is critical for antinociceptive effects induced by (+)-matrine and (+)-allomatrine. To further clarify the role of supraspinal KOR in (+)-matrine- and (+)-allomatrine-induced antinociception, we examined the ability of these alkaloids to activate G-proteins through the stimulation of KOR in membranes of the guinea pig cerebellum, which is known to be one of brain regions that contain high density of KORs (Fig. 4). The activation of G-proteins induced by (+)matrine and (+)-allomatrine in this area was examined by monitoring the binding to membranes of  $[^{35}S]GTP\gamma S$ . Conventional KOR agonists, U-50,488H and ICI-199,441, produced marked increase in [<sup>35</sup>S]GTP $\gamma$ S binding to membranes of the cerebellum region. Contrary to our expectation, neither (+)-matrine or (+)-allomatrine increased [ $^{35}$ S]GTP $\gamma$ S bindings in this area (Fig. 4), indicating that either (+)-matrineor (+)-allomatrine-induced supraspinal antinociception is not due to a direct stimulation of KORs by these alkaloids.

It was reported that the increased release of an endogenous  $\kappa$ -opioid peptide dynorphin A (1-17) at supraspinal site is, only in part, implicated in the expression of supraspinal antinociceptive effect induced by an endogenous  $\mu$ -opioid receptor (MOR) agonist endomorphin-2.<sup>9)</sup> Furthermore, a growing body of evidence suggests that the descending dynorphinergic system projecting to the spinal cord is found to be involved in antinociception induced by opioid receptor agonists.<sup>10)</sup> We have demonstrated that i.t. pretreatment with an antiserum against dynorphin A (1-17), which binds released dynorphin A (1-17) and inhibit its binding to KOR, significantly attenuates the antinociception induced by i.c.v.

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Fig. 2. Time Course Changes in the Antinociceptive Effects Induced by i.c.v. Administration of (+)-Matrine (Closed Circle, 100 nmol/Mouse) and (+)-Allomatrine (Closed Triangle, 100 nmol/Mouse) in the Mouse Tail-Flick (A) and Warm-Plate Assay (B)

The (+)-matrine- or (+)-allomatrine-induced antinociceptive effect was measured at 10, 20 and 60 min after the injection. Each point represents the mean with S.E.M. for 9-10 mice in each group.



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Fig. 4. Effect of Each KOR Ligands on the Binding of [35S]GTP γS to Membranes of the Guinea Pig Cerebellum

Membranes were incubated with  $50 \text{ pm} [^{35}\text{S}]\text{GTP}\gamma\text{S}$  and  $30 \,\mu\text{M}$  GDP with or without different concentrations (0.1–10  $\mu\text{M}$ ) of U-50,488H, ICI-199,411, (+)-matrine and (+)-allomatrine for 2 h at 25 °C, respectively. The data are expressed as a percentage of basal [ $^{35}\text{S}$ ]GTP $\gamma$ S binding measured in the presence of GDP and absence of agonist. The data represent the mean with S.E.M., at least, of three independent experiments





Fig. 5. (A) Effects of i.e.v. Pretreatment with an Antiserum against Dynorphin A (1-17) (Anti-Dyn) on the (+)-Matrine (100 mg/kg, s.c.)- and (+)-Allomatrine (180 mg/kg, s.c.)-Induced Antinociception in the Mouse Tail-Flick Test

The antinociceptive effects induced by s.c. (+)-matrine and (+)-allomatrine were measured at 15, 30, 45, 60 and 90 min after the injection. An i.c.v. pretreatment with Anti-Dyn was performed 1h before (+)-matrine or (+)-allomatrine injection. Each point represents the mean with S.E.M. of 6 mice. (B) Effects of i.t. pretreatment with an antiserum against dynorphin A (1-17) (Anti-Dyn) on the (+)-matrine (100 mg/kg, s.c.)-and (+)-allomatrine (180 mg/kg, s.c.)-induced antinociception in the mouse tail-flick test. The antinociceptive effects induced by s.c. (+)-matrine and (+)-allomatrine were measured at 30 and 60 min after the injection. An i.t. pretreatment with Anti-Dyn was performed 1h before (+)-matrine or (+)-allomatrine injection. Each point represents the mean with S.E.M. of 6—8 mice. The statistical significance of differences was assessed with two-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn multiple comparison test. Saline+(+)-matrine group vs. Anti-Dyn+(+)-matrine group, F(1, 12)=6.286,  $p {<} 0.05$ .

administration of endomorphin-2, indicating that the release of dynorphin A (1-17) acting on KOR in the spinal cord is, at least in part, related to MOR-mediating pain-control system. In addition to MOR-mediated antinociception, it is reported that activation of supraspinal KOR by U-50,488H causes the excitation of descending dynorphinergic neuron projecting to the spinal cord, subsequently induces the release of dynorphins acting on KORs in the spinal cord for producing antinociception.<sup>11)</sup> Thus, the next study was to investigate the involvement of dynorphin A (1-17) in (+)-matrine- or (+)-allomatrine-induced antinociception. In the mouse tail-flick test, i.c.v. pretreatment with an antiserum against dynorphin A (1-17) failed to affect the antinociceptive effect induced by s.c. treatment of either (+)-matrine (100 mg/kg) or (+)-allomatrine (180 mg/kg) (Fig. 5A). It is of interest to note that s.c.-administered (+)-matrine- and (+)-allomatrine-induced antinociceptive effect was significantly attenuated by i.t. pre-treatment of an antiserum against dynorphin A (1-17) in the tail-flick test (Fig. 5B). Considering these findings, it is likely that either (+)-matrine- or (+)-allomatrine, when given i.c.v., produces the antinociceptive effect through the descending dynorphinergic pain control pathway associated with the stimulation of spinal KORs.

In conclusion, we demonstrated here that s.c.-administered (+)-matrine and (+)-allomatrine-induced antinociceptive effect was significantly attenuated by i.t. pretreatment with an antiserum against dynorphin A. We, therefore, propose here that the descending inhibitory dynorphinergic neuron projecting to the spinal cord is, at least in part, implicated in the expression of either (+)-matrine- or (+)-allomatrine-induced antinociception.

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