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Dual Action Mechanisms of KK-3, a Newly Synthesized Leu-Enkephalin Derivative, in the Production of Spinal Analgesic Effects

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Abstract—The action mechanism for the production of spinal analgesia of KK-3, tyrosyl-N-methyl-γ-aminobutylyl-phenylalaninol, was examined by the tail pinch and tail flick methods. Intrathecal KK-3, 2.5, 5 and 10 nmol/mouse, dose-dependently produced an analgesic effect in both methods. In the tail pinch method, the analgesia was suppressed by 2 mg/kg but not by 1 mg/kg of naloxone; however, the analgesic effect was significantly antagonized by 1 and 2 mg/kg Mr2266, a κ-antagonist. Meanwhile, both naloxone and Mr2266 failed to block the analgesic effect of KK-3 in the tail flick test. Intrathecal capsaicin, 0.3, 3 and 15 nmol/mouse, also produced a dose-dependent analgesic effect in the tail flick test, whereas no appreciable analgesia could be found in the tail pinch test. Neither naloxone nor Mr2266 blocked the analgesic effect of capsaicin. The results indicate that KK-3 may possess two separate pharmacological mechanisms for the production of analgesic effects on the spinal level: one is the depletion of substance P following its release from the spinal cord, and the other is the mediation through κ-opioid receptors.

Studies from our laboratory have shown that intracerebroventricular injection of KK-3, a leucine-enkephalin analogue, produced a weak analgesic effect which was partially antagonized by naloxone in the tail pinch method (1), and also that intrathecal injection of the compound induced behaviors consisting of scratching, biting, licking in mice, resulting from the release of substance P from the spinal cord (2). Yaksh et al. reported that capsaicin, which is known to deplete substance P, produced a long lasting analgesic effect (3) as a consequence of such a depletion since the peptide has been suggested to be a pain transmitter in primary sensory neurons (4–6). However, they could find an analgesic effect using the hot-plate method, the thermal stimulation, but not in a mechanical stimulation such as the tail pinch method. In line with this, Kuraishi et al. (7) reported that there were differences in the transmission of pain signals via the endogenous peptides substance P and somatostatin, depending on the type of noxious stimulation. Furthermore, Tyers (8) differentiated the opiate receptors that participated in the production of the analgesic effect by the type of pain applied for evaluation. These literatures, taken together, suggest that quite different mechanisms, i.e., the depletion of pain transmitters and opioid receptors blocking, exist for the production of the analgesic effect, and it may be possible to clarify such mechanisms by the use of the corresponding method for the estimation.

In this context, we examined how the release of substance P by intrathecal KK-3 was involved in the production of the analgesic effect, and also the participation of opioid receptors in the effect, using the two different methods for the measurement of the analgesic effect.

Materials and Methods

Animals: Male mice of the dd strain weighing 18–20 g (Otsubo Exp. Animals, Nagasaki)

were housed as a group of 10 animals in a plastic cage. They were kept in a temperature controlled room at 22±1 °C and given normal laboratory diet and tap water ad libitum. After reaching 23–25 g they were employed for the experiments.

Compounds and administration schedules: KK-3 (tyrosyl-N-methyl-γ-aminobutylyl-phenylalaninol, synthesized and supplied from Prof. K. Kawasaki of Kobe Gakuin University), Mr2266 ((−)-2-(3-furylmethyl)-norethazocine, a gift from Boehringer Ingelheim), morphine (Takeda Pharm. Co.), naloxone (Sigma Pharm. Co.) and capsaicin (Nacalai Tesque) were used. Mr2266 was dissolved in 0.1 N HCl followed by adjusting the pH to 4–5 with an appropriate amount of NaOH solution, and capsaicin was dissolved in 5% Tween 80 and 5% ethanol. All other drugs were freshly prepared with saline. They were administered in a volume of 0.1 ml/10 g of body weight. Naloxone or Mr2266, 1 and 2 mg/kg, was injected 10 min before administration of intrathecal KK-3 at 10 nmol/mouse.

Measurement of antinociception: Analgesic effect was measured by the modified Haffner's method (9) or tail pinch (TP) test, using a 6-sec cut-off time to avoid tissue damage due to longer application, every 10 min for 60 min, and by the modified D'Amour and Smith method (10) or tail flick (TF) assessment using a tail flick analgesia meter (Muromachi, MK-330), as maximal response time to 10 sec, every 10 min for 60 min and at 90 min. The analgesic effect expressed as the area under the curve (AUC) was obtained by plotting the increase in response time (sec) on the ordinate and the time intervals on the abscissa.

Statistical analysis: The statistical significance of the differences was first determined by analysis of variance, followed by Dunnett's test for individual comparisons.

Injection procedure: Intrathecal (i.t.) injections were carried out according to the method described by Hylden and Wilcox (11). Briefly, a lumber puncture was performed using a 30 gauge needle directly connected to a microsyringe. The needle was inserted between L5 and L6, so that the dose was contained in a volume of 10 μl/mouse.

Results

Intrathecal KK-3, 2.5, 5 and 10 nmol/mouse, produced an analgesic effect in a dose-dependent manner, with a peak at 10 min and lasted about 60 min and 90 min in the TP and TF methods, respectively (Figs. 1 and 2).

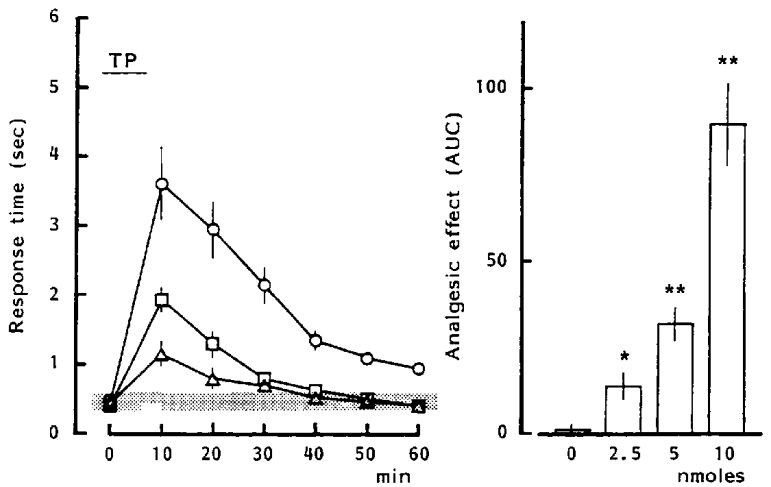

Fig. 1. Analgesic effect of intrathecal KK-3 in the tail pinch method. Left: Mice were treated with i.t. KK-3, 2.5 (△), 5 (□) and 10 (○) nmol/mouse, and the analgesic effect was measured by the tail pinch method, every 10 min for 60 min after the injection. Dotted area indicates the mean±S.E. response time in the saline control. Right: analgesic effect in the left panel transformed into the area under the curve (AUC). Each point indicates the mean±S.E. of 10 animals. *$P<0.05$, **$P<0.01$, compared with the saline control.

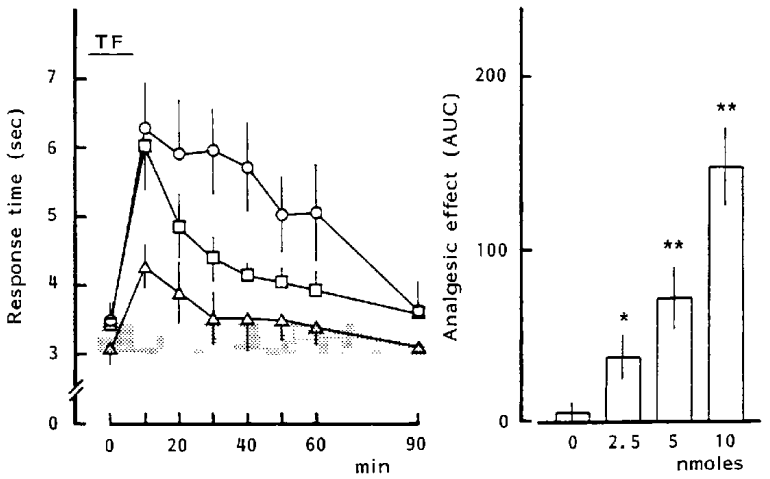

Fig. 2. Analgesic effect of intrathecal KK-3 in the tail flick method. Left: Mice were treated with i.t. KK-3, 2.5 (△), 5 (□) and 10 (○) nmol/mouse, and the analgesic effect was measured by the tail flick method every 10 min for 60 min and at 90 min after the injection. Dotted area indicates the mean±S.E. response time in the saline control. Right: The data are shown in the same way as in the right panel of Fig. 1. Each point indicates the mean±S.E. of 10 animals. *P<0.05, **P<0.01, compared with the saline control.

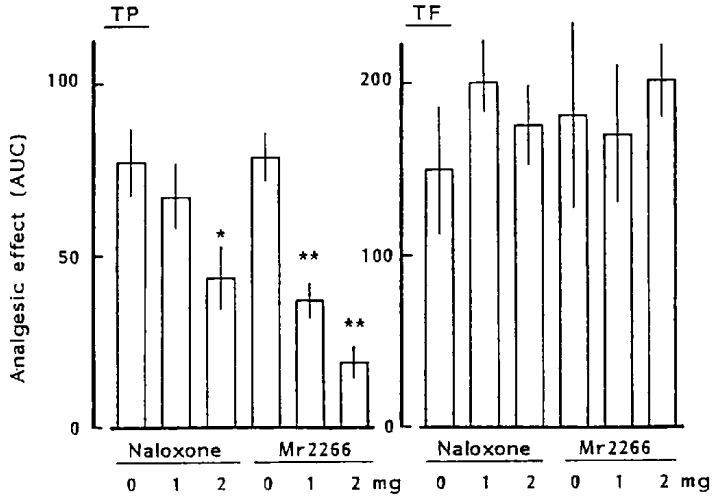

Fig. 3. Effect of naloxone and Mr2266 on intrathecal KK-3 induced analgesia. Mice were pretreated with naloxone and Mr2266, i.p., 10 min before 10 nmol/mouse of i.t. KK-3. The analgesic effect was measured by the tail pinch (TP) and tail flick (TF) methods and was expressed as the area under the curve (AUC). Figures are the dose of naloxone and Mr2266 (mg/kg) and 0, saline or vehicle alone. Each value indicates the mean±S.E. of 16 mice. *P<0.05, **P<0.01, compared with saline or vehicle control. For other details, refer to the footnote of Fig. 1.

In the TP method, KK-3 induced analgesia was not affected by 1 mg/kg naloxone, but was suppressed by 2 mg/kg of the antagonist; however, the analgesic effect was dose-dependently and significantly antagonized by the 10 min pretreatment with 1 mg/kg and 2 mg/kg Mr2266, a putative κ-antagonist (12). The suppressive effect of Mr2266 was greater

than that of naloxone (Fig. 3, left). On the other hand, both naloxone and Mr2266, 1 mg/kg and 2 mg/kg, failed to block the analgesic effect of KK-3 in the TF test (Fig. 3, right).

Capsaicin, 0.3, 3 and 15 nmol/mouse, treated mice showed a significant and dose-dependent analgesic effect in the TF test for 4 days after a single i.t. injection of the drug, whereas no appreciable analgesia could be found in the TP method at the higher dose, 15 nmol/mouse, of capsaicin (Fig. 4). Neither naloxone nor Mr2266, given 1 hr or 48 hr after 0.3 nmol/mouse of i.t. capsaicin treatment, blocked the analgesic effect of capsaicin

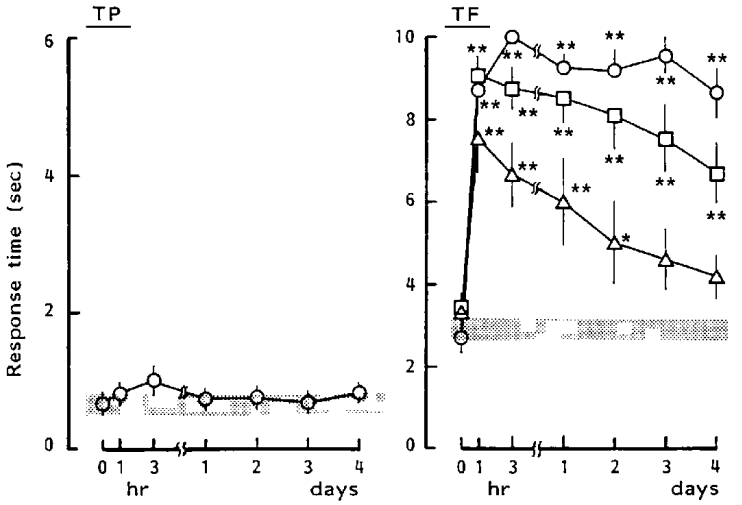

Fig. 4. Analgesic effect of intrathecal capsaicin in the tail pinch and tail flick methods. Mice were treated with i.t. capsaicin, 0.3 (△), 3 (□) and 15 (○) nmol/mouse, and the analgesic effect was measured by the tail pinch (TP) or the tail flick (TF) method, 1, 3 hr and every 24 hr after capsaicin injection for 4 days. The dotted area indicates the mean±S.E. response time in the saline control. Each point indicates the mean±S.E. of 10 animals. *P<0.05, **P<0.01, compared with the vehicle control.

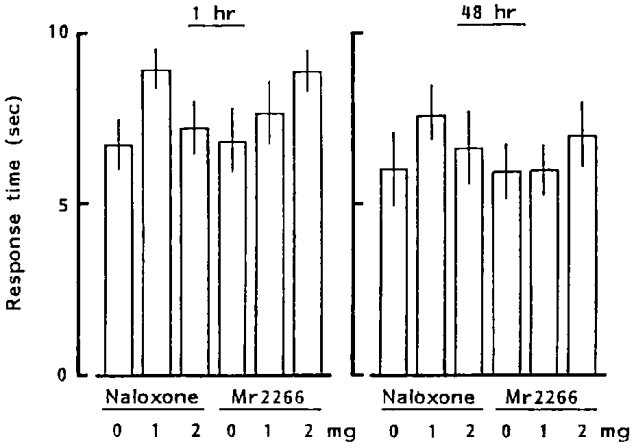

Fig. 5. Lack of effect of naloxone and Mr2266 on capsaicin induced analgesia in the tail flick method. Naloxone or Mr2266 was given i.p., 1 hr or 48 hr after i.t. capsaicin, 0.3 nmol/mouse, and the analgesic effect was measured by the tail flick method 30 min after the antagonist injection. The data are shown as the response time (sec). Figures are the dose of naloxone and Mr2266 (mg/kg) and 0, saline or vehicle alone. Each value indicates the mean±S.E. of 16 animals.

(Fig. 5).

Discussion

KK-3, given intrathecally, produced analgesic effects dose-dependently in both the TF and TP test, suggesting the compound acts on spinal cord for the effect.

For the explanation of spinal analgesic mechanism, there is considerable evidence that substance P participates in synaptic pain transmission in primary sensory neurons of the spinal cord, and therefore depletion of substance P or a competitive inhibition of substance P receptors by substance P antagonists produces analgesic effects (4–6). Alternatively, blocking the pain transmission by direct action of an opioid agonist on the spinal opioid receptors, including the activation of the descending pain-inhibitory system in the spinal cord, has been reported. Clinically, morphine produces a significant analgesic effect when administered epidurally, suggesting the production of analgesia at the spinal level.

In our previous paper, we reported i.t. injection of KK-3 released substance P from spinal cord in mice (2). This may suggest the possibility that an analgesic effect of i.t. KK-3 results from the depletion following the release of substance P from the spinal cord. Yaksh et al. reported that i.t. capsaicin produced a long-lasting analgesic effect in rats using the hot-plate and TF methods, but not noxious mechanical stimuli, suggesting the depletion of substance P from the spinal cord (3), although a conflicting result that a noxious stimulus by TP but not by TF released substance P from the spinal dorsal horn in rabbits has been reported (7). Likewise, we found the analgesia of i.t. capsaicin by the method in mice and that the effect was not antagonized by naloxone or Mr2266. Meanwhile, KK-3 exhibits an analgesic effect which is not antagonized by naloxone or Mr-2266 when the TF test is applied; and furthermore, the analgesic effect of i.t. KK-3 is likewise recognized by the TP test. Interestingly, the analgesic effect of KK-3 evaluated by the TP method is blocked by Mr2266 and, to a lesser extent, by naloxone. Since KK-3 is derived from leu-enkephalin, and it possesses opioid properties from the in vitro experiments (1), the production of the analgesic effect of KK-3 may be partly mediated through an opioid receptor, especially through the κ-opioid receptors, in the spinal cord. The results obtained in the literature indicate that KK-3 may possess two separate mechanisms for producing analgesic effects: one is the depletion of substance P following its release from the spinal cord, and the other is the mediation through opioid receptors, especially κ-receptors, in the spinal cord. It may be possible that the analgesic effect of KK-3 determined by the TF method may be mediated by opioid receptors; however, both naloxone and Mr2266 fail to suppress the effect. There is also no antagonism of such antagonists to capsaicin analgesia by the TF method. Moreover, Tyers demonstrated that the TF method was comparably specific for the evaluation of opioid μ-agonists and was less effective for detecting the analgesia of κ-agonists (8). This may exclude the possibility that the analgesic effect of KK-3 produced by the mediation of κ-receptors is detectable in the TF method.

Thus, KK-3 appears to possess dual pharmacological actions for the production of an analgesic effect at the spinal level, both mediated through opioid κ-receptors and due to the depletion as a consequence of the release of substance P.

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