N-methyl-D-aspartate (NMDA) and Non-NMDA Receptors are Involved in the Production and Maintenance of Nociceptive Responses by Intraplantar Injection of Bee Venom and Melittin in the Rat

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Whole bee venom (WBV) and its major component, melittin, have been reported to induce long-lasting spontaneous flinchings and hyperalgesia. The current study was designed to elucidate the peripheral and spinal mechanisms of N-methyl-D-aspartate (NMDA) and non-NMDA receptors by which intraplantar (i.pl.) injection of WBV and melittin induced nociceptive responses. Changes in mechanical threshold and flinching behaviors were measured after the injection of WBV (0.04 mg or 0.1 mg/paw) and melittin (0.02 mg or 0.05 mg/paw) into the mid-plantar area of a rat hindpaw. MK-801 and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium) were administered intrathecally (i.t. $10 \mu g$) or i.pl.($15 \mu g$) 15 min before or i.t. 60 min after i.pl. WBV and melittin injection. Intrathecal pre- and post-administration of MK-801 and CNQX significantly attenuated the ability of high dose WBV and melittin to reduce paw withdrawal threshold (PWT). In the rat injected with low dose, but not high dose, of WBV and melittin, i.pl. injection of MK-801 effectively suppressed the decrease of PWTs only at the later time-points, but the inhibitory effect of CNQX (i.pl.) was significant at all time-point after the injection of low dose melittin. High dose WBV- and melittin-induced spontaneous flinchings were significantly suppressed by i.t. administration of MK-801 and CNQX, and low dose WBV- and melittin-induced flinchings were significantly reduced only by intraplantarly administered CNQX, but not by MK-801. These experimental flinchings suggest that spinal, and partial peripheral mechanisms of NMDA and non-NMDA receptors are involved in the development and maintenance of WBV- and melittin-induced nociceptive responses.

Key Words: Whole bee venom, Melittin, Paw withdrawal threshold, Spontaneous flinching, NMDA and non-NMDA receptors

INTRODUCTION

Since Curtis et al (1959) first reported that an iontophoretic application of L-glutamate and L-aspartate produced a strong excitation of spinal neurons, evidence has accumulated that excitatory amino acids (EAAs) are candidate neurotransmitters responsible for nociceptive transmission in the spinal cord and peripheral sites. High density of EAA receptor binding sites is located in the superficial laminae of the dorsal horn where most nociceptive primary afferent fibers terminate (De Biasi & Rustioni, 1988; Merighi et al, 1991). Small myelinated and unmyelinated fibers also have glutamate immunoreactivity which increases further in inflammatory state (Westlund et al, 1992; Carlton & Coggeshall, 1999). Inflammation and nociceptive stimulation of primary afferent fibers produce a significant increase in the release of gultamate and aspartate

in the dorsal spinal cord and peripheral site (Jeftinija et al, 1991; Paleckova et al, 1992; Sluka & Westlund, 1993; Omote et al, 1998; deGroot et al, 2000). The majority of neurons activated by iontophoretic administration of EAA are located mainly in laminae I and II of spinal cord and receive C-fiber inputs from the peripheral receptive field (Schneider & Perl, 1985, 1988).

In the behavioral test in rats, intrathecal (i.t.) or intraplantar (i.pl.) administration of NMDA and non-NMDA agonists induces thermal and mechanical hyperalgesia which is alleviated by i.t. or i.pl. injection of NMDA and non-NMDA receptor antagonists, respectively (Raigorodsky & Urca, 1987; Zhou et al, 1996). NMDA and non-NMDA receptor agonists are also reported to be implicated in the development and maintenance of hyperalgesia and allodynia induced by nerve injury and inflammation with

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ABBREVIATIONS: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; EAA, excitatory amino acid; i.pl., intraplantar; i.t., intrathecal; NMDA, N-methyl-D-aspartate; PLA_2, phospholipase A_2; PWT, paw withdrawal threshold; WBV, whole bee venom; WDR, wide dynamic range.

different causes (Mao et al, 1992; Ren et al, 1992; Sluka et al, 1994; Kim et al, 1997).

Bee venom (BV) has dual effects; antinociceptive and nociceptive actions. In animal and clinical studies, BV injection into acupoint or painful sites reduced inflammatory somatic and visceral pain, and suppressed c-Fos protein expression in the spinal cord (Kwon et al, 2001a; 2001b; Lee et al, 2001). On the other hand, Lariviere and Melzack (1996) introduced the bee venom test in which i.pl. injection of bee venom (BV) into the rat hindpaw induced dose-dependent tonic pain with local inflammation and edema. In the subsequent behavioral and electrophysiological studies, BV-induced pain has been known to have characteristics of spontaneous flinching behaviors, thermal and mechanical hyperalgesia, allodynia, contralateral heat hyperalgesia and c-Fos expression in the spinal dorsal horn (Chen et al, 1998; Luo et al, 1998; Chen et al, 1999a, b, 2000; You et al, 2002). The time-courses of c-Fos expression and increased discharges of wide dynamic range (WDR) neurons parallel those of hyperalgesia and spontaneous flinchings, respectively (Chen et al, 1999b; You & Chen, 1999). WDR neurons without afferent inputs from C fiber are not activated by subcutaneous (s.c.) BV injection into the receptive field (Chen et al, 1998), and conduction block of afferent fibers by pretreatment with capsaicin prevents the development of hyperalgesia and spontaneous flinchings by s.c injection of BV and melittin (Chen & Chen, 2000; Shin & Kim, 2004). In the comparative studies on bee venom test and formalin test, bee venom test has been reported to be a more useful model in the study of pain than the formalin test (Chen et al, 1999b; You et al, 2002). Recent studies reported that melittin, a major component of BV, also induced long-lasting and dose-dependent spontaneous flinchings, hyperalgesia, allodynia and edema, and there was no substantial difference in the time-course and severeness of nociceptive responses induced by melittin and BV (Shin et al, 2004; Li & Chen, 2004). Shin & Kim (2004) reported that, in a behavioral and electrophysiological study, melittin-induced reduction of mechanical threshold and flinchings was caused by selective activation of capsaicin-sensitive primary afferent fibers in rat. All these findings suggest that most of BV-induced nociceptive responses are mediated through the melittin-induced selective activation of primary afferent C fibers.

Although it is clear that BV-induced nociceptive responses are mediated through selective activation of primary afferent C fibers by melittin (Shin & Kim, 2004), the mechanism by which BV and melittin cause rapid activation of C fibers has not clearly been understood. Inflammation induced by proinflammatory substances of bee venom may contribute to activation and/or sensitization of nociceptive afferent fibers, resulting in sustained hyperalgesia (Habermehl, 1981; Calixto et al, 2003). In the recent study from our laboratory, i.t. or i.pl. administration of L- and N-type, but not P-type, Ca^{2+} channel antagonists and intracellular Ca^{2+} antagonists strongly suppressed melittin-induced flinchings and mechanical allodynia (Lee et al, 2004). Spinal protein kinase C, P_{2X}-purinoceptor and descending facilitatory pathway from the rostral medial medulla have been known to contribute to BV-induced spontaneous nociception (Zheng & Chen, 2000; Li et al, 2000; Chen et al, 2003). You et al (2002) reported that peripheral NMDA receptor, but not non-NMDA receptors, plays a pivotal role in the development and maintenance of BV-induced increase in discharge of WDR neurons. However, there is a report that

non-NMDA receptors are implicated only in the induction of persistent firing of the dorsal horn WDR neurons by s.c BV injection (Chen et al, 1999a). To our knowledge, it is not known whether NMDA and non-NMDA receptors are involved in melittin-induced nociceptive responses. Current experiment was undertaken to investigate the spinal and peripheral mechanisms of NMDA and non-NMDA receptors in the production and maintenance of melittin- as well as BV- induced spontaneous flinchings and mechanical allodynia in rat.

METHODS

Male Sprague-Dawley rats (250~300 g) were used in this experiment. The Animal Care and Use Committee at Hanyang University approved all experimental protocols, and algesiometric assays were conducted under the ethical guidelines set forth by the International Association for the Study of Pain.

All rats were placed on an elevated metal mesh floor and allowed to acclimate for at least 30 min before behavioral testing. Von Frey filament was applied vertically to the mid-plantar surface of the right hindpaw in an ascending intensity order from underneath the floor. A bending force being able to evoke a brisk paw withdrawal in more than 50% of 6 trials was expressed as the paw withdrawal mechanical threshold (PWT, g). 26 g of bending force of von Frey filament was selected as the upper limit for testing, since stiffer filaments with bending force of more than 10% of body weight tends to passively raise the entire limb rather than to cause an active brisk withdrawal (Chaplan et al, 1994). Rats that sharply withdrew their paws, when von Frey filament with weak bending force below 26 g was applied, were not used in the experiment. A mirror was placed below the metal mesh floor at a 30° angle to allow an unobstructed counting of flinching. Changes in PWT at a given time-point and total number of flinchings for the initial 30 min were measured after the injection of whole bee venom (WBV, 0.04 or 0.1 mg/paw) and melittin (0.02 mg or 0.05 mg/paw) into the mid-plantar area of the right hindpaw. Only changes in PWT and flinching behaviors induced by low doses of WBV (0.04 mg/paw) and melittin (0.02 mg/paw) were used in the experiment in which peripheral action of NMDA and non-NMDA receptor antagonists was studied. We measured the total number of flinchings for the first 30 min, because more than 95% of flinchings were observed within the first 30 min after i.pl. injection of WBV or melittin. Because approximately 50% of dry bee venom is melittin, the dosage of melittin was determined to be one half the WBV dosage. To observe the effects of EAA receptor antagonists on the WBV- or melittin-induced nociceptive responses, NMDA (MK-801, Sigma) and non-NMDA (6-cyano-7-nitroquinoxaline-2,3-dione disodium, CNQX, Tocris) receptor antagonists were administered intrathecally or intraplantarly 15 min before or intrathecally 60 min after WBV and melittin injection. MK-801 and CNQX were administered intrathecally at the dose of 10 μg and intraplantarly at the dose of 15 μg. Since doses of NMDA and non-NMDA receptor antagonists higher than those used in this experiment could not be administered due to side effects, we investigated the effect of EAA receptor antagonists on the low dose WBV- and melittin-induced nociceptive responses. For i.t. administration of MK-801 and CNQX, chronic i.t. catheters were

inserted under the enflurane anesthesia by passing a PE-10 tubing through an incision in the atlanto-occipital membrane to a position 8.5 cm caudal to the cisterna at a level of the lumbar enlargement. Rats were allowed to recover for at least 5 days before being used in the experiment. All rats showing motor defects were not used in the experiment. All drugs were dissolved in 10 μl of saline. In the preliminary experiments, i.t. or i.pl. injection of 10 μl saline and intraperitoneal administration of MK-801 and CNQX (10 μg & 15 μg) did not induce any changes in PWT and spontaneous flinchings. Each rat was tested for a single antagonist.

The data are expressed as mean±SE and analyzed using ANOVA, followed by the Newman-Keuls test. P values less than 0.05 were considered significant. When experiments were completed, the rats were euthanized by an overdose of pentobarbital sodium.

RESULTS

Intraplantar administration of WBV (0.1mg/paw, n=13) produced rapid and strong reduction of PWT (Fig. 1) and an increase of flinching behaviors (Fig. 7). PWT was 3.1± 0.4 g at 10 min after WBV injection and thereafter, the reduced PWT very slowly increased to 5.7±0.5 g and 9.8± 1.5 g at 60 min and 180 min after WBV injection, respectively. The decreased PWT was recovered almost completely to normal level 24h after WBV injection. Intrathecal pre-administration of NMDA receptor antagonist, MK-801 (10 μg, n=10) significantly suppressed the ability of WBV to reduce PWT at all timepoints, except 24h after WBV injection (Fig. 1, p<0.01 or 0.001). PWTs of the rat pre-administered with MK-801 were significantly high, compared to corresponding PWTs of the rat injected with i.pl. WBV only. MK-801 had stronger effects on the PWTs at all time-points 30 min after i.pl. WBV injection than on the PWTs for the first 20 min. Intrathecal post-injection of MK-801 (n=8) 60 min after i.pl. WBV injection also
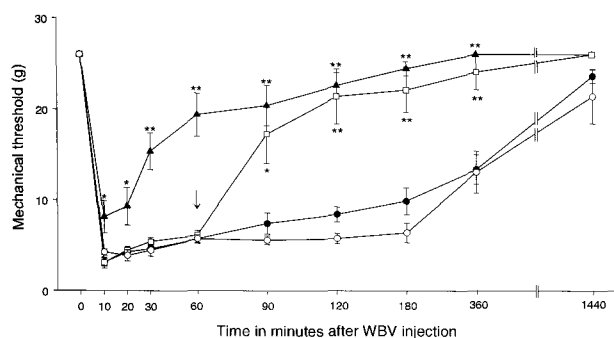
significantly attenuated WBV-induced reduction of PWT (Fig. 1). PWTs of the rats post-injected with MK-801 rapidly increased to 17.2±3.2 g (p<0.01) 30 min after i.t. post-treatment of MK-801, compared to 6.1±3.2g before MK-801 treatment. However, i.pl. administration of MK-801 (15 μg, n=8) 15 min before WBV injection did not have any significant effects on WBV-induced reduction of PWTs (Fig. 1). There were no substantial differences in PWTs of the rats injected with WBV only or with WBV and i.pl. MK-801, at all time-points after WBV injection.

In Fig. 2, the data on the changes of PWTs induced by i.pl. WBV injection were same as those used in Fig. 1. After i.t. pre-administration of the non-NMDA receptor antagonist, CNQX (10 μg, n=9), 15 min before i.pl. WBV injection, the ability of WBV to reduce PWT was very significantly attenuated at all time-points except 24h after i.pl. WBV injection (Fig. 2, p<0.001). PWTs of the rats pre-administered with i.t. CNQX were almost completely recovered to normal level 120 min after i.pl. WBV injection.

The decrease of PWT of the rat that received i.t. post-administration of CNQX (n=8) 60 min after i.pl. WBV injection was significantly less than that of the rat injected with i.pl. WBV only (Fig. 2, p<0.001). PWTs of the rat post-treated with CNQX were 18.6±2.9 g and 24.3±1.7 g at 90 min and 180 min after i.pl. WBV injection, whereas PWTs of WBV-injected rats were 7.3±1.2 g and 9.8±1.5 g, respectively. On the other hand, there was no significant difference in the decrease of PWTs of the rat administered with i.pl. WBV and CNQX or i.pl. WBV only at all time-points after i.pl. WBV injection (Fig, 2, n=8).

Intraplantar administration of melittin (0.05 mg/paw, n=12) induced rapid and sustained decrease of PWT (Fig. 3) and an increase of flinching behaviors (Fig. 7). The time-course and severity of decrease of PWT induced by WBV (0.1 mg/paw) and melittin (0.05 mg/paw) were very similar. As in the case of WBV-induced reduction of PWT, PWT rapidly reduced to 2.9±0.4 g 10 min after i.pl. melittin injection, thereafter, increased slowly to 5.4±0.4 g and 9.0±1.4 g at 60 min and 180 min after i.pl. melittin

Fig. 1. Effects of NMDA receptor antagonist, MK-801, on the changes in mechanical threshold following intraplantar injection of whole bee venom (WBV, 0.1 mg/paw, ●). Intraplantar injection of MK-801 (15 μg) 15 min before WBV injection did not have any effect on WBV-lowered mechanical threshold (○). However, intrathecal pre- (▲) or post-treatment (□) of MK-801 (10 μg) strongly suppressed the ability of WBV to reduce a mechanical threshold. Arrow indicates the time when MK-801 was intrathecally administered in post-treatment experiment. *; p<0.01, **; p<0.001, significant differences from the WBV-induced decrease in mechanical threshold.
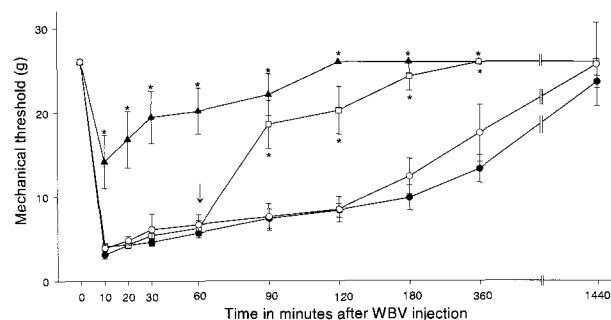

Fig. 2. Effects of the non-NMDA receptor antagonist, CNQX, on the changes in mechanical threshold following intraplantar injection of whole bee venom (WBV, 0.1 mg/paw, ●). WBV-induced decrease in mechanical threshold was not changed after intraplantar injection of CNQX (15 μg, ○) 15 min before WBV injection. However, the ability of WBV to reduce the mechanical threshold was greatly attenuated following intrathecal administration of CNQX (10 μg) 15 min before (▲) or 60 min after (□) WBV injection. Arrow indicates the time when CNQX was intrathecally administered in post-treatment experiment. *; p< 0.001, significant differences from the WBV-induced decrease in mechanical threshold.
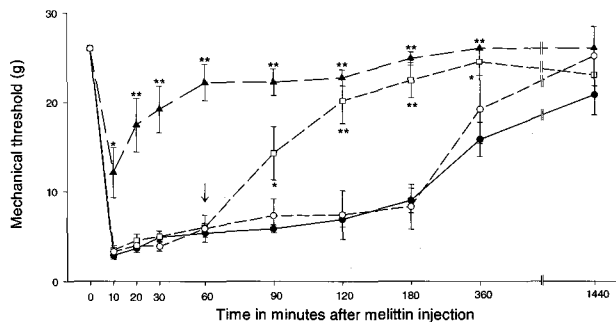
Fig. 3. Intraplantar injection of melittin (50 μg/paw) dramatically reduced mechanical threshold in the rat hind paw (●). The lowered mechanical threshold was not influenced after intraplantar administration of MK-801 (15 μg/paw) 15 min before injection of melittin (○). However, melittin-induced reduction of mechanical threshold was greatly attenuated following intrathecal pre- (▲) or post-treatment (□) of MK-801. Arrow indicates the time when MK-801 was intrathecally administered in post-treatment experiment *; $p < 0.01$, **; $p < 0.001$, significant differences from the melittin-induced decrease in the mechanical threshold.
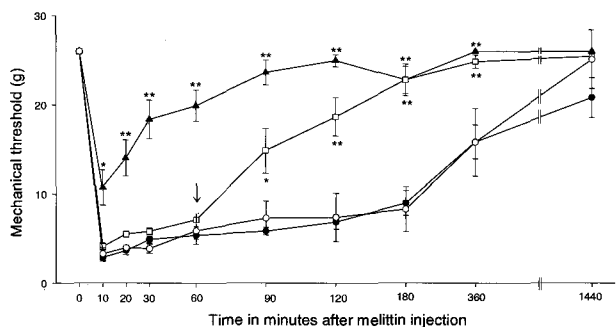

Fig. 4. Melittin-induced (50 μ g/paw) reduction of mechanical threshold (●) was significantly attenuated when non-NMDA receptor antagonist (CNQX, 10 μg) was intrathecally administered 15 min before (▲) or 60 min after (□) intraplantar injection of melittin. However, intraplantar injection of CNQX (15 μg/paw, ○) did not have any effect on the changes in mechanical threshold induced by melittin (50 μg/paw). Arrow indicates the time when CNQX was intrathecally administered in post-treatment experiment *; $p < 0.01$, **; $p < 0.001$, significant differences from the melittin-induced decrease in the mechanical threshold.

Fig. 5. Intraplantar administration of low dose WBV (0.04 mg/paw, ●) induced the decrease in the mechanical thresholds which were significantly attenuated by i.pl. pretreatment of MK-801(15 μg, ○) and CNQX (15 μg, ▲) without any inhibitory effect on the initial nociception within 30 min after WBV injection. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.005$, significant difference from the WBV-induced decrease in the mechanical theshold.

cantly different from each other.

Fig. 4 shows that the data on the changes of PWTs induced by i.pl. melittin injection were same as those used in Fig. 3. The i.t. administered non-NMDA receptor antagonist, CNQX (10 μg), strongly attenuated the ability of melittin to reduce PWT. In the rats i.t. pre-treated with CNQX (n=10), PWTs were significantly high, compared to PWTs of the rats i.pl. injected with melittin alone at all points except 24 h after melittin injection (Fig. 4, $p < 0.01$ or 0.001). The decreased PWT fully recovered to the control PWT (26 g) 6h after i.pl. melittin injection in the rat pretreated with CNQX. In the 9 rats post-treated with CNQX, PWT was 14.8±2.5 g at 90 min after i.pl. melittin injection thereafter, gradually increased to 24.9±0.7 g at 6h after i.pl. melittin injection (Fig. 4). In the rat injected with i.pl. melittin, i.pl. pre-administration of CNQX did not have any effect on the melittin- induced reduction of PWT.

Because i.pl. injection of more than 15 μg of antagonists, especially MK-801, caused side effect such as agitation, we could not increase the dose of antagonists above 15 μg. Instead of increasing the dose of antagonist, we investigated the effects of i.pl. injected MK-801 and CNQX (15 μg /paw) on the nociceptive responses induced by a low dose of WBV (0.04 mg/paw) and melittin (0.02 mg/paw). Intraplantar injection of WBV (0.04 mg) strongly reduced PWTs which were 1.9±0.4 g and 6.6±0.9 g at 10 min and 60 min after WBV injection, respectively (Fig. 5, n=12). The reduced PWT was recovered almost to the control level 24h after WBV injection. Intraplantar pre-injection of MK-801 (n=12) or CNQX (n=11) did not have any significant effects on the WBV-induced decreases of PWTs within 30 min after WBV injection. However, in the rats pretreated with MK-801 and CNQX, PWTs were significantly high compared to the rats injected with WBV only at all time-points since 120 min and 60 min after WBV injection, respectively (Fig. 5).

Low dose of melittin (0.02 mg/paw. i.pl.) also caused strong and sustained decreases in PWTs which were 3.1± 0.6 g and 4.9±0.4 g at 10 min and 60 min after melittin injection, respectively (Fig. 6, n=9). Intraplantar pre-injection of CNQX (15 μg/paw, n=13) significantly attenuated the ability of melittin to reduce PWT at all time-points except 24h after melittin injection. However, in the rats pretreated with MK-801 (n=9), PWTs were significantly

injection, respectively (Fig. 3).

The decreased PWT did not fully recover to the control PWT (26 g) 24h after i.pl. melittin injection. Melittin-induced decrease of PWT was greatly attenuated after i.t. pre-administration of NMDA receptor antagonist, MK-801 (10 μg, n=9), at all time- points except 24 h after i.pl. melittin injection (Fig. 3). PWT of the rat pre-treated with i.t. MK-801 was significantly high (12.1±2.9 g), compared to PWT of the control 10 min after i.pl. melittin injection, and was fully recovered 6h after melittin injection. In 9 rats which received i.t. administration of MK-801 (10 μg) 60 min after i.pl. melittin injection, the decreased PWT rapidly increased to 14.3±2.9 g and 22.4±2.0 g at 30 min and 120 min after i.t. post-treatment of MK-801. However, changes in PWTs of the rats i.pl. injected with melittin only or i.pl. administered with melittin and MK-801 were not signifi-
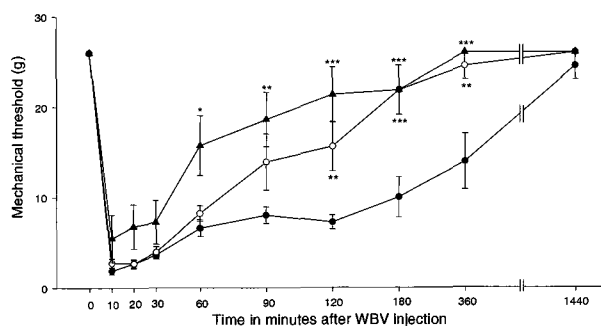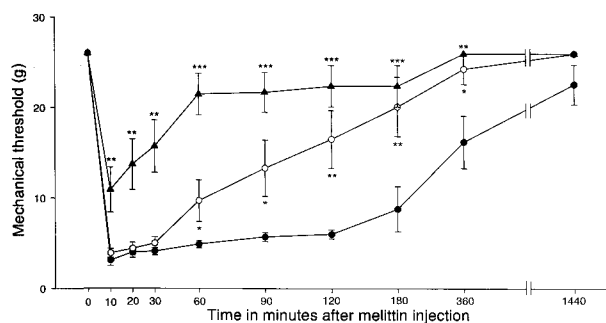
Fig. 6. Changes in the low dose melittin-induced (0.02 mg/paw, ●) mechanical thresholds following i.pl. pre-injection of NMDA receptor (MK-801,15 μg, ○) and non-NMDA receptor (CNQX, 15 μg, ▲) antagonists. Intraplantar injection of CNQX significantly attenuated the ability of melittin to reduce the mechanical threshold at all time-points. However, in the rat pre-treated with MK-801, the mechanical thesholds were significantly high compared to melittin-injected rats at the later part of observation period. *; $p < $ 0.05, **; $p < 0.01$, ***; $p < 0.001$, significant difference from the melittin-induced decrease in the mechanical theshold.

Fig. 8. Effects of intraplantarly administered NMDA and non-NMDA receptor antagonists (15 μg/paw) on the low dose whole bee venom (WBV, 0.04 mg/paw, A)- and melittin (MEL, 0.02 mg/paw, B)-induced flinching behaviors. CNQX, but not MK-801, significantly suppressed WBV- and melittin-induced flinchings. *; $p < $ 0.001, significant differences from the melittin- and WBV-induced flinchings.
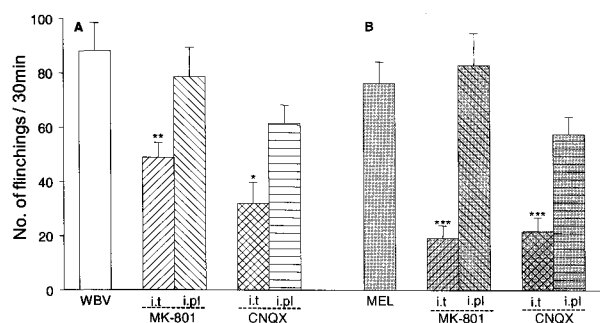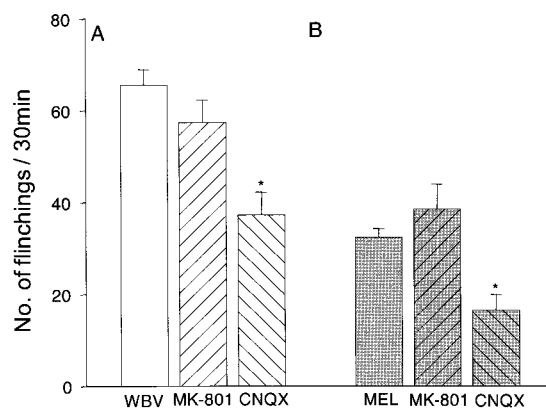

Fig. 7. Effects of NMDA and non-NMDA receptor antagonists on whole bee venom (WBV)- (A) and melittin (MEL)-induced (B) flinching behaviors. Intrathecal (i.t.) administration of MK-801 and CNQX significantly suppressed flinchings induced by WBV and melittin for the first 30 min. *; $p < 0.01$, **; $p < 0.005$, ***; $p < 0.001$, significant differences from the WBV- and melittin- induced flinchings.

high, compared to the rats injected with melittin only at all time-points since 60 min after WBV injection.

Flinching behaviors were not observed in the normal rat without i.pl. injection of WBV and melittin. However, i.pl. administration of high dose WBV (0.1 mg/paw, n=13) and melittin (0.05 mg /paw, n=12) greatly increased flinching behaviors (87.9 ± 10.4/30 min and 76.1 ± 8.0/30 min, respectively) which were very high immediately after administration of WBV and melittin and, thereafter, gradually decreased (Fig. 7). In the rats pre-treated with i.t. MK-801 (n=10) or CNQX (n=9), WBV-induced flinchings significantly decreased (Fig. 7A). After i.pl. pre-treatment of the rats with MK-801 (n=7) or CNQX (n=8), there was a tendency for i.pl. WBV-induced flinchings to decrease, however, these decreases were not significant. Intrathecal pre-treatment of MK-801 (n=9) and CNQX (n=9) strongly suppressed melittin-induced flinching behaviors (76.1 ± 8.0/30 min), which were decreased to 19 ± 4.7/30 min and

21.7 ± 5.1/30 min, respectively (Fig. 7B).

In the rats pre-treated with i.pl. MK-801 (n=7), i.pl. melittin injection caused a slight increase in flinchings (82.8 ± 11.9/30 min), compared to melittin-induced flinching without pretreatment of MK-801. Intraplantar pre-administration of CNQX (n=8) attenuated an increase in melittin-induced flinchings, however, this decrease did not reach the significant level.

Intraplantar injection of low doses of WBV (0.04 mg/paw, n=10) and melittin (0.02 mg/paw, n=27) caused flinching behaviors which were 65.6 ± 3.4/30 min and 32.3 ± 2.0/30 min, respectively (Fig. 8). After i.pl. pretreatment of rats with MK-801 (15g/paw), WBV- (n=12) and melittin-induced (n=8) flinchings were not significantly suppressed. However, WBV- (n=11) and melittin-induced (n=11) flinchings were reduced to almost one half of those induced by WBV or melittin, following i.pl. pretreatment of CNQX (15 μg/paw).

DISCUSSION

The current behavioral study shows that spinal NMDA and non-NMDA receptors positively contribute to the development and maintenance of mechanical allodynia and spontaneous flinchings by i.pl. injection of WBV and melittin into the rat hindpaw, and that in general, peripheral EAA receptors are involved in WBV- and melittin- induced nociceptions at the late part of whole time-course. In the present study, high dose WBV- (0,1 mg/paw) and melittin-induced (0.05 mg/paw) nociceptions were not affected by i.pl. injection of NMDA and non-NMDA receptor antagonist. It is suggested that the dose of peripherally administered MK-801 and CNQX (15 μg) might not be high enough to suppress the nociceptive responses induced by high doses of WBV and melittin. However, we could not administer highter dose of CNQX and especially MK-801, than 15 μg because of side effects. Since antinociceptive effects of drugs have inverse relationship to the intensity of nociceptive responses (Luttinger, 1985), we investigated the effects of i.pl. administered MK-801 and CNQX (15 μg)

on the nociceptive responses induced by low dose WBV (0.04 mg/paw) and melittin (0.02 mg/paw). Although i.pl. administration of CNQX attenuated low dose melittn-induced decrease of PWT and suppressed low dose WBV- and melittin-induced flinchings, MK-801 did not have any significant effect on the WBV- and melittin-induced flinchings, and attenuated the low dose WBV- and melittin- induced decrease of PWT only 60-120 min after melittin and WBV injection. The current results that EAA receptor antagonists, especially MK-801, had little or weak effects on the initial nociceptive responses induced within 30 min after WBV or melittin injection are a little different from that s.c. WBV-induced discharges of WDR neurons are strongly suppressed by i.pl. administered MK-801, but not by CNQX (You et al, 2002). However, the results of Chen et al. (1999a) that preteatment of CNQX (i.pl.) suppressed WBV-induced firing of WDR neurons are generally in line with our results. One possible reason for this difference may be due to the intensity of induced nociceptive responses or the dose of WBV used, and the effects of i.pl. administered MK-801 and CNQX can become more strong if the intensity of nociceptive responses is further reduced by injecting very low doses of WBV and melittin. The involvement of peripheral EAA receptors in the nociceptive transmission is further supported by the findings that hyperalgesia and allodynia induced by i.pl. or inta-articular injection of EAAs and formalin are significantly alleviated by i.pl. or inta-articular administation of NMDA and non-NMDA receptor antagonists (Zhou et al, 1996; Davidson et al, 1997; Lawand, 1997).

Another peripheral mechanism by which WBV and melittin induce nociceptive response is a sustained activation and/or sensitizaton of primary afferent fibers by inflammatory substances. WBV has pro-inflammatory substances such as histamine, mast cell degranulating peptide and phospholipase A, and various pro-inflammatory substances are released when an inflammation is induced by i.pl. injection of WBV and melittin. These inflammatory substances can activate and/or sensitize nociceptive afferent fibers, resulting in a sustained increase of afferent inputs to the spinal cord and resultant long -lasting hyperalgesia (Habermehl, 1981; Calixto et al, 2003).

In the present study, WBV- and melittin-induced reduction of PWT and flinching behaviors were strongly suppressed after i.t. pre- or post-administration of MK-801 and CNQX. An involvement of spinal NMDA and non-NMDA receptors in WBV- and melittin-induced nociception is in agreement with the results obtained from the study in which other pain models were used. In the behavioral and electrophysiological studies, i.t. administration of NMDA and non-NMDA receptor antagonists attenuates mechanical and thermal hyperalgesia induced by cutaneous inflammation and peripheral nerve injury, suggesting that spinal EAA receptors are implicated in the nociceptive responses with peripheral origins (Haley et al, 1990; Mao et al, 1992; Ren et al, 1992; Neugebauer et al, 1993; Sluka et al, 1994). Spinal NMDA- and non-NMDA receptors are known to be involved in WBV-induced contralateral heat hyperalgesia (Chen et al, 2000), and i.t. adminisiation of neurokinin 1/2 receptor antagonist, spantide, suppresses WBV-induced flinchings and thermal hyperalgesla (Zheng & Chen, 2001).

WBV- and melittin-induced nociceptive inputs can cause the release of EAAs as well as other neurotransmitters such as substance P and calcitonin gene-related peptide from the central endings of nociceptive afferent fibers in the spinal dorsal horn. These nociceptive neurotransmitters increase Ca^{2+} influx and formation of inositol-1,4,5- trisphosphate (IP_3), resulting in activation of protein kinase C (PKC) in the nociceptive dorsal horn neurons (Sladeczek et al, 1985; Mayer et al, 1987; Coderre, 1992; Mao et al, 1995). The activation of PKC increases NMDA current (Chen & Mae Huang, 1991), Ca^{2+} influx (Yang & Tsien, 1993), neuronal excitability (Manseau et al, 1998) and neurotransmitter release (Barber & Vasko, 1996), which results in further increase of PKC activity and stronger nociceptive responses. This positive feedback cycle can aggravate pain responses, and then hyperalgesia and allodynla can develop. The spinal pro-nociceptive function of PKC has been demonstrated in the bee venom test. Intrathecal pre- and post-injections of PKC inhibitor, chelerythrine, dose-dependently inhibit subcutaneous WBV-induced spontaneous flinchings and contralateral hyperalgesia in conscious rat (Li et al, 2000).

Bee venom also contains phospholipase A_2 (PLA_2) which is also located in superficial dorsal horn (Ong et al, 1999) and catalyzes the conversion of phosphatidylcholine to arachidonic acid (Coderre, 1992). Melittin by itself has an ability to activate PLA_2 (Habermehl, 1981). These arachidonic acid products produced by catalytic action of PLA_2 can activate and/or sensitize nociceptive neurons (Coderre, 1992). All these pro-nociceptive factors may act together and cause a sustained pain.

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