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Comparative Study on the Nociceptive Responses Induced by Whole Bee Venom and Melittin

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The present study was undertaken to confirm whether melittin, a major constituent of whole bee venom (WBV), had the ability to produce the same nociceptive responses as those induced by WBV. In the behavioral experiment, changes in mechanical threshold, flinching behaviors and paw thickness (edema) were measured after intraplantar (i.pl.) injection of WBV (0.1 mg & 0.3 mg/paw) and melittin (0.05 mg & 0.15 mg/paw), and intrathecal (i.t.) injection of melittin (6 μg). Also studied were the effects of i.p. (2 mg & 4 mg/kg), i.t. (0.2 μg & 0.4 μg) or i.pl. (0.3 mg) administration of morphine on melittin-induced pain responses. I.pl. injection of melittin at half the dosage of WBV strongly reduced mechanical threshold, and increased flinchings and paw thickness to a similar extent as those induced by WBV. Melittin- and WBV-induced flinchings and changes in mechanical threshold were dose- dependent and had a rapid onset. Paw thickness increased maximally about 1 hr after melittin and WBV treatment. Time-courses of nociceptive responses induced by melittin and WBV were very similar. Melittin-induced decreases in mechanical threshold and flinchings were suppressed by i.p., i.t. or i.pl. injection of morphine. I.t. administration of melittin (6 μg) reduced mechanical threshold of peripheral receptive field and induced flinching behaviors, but did not cause any increase in paw thickness. In the electrophysiological study, i.pl. injection of melittin increased discharge rates of dorsal horn neurons only with C fiber inputs from the peripheral receptive field, which were almost completely blocked by topical application of lidocaine to the sciatic nerve. These findings suggest that pain behaviors induced by WBV are mediated by melittin-induced activation of C afferent fiber, that the melittin-induced pain model is a very useful model for the study of pain, and that melittin-induced nociceptive responses are sensitive to the widely used analgesics, morphine.

Key Words: I.pl whole bee venom & melittin, I.t melittin, Nociception, C primary afferent fiber, Morphine

INTRODUCTION

Bee venom (BV) has been known to have dual effects. In oriental countries such as Korea and China, BV has been used for the treatment of chronic pain such as arthritis. However, it was not until recent times that systematic studies on the underlying mechanisms of BV-induced pain relief were done. In 1973, Zurier et al reported that chronic subcutaneous (s.c) injection of whole BV strongly inhibited the development of arthritis induced by s.c injection of complete Freund's adjuvant (CFA) in the rat. This beneficial effect of BV was blocked in the adrenalectormized rats, suggesting that pituitary-adrenal axis played a crucial role in the BV-induced pain relief (Zurier et al, 1973; Kwon et al, 2003). Lee and his colleagues reported that BV had antinociceptive and anti-inflammatory effects on the inflammatory pain induced by CFA, carageenan, zymosan and formalin in rats (Zurier et al, 1973; Kwon et al, 2001a, 2002, 2003; Lee et al, 2001). BV strongly suppressed cyclooxygenase 2 activity, c-Fos expression in the spinal cord, formation of interleukin (IL)-6 and tissue necrosis factor α, development of edema and hyperalgesia (Lee et al, 2001; Kwon et al, 2002, 2003). More beneficial effects were produced by subcutaneous injection of BV into the acupoint than into non acupoint in rats (Kwon et al, 2001a; Kim et al, 2003). BV acupuncture induced greater analgesic effect than traditional needle acupuncture in the knee osteoarthritic patients (Kwon et al, 2001b). From these experimental and clinical studies, they suggested that BV was very useful for the long-term treatment of arthritic inflammation and pain.

As mentioned above, BV also has an inhibitory effect on cyclooxygenase (COX) activity and pro-inflammatory cytokines. In *in vitro* experiment, aqueous partition of BV dose-dependently inhibited the lipopolysaccharide-induced increases in COX-2 activity, COX-2 mRNA expression and production of IL-1β and tissue necrosis factor-α (Nam et al, 2003).

On the other hand, BV also has a nociceptive action. In 1996, Lariviere and Malzack introduced a new BV model

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ABBREVIATIONS: CFA, complete freund's adjuvant; CSPAF, capsaicin-sensitive primary afferent fiber; COX, cyclooxygenase; NMDA, N-methyl-D-aspartate; PWT, paw withdrawal threshold; WBV, whole bee venom; WDR neuron, wide dynamic range neuron

of persistent pain characterized by local inflammation, edema, spontaneous nociceptive behaviors and tonic pain (Lariviere & Melzack, 1996). Intraplantar (i.pl.) injection of BV also increased c-Fos expression in the deep and superficial dorsal horn, and the time-course of c-Fos expression paralled that of mechanical and thermal hyperalgesia (Luo et al, 1998). Both BV-induced pain and c-Fos expression were suppressed by the s.c injection of morphine (Lariviere & Melzack, 1996; Luo et al, 1998). In the comparative study on the BV model and formalin test which is one of the most widely used pain models, Chen et al (1999a) suggested that the BV model was more useful in the study of pain than the formalin test. BV-induced mechanical and thermal hyperalgesia was very stable nociceptive response which accompanied striking edema and redness, but not any tissue damage. In contrast, the i.pl. injection of formalin induced biphasic spontaneous nociceptive behaviors, but not hyperalgesia, and rather caused permanent hypoalgesia due to tissue damage. BV has been known to selectively activate capsaicin-sensitive primary afferent fibers (CSPAF) (Chen & Chen, 2000) and then cause sustained discharges of wide dynamic range (WDR) neurons with C fiber inputs, but not without C fiber volleys from the peripheral receptive field (Chen et al, 1998; You et al, 2002). An activation of the peripheral N-methyl-D-aspartate (NMDA), but not non-NMDA, receptors was implicated in the BV-induced firings of WDR cells (Chen et al, 1999b; You et al, 2002).

Of the various constituents, melittin is the major one which comprises about 50% of dry bee venom, and the other important ingredients are apamin, adolapin, hyaluronidase, phospholipase A and mast cell degranulating peptide (Minton, 1974; Habermehl, 1981). Melittin has been reported to cause hemolysis, inflammation, activation of phospholipase C and A_2, and an increase in Ca^{2+} influx (Shier, 1979; Choi et al, 1992; Fletcher & Jiang, 1993). Activated phospholipase C increases an intracellular Ca^{2+} concentration by the formation of inositol-1,4,5-trisphosphate (IP3) and the production of diacylglycerol, resulting in the activation of protein kinase C (PKC) (Choi et al, 1992). Phospholipase A_2 also contributes to the production of pain by increasing the formation of arachidonic acids (Shier, 1979). However, the precise constituents responsible for the nociceptive action of BV and the characteristics of melittin-induced pain are not clear. The present study was undertaken to investigate the role of melittin in BV-induced pain behaviors, to characterize the melittin-induced pain and to study the effect of morphine on the melittin-induced pain behaviors.

METHODS

Sprague-Dawley male 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.

Behavioral experiments

All rats were placed on an elevated metal mesh floor and allowed to acclimate for at least 30 min before behavioral testing. von Frey hair was applied vertically to the mid-plantar surface of the hindpaw in an ascending intensity order from underneath the floor. A bending force being able to evoke brisk paw withdrawal in more than 50% of 10 trials was expressed as the paw withdrawal mechanical threshold (PWT, g). A mirror was placed below the metal mesh floor at a 30° angle to allow an unobstructed counting of flinching. Changes in paw thickness (mm) were measured by using caliper and expressed as percentage changes in the control state without any treatment. Changes in mechanical threshold, total number of flinchings and paw thickness were measured after the injection of whole bee venom (WBV) (0.1 mg & 0.3 mg) and melittin (0.05 mg & 0.15 mg) into mid-plantar area of the hindpaw of normal rats. WBV and melittin (Sigma) were dissolved in 10 μl of saline. Because approximately 50% of dry bee venom is melittin, the dosage of melittin was determined to be one half the WBV dosage. The effects of morphine on melittin-induced changes in mechanical threshold and flinching behaviors were studied by administering morphine intraperitoneally (i.p., 2 mg & 4 mg), intrathecally (i.t., 0.2 μg & 0.4 μg) and i.pl. (0.3 mg) 10 min before melittin injection. In another behavioral experiment, we investigated whether the i.t. administration of melittin induced the same nociceptive behaviors as those produced by i.pl. injection of melittin. For the i.t. administration of melittin, chronic intrathecal 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 lumber enlargement. Rats were allowed to recover for at least 5 days before being used in the experiment. After i.t. administration of melittin (6 μg in 10 μl of saline), changes in nociceptive behaviors were measured by the aforementioned method. Vehicle (saline) for BV, melittin and morphine was injected to confirm its effect on mechanical threshold and spontaneous flinching behaviors.

Extracellular recording of dorsal horn cell responses

Rats were anesthetized with pentobarbital sodium (50 mg/kg,i.p.), and anesthesia was maintained by intravenous infusion of pentobarbital sodium (10 mg/kg/h). A tracheotomy was performed, and the rats were artificially ventilated by a small animal ventilator (Model 683, Harvard Apparatus, USA). End-tidal CO_2 level was maintained between 3.5 and 4.5% by adjusting respiratory rate and volume (End-tidal CO_2 analyzer, Capstar-100, IITC Inc.). Rectal temperature was maintained near 37°C with a homeothermic heating blanket (Harvard Apparatus, USA). After exposing a lumbar enlargement between T13 and L3 and sciatic nerve at mid-thigh level, rats were placed in a stereotaxic apparatus. Extracellular activities of WDR neurons evoked by i.pl. injection of melittin (0.15 mg) were recorded through the carbon filament microelectrode. In this experiment, WDR cells receiving C fiber inputs from the periphery and WDR neurons with only A fiber volleys were used. A and C fibers were electrically activated by a single stimulus (0.1 msec, 10T) or 4 train stimuli (50 Hz, 0.5 msec). Stimulus intensity of single or train stimuli was 10 or 100 times the threshold (1T) for activation of Aβ fiber, respectively. All evoked activities were amplified (WPI, DAM80, U.S.A) and fed into a window discriminator (Frederic Haer & Co, USA) whose outputs were used for compilation of the post-stimulus time histogram. After i.pl. injection of melittin (0.15 mg) into the most sensitive area

of the receptive field, changes in the discharge rate were recorded from WDR cells with C fiber inputs, and WDR neuron with only A fiber inputs. In order to confirm the origin of melittin-induced discharges of WDR cells, a small piece of cotton (5×5mm) soaked in lidocaine (2%) was placed onto the exposed sciatic nerve at mid-thigh level after i.pl. melittin-induced discharge reached a steady level, and the obtained result was compared to those without lidocaine treatment. In the preliminary experiments, i.pl. injections of 10 μl of saline were identified as not having any significant effects on melittin-induced pain behaviors and discharge rate of WDR neuron. The data are expressed as mean±SE and analyzed using ANOVA followed by the Newman-Keuls test. P value less than 0.05 were considered significant. When the experiments were completed, the rats were euthanized by an overdose of pentobarbital sodium.

RESULTS

Behavioral study

The PWT of a normal rat was approximately 26 g. Intraplantar injection of melittin (0.05 mg, n=13) and WBV (0.1 mg, n=13) dramatically reduced mechanical thresholds, which reached a peak reduction of 3.1±0.4 g and 3.4±0.5 g, respectively, within 10 min (Fig. 1).

The decreased mechanical threshold recovered very slowly to 5.7±0.4 g and 5.6±0.5 g 60 min after the injection of melittin (0.05 mg) and WBV (0.1 mg), respectively. The recovery rate of decreased PWT was not great until 180 min after WBV and melittin injection. In general, there was no difference in PWT between the normal and injected rats 24h after injection. High doses of melittin (0.15 mg, n=14) and WBV (0.3 mg, n=10) also caused a great decrease in PWTs, which were 3.4±0.3 g and 3.3±0.7 g 10 min after the i.pl. injections, respectively. We could not find any significant differences between the maximum decrease in PWT induced by low and high doses of melittin and WBV for the initial 60 min. However, the high dosage-induced decrease in PWT was more sustained than that caused by low dosage. The PWTs were 9.4±1.2 g and 12.7±2.1 g 2 days after i.pl. injection of high doses of melittin (0.15 mg) and WBV (0.3 mg), respectively, and did not completely recover 5 days after melittin (24.9±4.2 g) and WBV (23.6±4.1 g) injection. The time courses of changes in PWTs and the efficacy induced by low or high doses of melittin and WBV were very similar.

Flinching behaviors of the hind paw were not observed in the normal rat. However, i.pl. injection of low doses of melittin (0.05 mg, n=13) and WBV (0.1 mg, n=13) caused a great increase in the number of flinchings, which were 23.6±2.4 and 23.7±2.2 during initial 5 min, respectively (Fig. 2). The increased flinching behaviors gradually decreased to 1.5±0.5 and 6.3±1.4 30 min after i.pl. injection. High doses of melittin and WBV caused greater increase in flinching behaviors than low dose, and the efficacy of high dose was more long-lasting. The numbers of flinching behaviors induced by high doses of melittin (0.15 mg, n=14) and WBV (0.3 mg, n=10) were 34.6±4.6 and 29.0±4.1 for the initial 5 min and decreased to 15.7±0.5 and 20.3±2.3 30 min after i.pl. injection, respectively.

Intraplantar injection of melittin and WBV increased paw thickness, which reached the maximun level 60 min after i.pl. injection (Fig. 3). Low doses of melittin (n=10) and

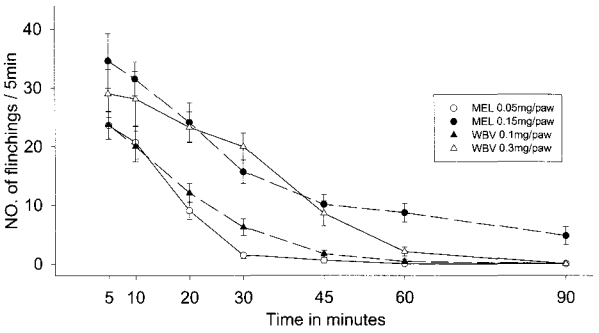

Fig. 2. Intraplantar injection of melittin (MEL, 0.05 mg & 0.15 mg/paw) and whole bee venom (WBV, 0.1 mg & 0.3 mg/paw) dose-dependently produced flinching behaviors in the rat. Each value is represented as the total number of flinchings measured for 5 min time block before each time point.

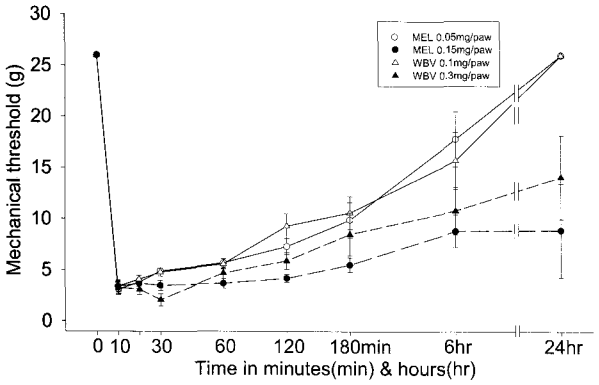

Fig. 1. Comparison of changes in mechanical thresholds induced by the intraplantar injection of whole bee venom (WBV, 0.1 mg, △ & 0.3 mg/paw, ▲) and melittin (MEL, 0.05 mg, ○ & 0.15 mg/paw, ●) in the rat. Each value is represented as mean±S.E.

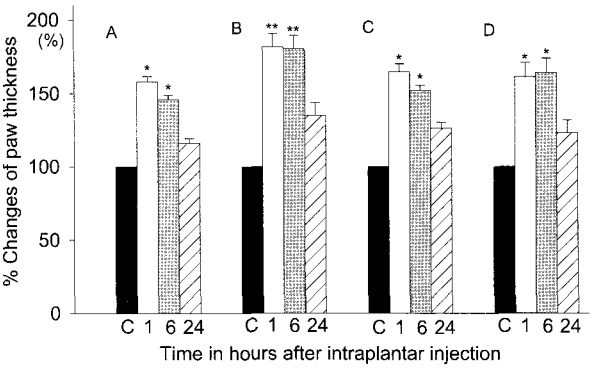

Fig. 3. Changes in paw thickness after intraplantar injection of melittin (0.05 mg/paw<A> & 0.15 mg/paw<B>) and whole bee venom (0.1 mg/paw<C> & 0.3 mg/paw<D>). Data are represented as percent changes of the control (C, 100%). *; $p<0.05$, **; $p<0.01$, significant differences from the control.

WBV (n=11) increased paw thickness by 58.0±3.8% and 64.9±5.3%, respectively, 1 hr after i.pl. injection. Paw thickness also increased by 81.6±9.0% and 61.4±9.6% 1 hr after i.pl. injection of high doses of melittin and WBV, respectively. High dose-induced increase in paw thickness changed little even 6 hr after i.pl. injection, whereas there was a small but not significant decrease in paw thickness 6 hr after i.pl. injection of low doses of melittin (45.7±3.4%, n=9) and WBV (52.3±3.4%, n=12).

Intrathecal administration of melittin (6 μg) decreased PWT (Fig. 4, n=7) and induced flinching behaviors (Fig. 5), but did not have any effect on paw thickness. PWT decreased to 11.3±2.7 g within 10 min after i.t administration and remained in a decreased state until 60~90 min. The decreased PWT fully recovered about 6 hr after i.t administration (Fig. 4). The number of flinchings induced by i.t melittin was 17.0±6.1 for the initial 5 min and thereafter decreased rapidly to 3.4±3.2 20 min after i.t administration (Fig. 5). In preliminary experiments, doses of melittin lower than 6 μg induced a weak effect, whereas higher doses caused side effects such as severe agitation.

The ability of melittin to reduce PWT was profoundly attenuated at all time points in the rat treated with morphine (2 mg, n=7 & 4 mg/kg, n=8) (Fig. 6). The decreased PWT completely recovered to the normal level 60 min after i.pl. injection of melittin in the rat administered with morphine (4 mg/kg, i.p), but it took about 24h for the decreased PWT to recover fully in the rat without morphine administration.

I.pl. injection of morphine 10 min before melittin injection significantly attenuated the ability of melittin to reduce mechanical threshold only at 120 min (14.5±2.6 g for morphine group / 8.3±1.0 g for melittin group) and 180 min (15.6±2.9 g for morphine group / 9.1±1.4 g for melittin group) after melittin injection (n=10, $p<0.05$, Fig. 7). Following administration of morphine 60 min after i.pl. melittin injection, melittin-induced decrease in mechanical threshold were significantly, but not strongly attenuated except at time-points of 120 min and 24 h after melittin injection (n=8, Fig. 7)

I.t. administration of 0.2 μg of morphine did not cause a significant attenuation of the melittin-induced decrease in mechanical threshold except at 180 min after i.pl. melittin injection (n=9, Fig. 8). But, there was a tendency that mechanical thresholds of 0.2 μg of morphine-treated group were higher than those of melittin-injected group. However, high dose (0.4 μg) of morphine strongly attenuated the decrease in mechanical threshold by i.pl. melittin injection (n=14, $p<0.001$, Fig. 8). In morphine-injected

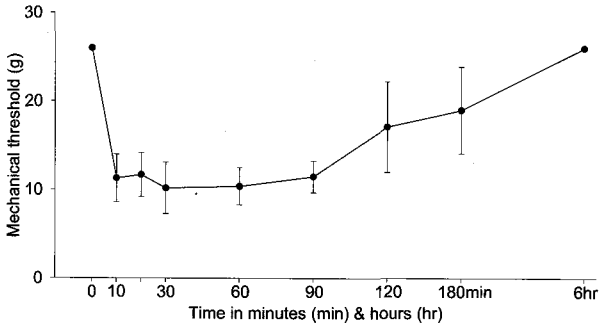

Fig. 4. Intrathecal administration of melittin (6 μg) induced sustained decrease in mechanical threshold, that gradually recovered about 90 min after melittin treatment.

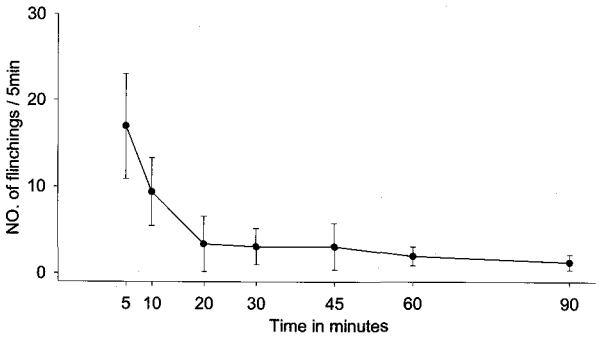

Fig. 5. Intrathecal injection of melittin (6 μg) caused flinchings of the hind paw in the rat. Each value is the number of flinchings measured for a 5 min time block before each time point.

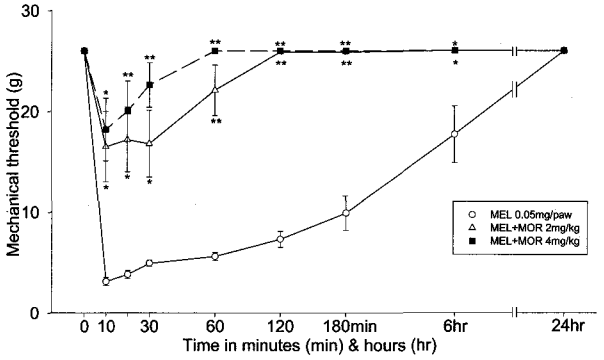

Fig. 6. Intraperitoneal administration of morphine (MOR, 2 mg/kg and 4 mg/kg) reduced melittin (MEL)-induced decreases in mechanical threshold. Data are expressed as mean±S.E. *; $p<0.03$, **; $p<0.001$, significant differences from the melittin-treated group.

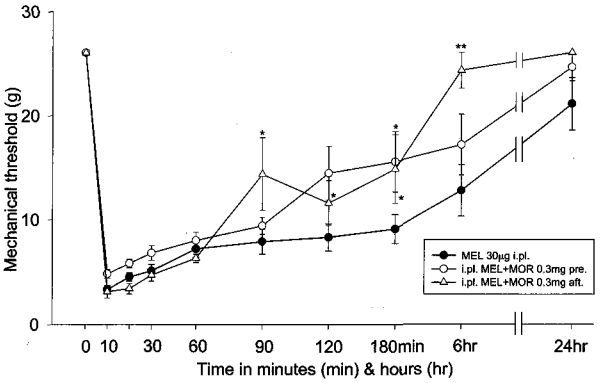

Fig. 7. Intraplantarly administered morphine (MOR, 0.3 mg/paw) 10 min before (-○-) or 60 min after (-△-) intraplantar injection of melittin (MEL, 30 μg/paw, -●-) had limited inhibitory effects on the melittin-induced reduction of mechanical threshold. Data are expressed as mean±S.E. *; $p<0.05$, **; $p<0.01$, significant differences from the melittin-injected group.

group, mechanical thresholds were 15.5±2.6 g, 20.2±2.2 g and 24.7±1.3 g 10 min, 60 min and 6 h after i.pl. melittin injection, whereas those of melittin-injected group were 3.3 ±0.4 g, 7.2±0.8 g and 13.8±2.5 g at respective time points, respectively.

Melittin-induced flinchings were strongly suppressed by the administration of morphine (Fig. 9). Melittin-induced increase in flinching behaviors (61.1±5.1/30 min) significantly decreased to 15.0±3.4/30min after i.p. injection of 12 mg/kg morphine, (n=15, $p<0.001$). I.pl. or i.t. administration of morphine also strongly inhibited the increase in flinching behaviors, and the numbers of flinchings were

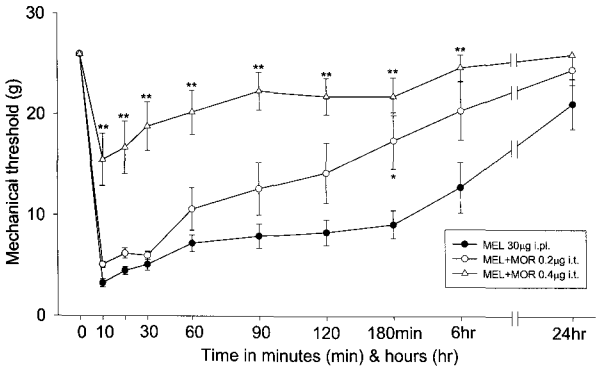

Fig. 8. Intrathecal administration morphine (MOR, 0.2 μg, -○-; 0.4 μg, -△-) dose-dependently attenuated the ability of melittin to reduce mechanical threshold (MEL, -●-). Data are expressed as mean±S.E. *; $p<0.05$, **; $p<0.001$, significant differences from the melittin-injected group.

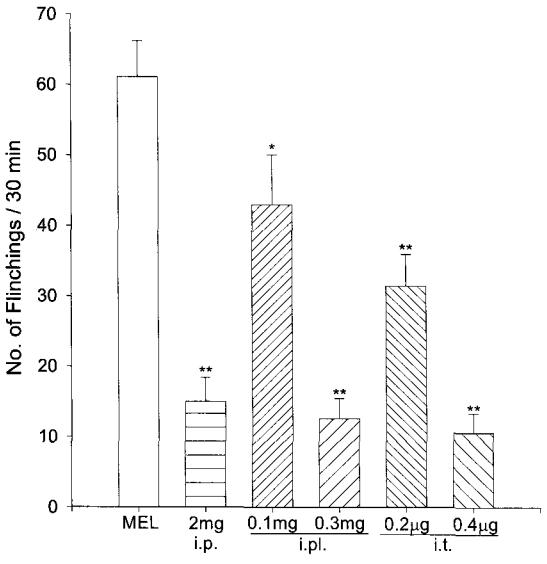

Fig. 9. Intraplantar injection of melittin (MEL, 30 μg/paw) induced the increase in the flinching behaviors which were strongly inhibited by intraperitoneal (i.p., 2 mg), intraplantar (i.pl., 0.1 mg & 0.3 mg or intrathecal (i.t., 0.2 μg & 0.4 μg) administration of morphine. Data are expressed as mean±S.E. *; $p<0.05$, **; $p<0.001$, significant differences from the melittin-induced increase in flinching behaviors.

12.6±2.9/30 min (n=9, $p<0.001$) and 10.6±2.8/30 min (n=13, $p<0.001$) after i.pl. (0.3mg/paw) or i.t. (0.4 μg) administration of morphine, respectively.

Electrophysiological study: In the 19 rats, changes in extracellular activities were recorded from WDR neurons with or without C fiber inputs from the peripheral receptive field (RF) after i.pl. injection of melittin (0.15 mg/paw) into RF. Melittin injection greatly increased the discharge rates of WDR neurons with C fiber inputs from the peripheral RF (Fig. 10B), whereas the discharge rates of WDR neurons without C fiber inputs did not change even after i.pl. injection of melittin (Fig. 10A), suggesting that melittin selectively activated C primary afferent fibers. The discharge rate of WDR neurons with C fiber inputs was 1213.1 ±133.3 for the first 1 min after i.pl. injection of melittin. The increased discharge rate gradually decreased to 596.8 ±128.9/min at 5 min and was almost zero at 30 min after i.pl. injection of melittin. Fig. 10C shows that topical

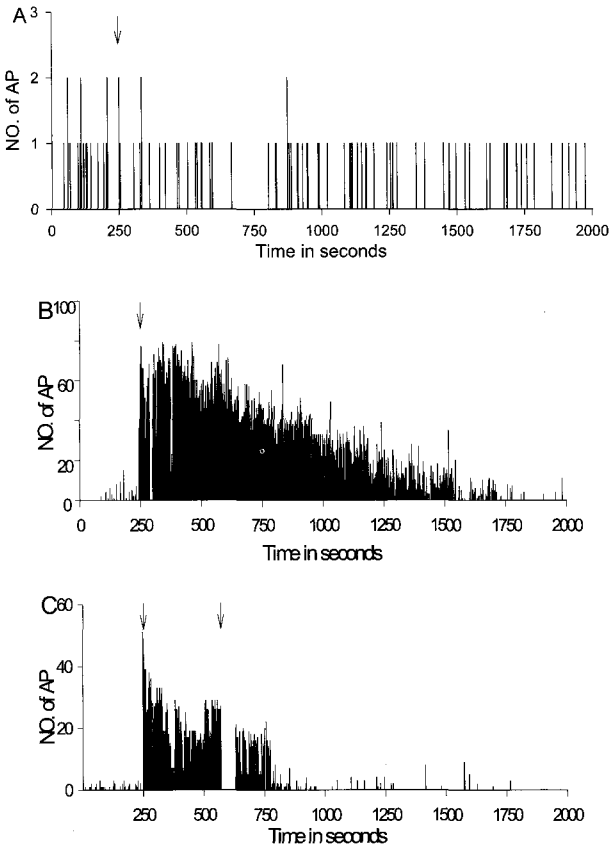

Fig. 10. Discharges of wide dynamic range neurons with (B), but not without (A), C fiber inputs from the peripheral receptive field were increased by intraplantar injection of melittin (0.15 mg/paw). Topical application of lidocaine (2%) onto sciatic nerve at mid-thigh level strongly blocked melittin-induced increases in discharges of dorsal horn neurones (C). Arrows indicate the time when melittin was injected into the receptive field and lidocaine was applied onto the sciatic nerve at 2nd arrow in C. Recording of wide dynamic range cell activity was stopped during lidocaine application to prevent the inflow of electrical noises. No. of AP is the number of action potentials produced by melittin-induced activation of afferent fibers. Bin time was 1,000 msec.

application of lidocaine onto sciatic nerve at mid-thigh level almost completely blocked melittin-evoked increases in discharges of WDR neurons. We also obtained similar results from another 5 experimental cases.

DISCUSSION

The results obtained from the present behavioral and electrophysiological experiments suggest that most of WBV-induced nociceptive responses was mediated through melittin-induced activation of primary afferent C fibers. Melittin-induced nociceptive responses were dose-dependent, very stable, sustained and had very rapid onset. Nociceptive behaviors such as flinching and licking, and discharges of WDR neuron were induced immediately after i.pl. injection of melittin. PWT appeared to be maximally reduced within 5 min after i.pl. injection of melittin, although we represented data measured 10 min after i.pl. injection. The reduced PWT was very long-lasting and recovered very slowly. All these melittin-induced characteristics of nociceptive responses were also observed in the rat injected with WBV. WBV has already been reported to induce long-lasting mechanical and thermal hyperalgesia (Lariviere & Melzack, 1996; Chen et al, 1999a; You et al, 2002) and contralateral heat hyperalgesia (Chen et al, 2000, 2001), to increase c-Fos expression in the spinal dorsal horn (Luo et al, 1998), and to enhance the discharge rate of WDR neuron with C fiber inputs from the peripheral receptive field (Chen et al, 1998; You & Chen, 1999).

Another common feature was that there was no substantial difference in the time course and severeness of nociceptive responses induced by melittin and WBV. The WDR neuron only with, but not without, C fiber input from the peripheral receptive field was activated by i.pl injection of melittin. In the behavioral test, topical application of capsaicin (1%) to the sciatic nerve almost completely blocked melittin-induced flinching behaviors and decrease in mechanical threshold (Shin & Kim, 2004). Chen and Chen (2000) also reported that capsaicin-sensitive primary afferent fibers played a pivotal role in the development of thermal and mechanical hyperalgesia, contralateral hyperalgesia and spontaneous nociception by i.pl. injection of WBV. Subcutaneous injection of WBV did not produce a long-lasting increase in the firing of WDR neuron which received only A fiber afferent volleys from a peripheral receptive field (Chen et al, 1998).

Melittin-induced discharges of WDR neuron with C fiber inputs were completely suppressed after conduction blockade of the sciatic nerve with lidocaine, suggesting that the increased firings of WDR neuron are resulted from the activation of peripheral afferent fibers. Lidocaine has been reported to suppress the conduction of nociceptive signals produced by WBV at the peripheral receptive field (Chen et al, 1998). Putting all these experimental results together, it is evident that melittin-induced nociceptive responses have all the same characteristics as those induced by WBV. These experimental evidences support the view that melittin is a major component that is implicated in the production of pain by i.pl. injection of WBV or bee stings, and the melittin model of pain is very useful for the study of the pain mechanism.

Intrathecal administration of melittin also reduced the mechanical threshold of peripheral receptive field and induced flinching behaviors, but did not produce an edema. The reduced mechanical threshold by i.pl. injection of melittin was more sustained and stable than that induced by intrathecal administration. It was not easy to determine an appropriate dosage that induced sustained nociceptive responses, because a little higher or lower dosage induced side effects such as agitation or too weak nociceptive responses, respectively.

Chen and his colleagues compared the characteristics of nociceptive responses induced by subcutaneous injection of formalin and WBV, and suggested that the WBV model of pain had more advantages over the formalin test in the study of pain. Formalin injection induced a biphasic spontaneous pain followed by a permanent hypoalgesia, resulting from tissue damage, and did not produce hyperalgesia by mechanical and thermal stimulation of the injected site. On the other hand, i.pl WBV induced a profound decrease in the mechanical and thermal threshold, followed by long-lasting hyperalgesia depending on the dosage and did not cause any tissue damage (Chen et al, 1998, 1999a). It is apparent from the present experiment that melittin activated C afferent fibers at peripheral site, resulting in very long-lasting nociceptive responses with a decreased mechanical threshold. This view can be supported by experimental evidence that topical application of capsaicin to the sciatic nerve almost completely prevented the development of mechanical hyperalgesia and flinching behaviors after i.pl injection of WBV (Chen & Chen, 2000) and the increase in discharges of WDR neuron following melittin injection (Shin and Kim, 2004).

However, the peripheral mechanism by which melittin induced a sustained nociceptive responses can not clearly be understood. It appears that the melittin-induced initial nociceptive responses might be caused by direct activation of primary afferent fibers in the injected site. This proposal is evidenced by the results that flinching behaviors and discharges of WDR neuron were increased immediately after i.pl. injection of melittin. Chen et al (1999b) and You et al (2002) reported that the activation of peripheral NMDA receptor contributes to the development of WBV-induced nociceptive responses. They described that subcutaneous injection of NMDA, but not non-NMDA, receptor antagonists, such as dl-2-amino-5-phosphonovaleric acid (AP5) and MK-801, strongly inhibited WBV-induced mechanical hyperalgesia and discharges of dorsal horn neuron. However, i.pl. injection of 10 μg of MK-801 did not have any effect on the melittin-induced decreases in mechanical threshold and flinching behaviors in our experiment (Shin et al, 2003). This discrepancy may be due to difference in the dose of MK-801. The dose of MK-801 (about 33 μg) administered by You et al (2002) was high enough to cause severe side effects such as agitation in our experiment.

Melittin has been known to increase Ca^{2+} influx in rat pheochromocytoma PC12 cells, activities of phospholipase A_2 and C, lipoxygenase activity in human leukocytes and platelets, and the release of lysosomal enzymes (Shier,1979; Salari et al, 1985; Choi et al, 1992). If the function of all these factors are still effective in the primary afferent nerve terminals, the increased Ca^{2+} influx and formation of IP3 (inositol-1,4,5-trisphosphate) by catalytic action of phospholipase C can increase the activity of protein kinase C (PKC), and phospholipase A_2 catalyzes the conversion of phosphatidylcholine into arachidonic acid (Shier, 1979; Choi et al, 1992). The activated PKC together with lipoxygenase products and arachidonic acid products may induce pain

reaction in peripheral nociceptors. In the behavioral test, we observed that i.pl. injection of N- and L-type Ca^{2+} channel antagonist (ω-conotoxin GVIA & verapamil) strongly inhibited melittin-induced flinching behaviors and a decrease in mechanical threshold (unpublished data). This results suggest that Ca^{2+} influx through voltage-dependent N- and L-type Ca^{2+} channels triggers the nociceptive responses in the peripheral site.

Melittin-induced sustained inputs from the peripheral site can activate and sensitize nociceptive spinal WDR neuron, which may result in the hyperalgesia. In another experiment, we investigated the role of spinal NMDA and non-NMDA receptor and Ca^{2+} channels in the melittin-induced nociception. Melittin-induced flinching behaviors were significantly reduced, and the decrease in mechanical threshold was strongly blocked after intrathecal pre- and post-administration of NMDA (MK-801), and non-NMDA (CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione disodium) receptor antagonist (Shin et al, 2003), and Ca^{2+} channel antagonist (ω-conotoxin GVIA & verapamil) (unpublished data). Intrathecal administration of neurokinin 1/2 receptor antagonist (spantide) and PKC inhibitor (chelerythrine chloride) produced dose-dependent suppressive effects on WBV-induced flinching behaviors (Li et al, 2000; Zheng & Chen, 2001). However, spantide had a limited inhibitory effect on the thermal hyperalgesia and no effect on mechanical hyperalgesia. Taken together, all these findings suggest that the activation of Ca^{2+} channel, PKC, NMDA and non-NMDA receptors contributes to the induction and maintenance of melittin-induced nociception in the spinal cord.

Morphine and other opioid substances are widely used and strong analgesics, but their repeated administration causes the development of tolerance characterized by their reduced ability to induce analgesic action (Shin, 1998). The mechanisms by which opioid substances inhibit nociceptive responses are summerized as follows: increase in the conductance of K^+ channel which results in membrance hyperpolarization (Williams et al, 1982) and decrease in Ca^{2+} influx which reduces the release of neurotransmitters from presynaptic afferent fibers and suppresses pain reactions in nociceptive neurons (Werz & Macdonald, 1983; Cherubini & North, 1985). In the present experiment, i.pl. or i.t. administration of morphine strongly inhibited spontaneous flinchings and the ability of melittin to reduce mechanical threshold. These inhibitory effects on the melittin-induced nociception can result from the decrease in membrane excitability and in the release of nociceptive neurotransmitters. Recent studies reported that opioid substances had peripheral antinociceptive action as well as spinal and supraspinal effects. The peripheral analgesic effect is stronger in painful inflammatory conditions than in acute model of pain (Czlonkowski et al, 1993; Hassan et al, 1993; Stein, 1993). In the present study, i.pl. administration of morphine at a dose which did not have systemic effect had mild and limited analgesic effect on melittin-induced nociception. I.pl. post-treatment of morphine 60 min after i.pl. melittin injection produced significant, but not strong, antinociceptive action, which was not greatly different from the antinociception induced by pre-treatment of i.pl. morphine at same dose. Taking all these experimental results into consideration, melittin induces sustained and opioid-sensitive nociceptive responses which can be used as a helpful pain model for the study of pain mechanism.

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